

**EFFECTS OF CHANGES IN THE RATE AND RHYTHM OF
STIMULATION ON EXCITATION-CONTRACTION COUPLING
IN MAMMALIAN VENTRICULAR MUSCLE**

**A Thesis
Presented to the
University of Manitoba**

**In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy**

**by
Ronald Allan Bouchard
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MAMMALIAN VENTRICULAR MUSCLE

BY

RONALD ALLAN BOUCHARD

A thesis submitted to the Faculty of Graduate Studies of
the University of Manitoba in partial fulfillment of the requirements
of the degree of

DOCTOR OF PHILOSOPHY

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This work is dedicated to the memory of my father,

Robert Allan Bouchard

He had thought more than other men and in the matters of the intellect he had that calm objectivity, that certainty of thought and knowledge, such as only truly intellectual men have, who have no axe to grind, who never wish to shine or talk others down, or to appear always in the right.

Hermann Hesse, *Steppenwolf*, 1929

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ABSTRACT

The aim of this work was to test the hypothesis that rate-dependent changes in the strength of contraction by isolated mammalian cardiac muscle were related to accompanying alterations in both the magnitude of intracellular Ca^{2+} loading and release, and shifts in the dominant mode of operation of the sarcolemmal Na^+ - Ca^{2+} exchange process. We described the quantitative relationship between Ca^{2+} availability within the sarcoplasmic reticulum (SR) and the strength of contraction during alterations in the rate and rhythm of stimulation in solutions of varying ionic composition and temperature. In control solutions containing 140 mM $[\text{Na}^+]_o$ and 2.5 mM $[\text{Ca}^{2+}]_o$, a graded increase of stimulation frequency resulted in a stepwise increase in the peak of isometric contraction, which was associated with a modest reduction of both time to peak force and time required for complete relaxation of the twitch. The positive staircase of contraction was accompanied by a smaller, but significant, increase in the amount of Ca^{2+} available for release from SR stores, which was estimated independently with rapid cooling contracture and postrest contraction measurements. Restitution curves constructed from the contractile response to both premature and postrest stimulation at different coupling intervals and basal stimulation frequencies indicated that the rate constant for unidirectional Ca^{2+} transport within the SR during the diastolic interval was enhanced during high-frequency stimulation. Lowering $[\text{Na}^+]_o$ to 70 mM produced inotropic changes in the steady-state which were consistent with a $[\text{Na}^+]_o$ -dependent increase of SR Ca^{2+} sequestration and release due to inhibition of sarcolemmal Ca^{2+} extrusion. Following reduction of $[\text{Na}^+]_o$, a graded increase of stimulation frequency lead to a marked negative staircase of contraction, while steady-state cooling contracture amplitude and postrest contraction remained unaltered or slightly increased. Experiments designed to probe the underlying mechanism of this apparent uncoupling of SR Ca^{2+} availability and release suggested that it was not related to a slowed rate constant for Ca^{2+} transport between intracellular compartments, or the absolute size of the intracellular Ca^{2+} storage pool, but was more likely to be a consequence of Ca^{2+} -induced inactivation of the SR Ca^{2+} -release process. In the presence of low- $[\text{Na}^+]_o$ solution,

application of the neutral plant alkaloid ryanodine (10 nM) or the methylxanthine compound caffeine (3 mM) depressed contraction and restored the positive staircase, the slope of which was dramatically enhanced in the presence of ryanodine. Inhibition of sarcolemmal Ca^{2+} influx by the dihydropyridine Ca^{2+} channel antagonist nifedipine (2 μM) depressed contractile strength in a rate-dependent fashion, while opposite effects were demonstrated with similar concentrations of the racemic Ca^{2+} channel agonist BAY K 8644 (1 μM). Based on the forgoing data as well as on the results of other experiments conducted on ventricular muscle obtained from rat and guinea-pig ventricle, a 3-compartment model of intracellular Ca^{2+} transport was developed. The experimental results were consistent with the interpretation that rate-dependent changes of both contraction and intracellular Ca^{2+} loading in mammalian ventricle require the presence of a functional Na^+ - Ca^{2+} exchange process. The possibility is discussed that net cellular Ca^{2+} gain related to the activity of the exchanger may affect the strength of contraction during high-frequency stimulation by either altering the trigger for intracellular Ca^{2+} release, or in a more direct fashion by making Ca^{2+} available to the myofilaments. This working hypothesis was used successfully to attribute contractile changes in a previously established model of diabetic cardiomyopathy to defective Ca^{2+} handling by the SR.

PREAMBLE

Every experiment has a history, and so it is with those described within the text of this Thesis. Experiments performed with the dihydropyridine Ca^{2+} channel agonist BAY K 8644, described in SECTION 1, formed a part of an ongoing project which began well before my arrival in the laboratory. I am indebted to Drs. L.V. Hryshko and J.K. Saha for contributing valuable electrophysiological data and offering many interesting and useful suggestions during this time.

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LIST OF ABBREVIATIONS

$[\text{Na}^+]_o$	Extracellular concentration of sodium ions
$[\text{K}^+]_o$	Extracellular concentration of potassium ions
$[\text{Ca}^{2+}]_o$	Extracellular concentration of calcium ions
[free Ca^{2+}]	Concentration of free calcium ions
a_{Na}^i	Intracellular free sodium activity
a_{Na}^o	Extracellular free sodium activity
a_{Ca}^i	Intracellular free calcium activity
a_{Ca}^o	Extracellular free calcium activity
\hat{u}_{Na}	Electrochemical gradient for sodium
\hat{u}_{Ca}	Electrochemical gradient for calcium
I_{Na}	Tetrodotoxin-sensitive sodium current
I_{Ca}	Dihydropyridine-sensitive calcium current
I_{to}	Transient outward potassium current(s)
I_{NaCa}	Putative Na^+ - Ca^{2+} exchange current
E_m	Resting membrane potential
E_{Na}	Sodium reversal potential
E_{Ca}	Calcium reversal potential
E_{NaCa}	Na^+ - Ca^{2+} exchange reversal potential
EC-Coupling	Excitation-contraction coupling
NIEA	Negative inotropic effect of activation
PIEA	Positive inotropic effect of activation
RC	Rested-state contraction
SR	Sarcoplasmic reticulum
RCC	Rapid cooling contracture

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GENERAL INTRODUCTION

The field of cardiac physiology has become enriched with the rapidly growing body of scientific literature concerning the cellular and molecular function of various tissues of the heart. This is particularly evident when one considers the role of Ca^{2+} ions in the coupling of membrane excitation to contraction in different types of striated muscle, or the precise biophysical properties of proteins embedded in the plasma membrane responsible for governing the movements of various ion species both into and out of the cell. The importance of gaining an understanding of the biophysical basis of cardiac function is underscored by the fact that virtually every important physiological, pharmacological or pathological change in cardiac function originates from changes in the physical and chemical processes which are responsible for contraction (Katz, 1977). The aim of this review is to introduce the different cellular pools from which Ca^{2+} may be derived for the contraction of mammalian cardiac muscle, as well as the necessary interaction between these functional compartments during successive cardiac cycles. The main focus of the discussion will be on the qualitative relationship between Ca^{2+} located intracellularly and peak isometric contraction, and in particular, on those cellular processes thought to govern the amount of Ca^{2+} present within each of the individual compartments at various points in the cycle of contraction to relaxation.

The ability of the heart to propel blood through the vascular bed is governed to a large extent by a number of important geometrical factors which affect the function of the intact heart as well as various neural and hormonal influences, and all of these factors must be either taken into account or eliminated when investigating the cellular mechanisms underlying contraction. Complications introduced by cell shortening can be reduced significantly by conducting experiments under isometric conditions, although it is of more than passing interest that an increasing number of studies have reported a similar dependence of isometric and isotonic contraction to a wide range of experimental and pathological conditions. As elegantly reviewed by Katz (1977), *in situ* cardiac performance can be strongly influenced both by alterations of the intrinsic contractile properties of

the heart and by length-dependent changes of contractility following manipulations of preload. Recent experiments, however, have indicated that in addition to the degree of myofilament overlap, changes in myofilament Ca^{2+} -sensitivity (Fabiato and Fabiato, 1976) or degree of muscle activation (Jewell, 1977; Allen and Blinks, 1978) are involved in Starling's law of the heart, even after compensating for "muscle deactivation" that results from variable amounts of shortening which take place during isometric contractions (Jewell and Wilke, 1960). Thus, it appears that a significant degree of interplay takes place between the cellular processes mediating the length-tension relationship and those involved in transiently elevating myoplasmic Ca^{2+} concentration during contraction.

In the remainder of this review, I shall exclude length-dependent changes in contraction and extrinsic influences on contractility, and focus primarily on length-independent contractile phenomenon and the mechanisms which link membrane excitation to contraction (excitation-contraction coupling). Results obtained with mammalian cardiac tissues will be emphasized throughout the discussion, and the main thrust will be on the cellular mechanisms involved in the delivery of Ca^{2+} to the myofilaments. SECTION 1 deals with the obligatory role of Ca^{2+} in the contraction of mammalian cardiac muscle and the different sources from which this Ca^{2+} may originate. SECTION 2 contains a review of the effects of changes in the rate and rhythm of stimulation on the processes governing the rise of intracellular Ca^{2+} during contraction. In the final section, a working hypothesis, or model, of excitation-contraction coupling (EC-Coupling) will be introduced which has evolved over the last several years in this laboratory. The model was proposed originally to account for the mechanical and electrophysiological effects of strontium ions on canine ventricular muscle (King and Bose, 1984), and since that time has been extended specifically to include the interval-dependence of contraction in mammalian cardiac tissues (Bose, King, Hryshko and Chau, 1988; Bouchard and Bose, 1989) and the sensitivity of the interval-force relation to various inotropic and pathological conditions (Bose, Kobayashi, Bouchard and Hryshko, 1988; Hryshko, Bouchard, Chau and Bose, 1989; Bouchard, Hryshko, Saha and Bose, 1989; Saha, Hryshko, Bouchard, Chau and Bose, 1989; Bouchard and Bose, 1991). Hypotheses relating to the

cellular control of contraction in ventricular muscle and their relation to intracellular Ca^{2+} handling will be presented in this section.

1.0 Dependence of Contraction on Myoplasmic Ca^{2+}

As early as 1882, Sidney Ringer demonstrated the importance of extracellular Ca^{2+} for the contraction of isolated cardiac muscle preparations. This finding was interpreted at the time as an indication of the Ca^{2+} -sensitivity of the cell membrane, and it was not until 1907 that Lock and Rosenheim reported that action potentials recorded from isolated mammalian hearts persisted long after the disappearance of contraction following the removal of extracellular Ca^{2+} . The ability of Ca^{2+} to activate contraction was demonstrated in 1947 by Heilbrun and Wiercinski who showed that microinjection of Ca^{2+} into the myoplasm of mammalian cardiac muscle resulted in the "initiation of contraction". Not long afterwards, repetitive stimulation was correlated with an increase of net Ca^{2+} uptake into the cells of various skeletal (Bianchi and Shanes, 1959) and cardiac (Winegrad and Shanes, 1962; Niedergierke, 1963; Langer, 1965) muscle preparations. The resting uptake of Ca^{2+} was estimated in these experiments to be $\sim 9 \text{ nM/cm}^2/\text{sec}$ during resting conditions and $\sim 100 \text{ nM/cm}^2/\text{sec}$ during steady-state stimulation.

During the course of the last decade, a number of biochemical probes have been developed for the purpose of estimating the level of free myoplasmic Ca^{2+} during contraction. The first such compound was aequorin, a bioluminescent Ca^{2+} -sensitive protein extracted from the jellyfish, *aequorea forkalea* (Shimomura, Johnson and Saiga, 1962). This compound was first used to quantify intracellular Ca^{2+} transients in isolated ferret papillary muscle by Allen and Blinks in 1978. However, difficulties in calibrating the aequorin signal, the poor sensitivity to low levels of myoplasmic Ca^{2+} , and the non-linearity of its dependence on Ca^{2+} concentration led ultimately to the development of the second generation Ca^{2+} indicators, typified by fura-2 and indo-1. First described by Grynkiewicz, Poenie and Tsien (1985), these indicators are easily loaded into cells either as membrane permeant esters which are subsequently hydrolyzed into free acids by

intracellular esterases or in a more direct fashion by inclusion in microelectrode filling solutions as potassium-salts. Although both fura-2 and indo-1 have been employed frequently in the last few years to assess intracellular [free Ca^{2+}], the homogeneous intracellular distribution of indo-1 once loaded into the cell, compared with the significant compartmentalization of fura-2 into the mitochondria (Steinberg, Bilezikian and Al-Awqati, 1987), suggests that signals obtained with the former compound may be a better indication of time-dependent shifts in myoplasmic Ca^{2+} during contraction. Values calculated from different laboratories with respect to resting intracellular [free Ca^{2+}] are shown in Table 1. Most estimates of this nature have been derived with either fura-2 or indo-1 and show a surprisingly small variation around a resting [free Ca^{2+}] of ~ 100 nM, which is similar to the 90 nM predicted by the pCa-tension curve (Fabiato, 1981). The data indicate that within a given species investigated, no clear correlation exists between the level of resting intracellular [free Ca^{2+}] and extracellular [total Ca^{2+}] or the temperature of the bathing solution. Not surprisingly, resting intracellular [free Ca^{2+}] is appreciably greater in spontaneously beating cells than in non-stimulated preparations. This observation can be explained by the relatively slow time constant calculated for the decay of peak transients following spontaneous depolarization ($t_{1/2} > 100$ ms in Clusin and Lee, 1987; Peeters, Hlady, Bridge and Barry, 1987), and the continual leak of Ca^{2+} which occurs from intracellular stores during rest, which is greater in stimulated than non-stimulated preparations (Gibbons and Fozzard, 1971; Kurihara and Sakai, 1985; Bridge, 1986; Kort and Lakatta, 1988a,b).

Although a considerable amount of experimental evidence supported the postulate that Ca^{2+} was involved in EC-Coupling it was not until 1978 that Allen and Blinks, using aequorin bioluminescence, confirmed that contraction was initiated by the increase of myoplasmic Ca^{2+} . As is the case with estimates of resting intracellular [free Ca^{2+}], the precise concentration of myoplasmic Ca^{2+} reached during contraction within a given species is only modestly dependent on the experimental conditions under which it is assessed. Table 2 illustrates several estimates of peak intracellular [free Ca^{2+}] attained during contraction elicited at various rates of stimulation, extracellular [total Ca^{2+}], and membrane potentials. Despite the expected variability in the peak of

Table 1. Estimates of resting intracellular [free Ca²⁺]

Species	[free Ca ²⁺] _i	[total Ca ²⁺] _o	T	Indicator	Ref.
A. Rat					
	90 nM	2.5 mM	22°C	T-pCa	Fabiato, 1981
	121 nM	2.0 mM	37°C	Quin 2	duBell & Houser, 1989
	134 nM	1.0 mM	35°C	Fura 2	Wier et al, 1987
	100 nM	1.0 mM	35°C	Fura 2	Cannell et al, 1987
	100 nM	2.0 mM	22°C	Fura 2	Nabauer et al, 1989
	81 nM	1.3 mM	37°C	Fura 2	Thomas et al, 1989
	120 nM	1.0 mM	37°C	Fura 2	Li et al, 1989
B. Guinea-pig					
	100 nM	2.0 mM	37°C	Quin 2	duBell and Houser, 1989
	186 nM	2.5 mM	22°C	indo 1	Leblanc and Hume, 1990
C. Ferret					
	58 nM	2.0 mM	37°C	Quin 2	Chapman, 1986
	104 nM	2.0 mM	37°C	NMR ¹⁹ F	Marban et al, 1987
D. Cat					
	57 nM	2.0 mM	37°C	Quin 2	duBell and Houser, 1987
E. Rabbit					
	200 nM	2.0 mM	30°C	indo 1	Lee et al, 1987
F. Chick Embryonic*					
	208 nM	1.8 mM	37°C	indo 1	Clusin and Lee, 1987
	328 nM	1.8 mM	37°C	indo 1	Peeters et al, 1987

[free Ca²⁺]_i, Free resting intracellular [Ca²⁺]_i; [total Ca²⁺]_o, Total extracellular [Ca²⁺]_o; T, Bath temperature (°C); T-pCa, Values extrapolated from the Tension-pCa curve; * End-diastolic [Ca²⁺]_i

the Ca^{2+} transient under these conditions, there is fair agreement regarding the kinetics of the Ca^{2+} transient and its temporal relationship with accompanying action potentials and contraction. In many of the reports cited in the literature, the following characteristics of the Ca^{2+} transient appear to be consistent from one preparation to the next: the rise of [free Ca^{2+}] is (a) initiated by depolarization of the membrane, with a delay of approximately 5-18 ms (Wier, 1980; Cannell, Berlin and Lederer, 1987; Lee and Clusin, 1987), (b) precedes the onset of contraction by ~30 ms (Allen and Blinks, 1978; Peeters et al, 1987; Lee, Smith, Mohabir and Clusin, 1987), (c) peaks around 40 ms after cell activation (Cannell et al, 1987; duBell and Houser, 1989; Thomas, Sass, Tun-Kirchmann and Rubin, 1989), and (d) decays monoexponentially over the course of 100-200 ms (Wier, 1980; Cannell et al, 1987; Lee et al, 1987; Peeters et al, 1987; Thomas et al, 1989; see also Allen and Blinks, 1978). Under relatively controlled experimental conditions, a direct relationship has been described between the peak of the Ca^{2+} transient and the concentration of Ca^{2+} present in the bathing medium (Allen, Eisner, Piolo and Smith, 1984; Orchard and Lakatta, 1985), and Yue, Marban and Wier (1986) have demonstrated a steep relationship between steady-state intracellular [free Ca^{2+}] and the strength of contraction, with a Hill coefficient of >4.0. Other investigators have shown a strong co-dependence of contraction and peak myoplasmic Ca^{2+} on conditioning interval, including depressed contractions associated with premature stimulation (Wier, 1980; Wier and Yue, 1986) and potentiated contractions immediately following the extrasystolic contraction (Wier, 1980; Wier and Yue, 1986), resumption of stimulation after brief periods of rest (Wier, 1980), and alterations in the rate of rhythmic stimulation (Orchard and Lakatta, 1985; Lee and Clusin, 1987). An interesting observation was made by Beuckelmann and Wier (1988; see also Leblanc and Hume, 1990) who showed that the peak magnitude of the Ca^{2+} transient was increased ~2-fold after switching from voltage-clamp to current-clamp mode to allow for the production of normal action potentials. Altering the voltage control of the membrane itself was not responsible for the depressing the peak of the Ca^{2+} transient, as essentially similar observations were made when the membrane was held near the resting membrane potential (-68 mV) in the presence of tetrodotoxin, suggesting that ionic currents in the initial 5-30 ms of the action potential (other than

Table 2. Estimates of peak intracellular [free Ca²⁺]

[free Ca ²⁺] _i	[total Ca ²⁺] _o	V _h	T	F _s	Indicator	Ref.
A. Rat						
2.0 μM	2.0 mM	E _m	37°C	0.2 Hz	Aequorin	(1)
0.8 μM	1.0 mM	-75	35°C	1.0 Hz	Fura 2	(2)
0.6 μM	2.0 mM	-60	22°C	0.2 Hz	Fura 2	(3)
0.3 μM	1.2 mM	E _m	37°C	0.4 Hz	Fura 2	(4)
1.0 μM	1.0 mM	E _m	35°C	0.3 Hz	Fura 2	(5)
0.8 μM	3.0 mM	-70	22°C	0.2 Hz	Fura 2	(6)
B. G-pig						
0.6 μM	2.5 mM	-40	23°C	?	Fura 2	(7)
0.6 μM	2.5 mM	-40	23°C	1.0 Hz	Fura 2	(8)
1.0 μM	2.5 mM	E _m	23°C	1.0 Hz	Fura 2	(8)
0.8 μM	2.0 mM	E _m	22°C	0.3 Hz	indo 1	(9)
0.8 μM	3.0 mM	-70	22°C	0.2 Hz	Fura 2	(6)
1.4 μM	2.5 mM	E _m	22°C	0.5 Hz	indo 1	(10)
1.0 μM	2.5 mM	-40	22°C	0.5 Hz	indo 1	(10)
C. Ferret						
0.3 μM	1.0 mM	E _m	30°C	0.7 Hz	Aequorin	(11)
0.7 μM	3.0 mM	E _m	30°C	0.7 Hz	Aequorin	(11)
1.0 μM	1.0 mM	E _m	30°C	0.5 Hz	Aequorin	(12)
1.5 μM	1.0 mM	E _m	30°C	1.5 Hz	Aequorin	(13)
0.3 μM	2.0 mM	E _m	30°C	1.0 Hz	¹⁹ F NMR	(14)
D. Frog						
2.0 μM	1.8 mM	E _m	21°C	0.5 Hz	Aequorin	(15)
E. Chick Embryonic*						
0.8 μM	1.8 mM	E _m	37°C	~2.0 Hz	indo 1	(16)
0.6 μM	2.0 mM	E _m	37°C	~2.0 Hz	indo 1	(17)

[free Ca²⁺]_i, Free intracellular [Ca²⁺] reached during contraction; [total Ca²⁺]_o, Total extracellular [Ca²⁺]; V_h, Holding potential during voltage-clamp (mV) or *in vivo* resting membrane potential (E_m); T, Bath temperature (°C); F_s, Frequency of stimulation (Hz); *, Spontaneous activity

(1) Orchard and Lakatta, 1985; (2) Cannell et al, 1987; (3) Nabauer et al, 1989; (4) Thomas et al, 1989; (5) Li et al, 1989; (6) Callewaert et al, 1989; (7) Barceñas-Ruiz et al, 1987; (8) Beukelmann and Wier, 1988; (9) Bers et al, 1989; (10) Leblanc and Hume, 1990; (11) Yue, 1986; (12) Wier, 1986; (13) Yue, 1987; (14) Marban et al, 1987; (15) Allen and Blinks, 1978; (16) Peeters et al, 1987; (17) Lee et al, 1987.

those flowing through voltage-gated Ca^{2+} channels) may be involved in the release of Ca^{2+} from intracellular stores.

The significant delay preceding the rise of myoplasmic Ca^{2+} following the upstroke of the action potential and its temporal relation to contraction raise the issue of where the Ca^{2+} that activates the myofilaments originates from. Compartmentalization of Ca^{2+} into distinct pools located either inside or outside of the cell membrane has been frequently incorporated into models or working hypotheses of EC-coupling (Koch-Weser and Blinks, 1963; Wood, Hepner and Weidmann, 1969; Morad and Goldman, 1973; Langer, 1982; Wohlfart and Noble, 1982; Chapman, 1983; Schouten, van Deen, de Tombe and Verveen, 1987; Boyett, Hart, Levi and Roberts, 1987). This has been done in an attempt to quantify the degree of interaction between different sources of Ca^{2+} and their possible contribution to contraction, and to a large extent was necessitated by the observation that a change in the duration or height of the plateau phase of one action potential has an inotropic effect predominantly on the following contraction and not on the concomitant contraction (Wood, Hepner and Weidmann, 1969; Antoni, Jacob and Kaufmann, 1969; Sumner, 1970; Morad and Cleeman, 1987).

1.1 Trans-sarcolemmal Ca^{2+} Entry During Contraction

Evidence for the direct activation of the myofilaments due to Ca^{2+} entry across the sarcolemma stems directly from Ringer's observation that without the presence of Ca^{2+} ions in the perfusion solution, no contraction can be elicited. This has been confirmed recently in isolated rat, rabbit, and guinea-pig ventricular myocytes, where an abrupt (<50 ms) removal of Ca^{2+} from the perfusate has been found to result in an immediate termination of either cell shortening (Rich, Langer and Klassen, 1988) or force production (Sheperd and Fisher, 1990).

Calculations performed to determine the amount of Ca^{2+} entering the cell during the action potential and the amount of Ca^{2+} required to directly activate the myofilaments constitute the strongest evidence in support of the proposal that trans-sarcolemmal Ca^{2+} entry itself is

responsible for the initiation of contraction. Toward this end, time-dependent changes of extracellular [total Ca^{2+}] have been used to infer the change of intracellular [total Ca^{2+}] during the course of both single excitations and those averaged over the course of several beats. Net transient depletions of extracellular [total Ca^{2+}] have been recorded with double-barreled Ca^{2+} -sensitive microelectrodes (Bers, 1983), the Ca^{2+} -sensitive absorption dyes antipyrilazo III (Hilgemann, Delay and Langer, 1984; Cleeman, Pizarro and Morad, 1984), and tetramethylmurexide (Hilgemann et al, 1983; Hilgemann, 1986a,b), and in a more direct fashion using various voltage-clamp techniques on multicellular ventricular preparations as well as freshly dispersed ventricular myocytes (New and Trautwein, 1972; Isenberg and Klockner, 1982; Marban and Tsien, 1981; Isenberg, 1982; London and Krueger, 1986; Egan, Noble, Noble, Powell, Spitzer and Twist, 1989; see also Fabiato, 1983; Bridge, Smolley and Spitzer, 1990). Estimates of net cellular Ca^{2+} movements obtained with the three different approaches in a variety of preparations have been provided in Table 3. The task of normalizing values obtained with various methods to a common reference point is made technically difficult by the rather wide variation in experimental conditions used by different investigators. Moreover, most of the values illustrated in Table 3 have been calculated as a function of transient alterations of extracellular [total Ca^{2+}] for a given stimulation sequence. Bers (1983) has demonstrated that a linear relationship does not exist between extracellular [total Ca^{2+}] and net quantitative depletion of Ca^{2+} from the extracellular space, thus excluding direct extrapolation of values obtained with varying concentrations of extracellular Ca^{2+} . Following normalization to intracellular [total Ca^{2+}] per liter of cell water accessible to Ca^{2+} , Fabiato (1983) has shown that the amount of Ca^{2+} necessary to activate the myofilaments is increased by ~ 1.3 orders of magnitude following compensation for Ca^{2+} binding to numerous ligands present within the myoplasm under physiological conditions. A computer program has been developed (Fabiato and Fabiato, 1979; see also Fabiato, 1983 for corrections to this program) which has allowed the determination of intracellular [free Ca^{2+}] from the total concentration of Ca^{2+} present within the cytosolic compartment at increments of 0.01 pCa.

Table 3. Estimates of Ca^{2+} entry during the action potential

net flux/beat	[total Ca^{2+}] _o	Method	Ref.
A. Rabbit V.			
4.66 μM	0.3 mM	ME	Bers, 1983
17.0 μM	0.5 mM	ME	Bers, 1983
45.0 μM	2.0 mM	ME	Bers, 1983
11.6 $\mu\text{M}^{-\text{kg}}$	2.0 mM	ME	Bers, 1983*
27.6 μM^{-1}	2.0 mM	ME	Bers, 1983*
B. Guinea-pig V.			
10.0 μM^{-100}	2.5 mM	SCVC	London & Krueger, 1986
60.0 μM^{-2000}	2.7 mM	SCVC	Bridge et al, 1990
C. Rat V.			
7.0 μM^{-10}	3.6 mM	SCVC	Isenberg, 1980
10.0 μM^{-20}	3.6 mM	SCVC	Isenberg, 1980
D. Cat V.			
0.5-5 μM^{-100}	1.8 mM	MCVC	New & Trautwein, 1972
E. Frog V.			
5-15 μM	0.2 mM	AP III	Cleeman et al, 1984
6.5 μM^{-1}	1.0 mM	ME	Dresdner et al, 1985
F. Rabbit A.			
0.6 $\mu\text{M}^{-\text{kg}}$	0.25 mM	TMM	Hilgemann, 1986
2.5 $\mu\text{M}^{-\text{kg}}$	1.0 mM	TMM	Hilgemann, 1986
G. Guinea-pig A.			
20 $\mu\text{M}^{-\text{kg}}$	1.0 mM	TMM	Hilgemann et al, 1984
H. Bovine Purkinje fibre			
0.7 μM^{-10}	?	MCVC	Marban & Tsien, 1981*
1.0 μM^{-20}	?	MCVC	Marban & Tsien, 1981*
25 μM^{-100}	?	SCVC	Isenberg & Klockner, 1980

$\mu\text{M}^{-\text{kg}}$, net flux/kg wet weight; μM^{-1} , net flux/litre cell water accessible to Ca^{2+} diffusion; μM^{-10} , net flux/x-ms integration of i_{Ca} ; ME, Ca^{2+} -sensitive microelectrode; MCVC, multicellular voltage-clamp; SCVC, single cell voltage-clamp; AP III, antipyrilazo III; TMM, tetramethylmurexide; *, values calculated by Fabiato, 1983.

In an earlier report, Solaro, Wise, Shiner and Briggs (1974) defined the requirements for purified canine cardiac myofibrils to be activated directly by alterations of intracellular [total Ca^{2+}]. The intracellular [free Ca^{2+}] required for 20% of maximal tension was calculated to be 1.15 μM , with 95% maximal activation of the myofilaments at 9.0 μM intracellular [free Ca^{2+}]. Similar results were obtained when the intracellular [free Ca^{2+}]-dependence of myofibrillar ATPase activity was determined. Calcium binding to "metabolism-independent" intracellular ligands was not compensated for in this study, and the authors acknowledged that previous estimates of Ca^{2+} requirements for direct activation of the myofilaments were generally high, with values ranging from 50-100 $\mu\text{M}/\text{kg}$ wet wt. reported in previous literature (Gertz et al, 1967; Katz, 1970). Following consideration for the ability of numerous intracellular buffers to bind Ca^{2+} based on their apparent stability constants, Fabiato (1983) has recalculated the pCa required to produce 20% maximal twitch force to be equal to 5.91, or $\sim 2 \mu\text{M}$ [free Ca^{2+}]. With regards to the ability of trans-sarcolemmal Ca^{2+} to directly stimulate contraction, measurements of extracellular Ca^{2+} depletions from the experiments of Bers (Bers, 1983; 1987; Bers and MacLeod, 1986) are useful to compare against the calculations of Fabiato (1983). This is because the estimated extracellular [total Ca^{2+}] depletions are in the higher range of those calculated with similar techniques (Hilgemann et al, 1983; Morad and Cleeman, 1987; Hilgemann, 1986a,b), and the previous demonstration that contractile strength in the rabbit is only modestly dependent on the magnitude of Ca^{2+} release from intracellular stores (Fabiato and Fabiato, 1978; 1982; Sutko and Willerson, 1980; Kort and Lakatta, 1984; Bers, 1985; 1989; Horackova, 1986). The amount of Ca^{2+} calculated to enter the cell during 1.0 Hz stimulation was 11 $\mu\text{M}/\text{kg}$ wet wt. for muscles bathed in 2.0 mM extracellular [total Ca^{2+}], which gives an increase of intracellular [total Ca^{2+}] of 27.6 $\mu\text{M}/\text{litre}$ cell water. Added to the estimate of resting [total Ca^{2+}] of pCa 7.0 yields a pCa of 6.35 (0.35 μM), which is insufficient to activate the myofilaments directly (pCa of 5.91 is required for 20% maximal activation). For guinea-pig atrial muscle bathed in 1.0 mM extracellular [total Ca^{2+}] (0.5 mM [free Ca^{2+}]), Hilgemann et al (1984) have estimated a net cell gain of Ca^{2+} per contraction to be $\sim 20 \mu\text{M}$ [total Ca^{2+}]/kg wet wt. The largest net Ca^{2+} fluxes in this study were demonstrated following

depletion of intracellular Ca^{2+} stores by ryanodine, and hence in the absence of appreciable release of Ca^{2+} from the sarcoplasmic reticulum (SR). Due to the inhibition of intracellular Ca^{2+} release, it has been suggested that this result may more accurately represent the ability of trans-sarcolemmal Ca^{2+} entry to directly activate contraction (Hilgemann et al, 1984), than conditions in which intracellular release mechanisms are intact. This is consistent with the demonstration by Morgan and Blinks (1982) that the requirement of the myofilaments for Ca^{2+} , assessed by the amplitude of the Ca^{2+} transient during contraction, depends to a large extent on the rate of uptake of Ca^{2+} by the SR. Further support for this conclusion is provided by the observation that application of ryanodine to a number of different cell types either has no significant effect on peak Ca^{2+} current (Mitchell, Powell, Terrar and Twist, 1985; Boyett, Kirby and Orchard, 1988) or modestly reduces it (Tseng, 1988; Giles and Shimoni, 1989; see also, Cohen and Lederer, 1988). Using antipyrilazo III to monitor changes in extracellular [total Ca^{2+}], Morad and Cleeman (1987), have also failed to demonstrate a direct relationship between the strength of contraction and either the magnitude of extracellular Ca^{2+} depletions or the entry of Ca^{2+} through voltage-gated Ca^{2+} channels during alterations in the rhythm of stimulation in isolated cat ventricular muscle. In similar preparations of frog ventricle, which have poorly developed longitudinal and junctional SR (Sommer and Johnson, 1970), alterations of conditioning interval produced parallel effects on the force of contraction, peak Ca^{2+} current, and magnitude of extracellular Ca^{2+} depletion. An interesting observation made in this study was that maximal depletion signals were ~6-fold greater in strips of frog ventricle ($12 \mu\text{M}$ [total Ca^{2+}]/contraction), than equivalent depletions demonstrated in cat ventricular muscle ($2 \mu\text{M}$ [total Ca^{2+}]/contraction). These results led the authors to conclude that although Ca^{2+} entry through voltage-gated channels played an important role in the activation of contraction in mammalian and amphibian ventricular muscle, species with extensively developed SR appeared to depend more on intracellular Ca^{2+} release than trans-sarcolemmal Ca^{2+} entry for direct myofilament activation.

1.2 Ca^{2+} Release From Intracellular Stores

Evidence from voltage-clamp studies: One of the first direct indications that Ca^{2+} located within an intracellular compartment was involved in force production came from the pioneering voltage-clamp studies of Wood, Hepner, and Weidmann (1969). Although other electrophysiological (Kavalier, 1959; Morad and Trautwein, 1968; Antoni, Jacob and Kaufmann, 1969) and mechanical (Braveny and Kruta, 1958; Koch-Weser and Blinks, 1963) investigations provided strong evidence in support of a "memory" process linking the strength of a given contraction with the residual effects of preceding membrane depolarization or contraction amplitude, this was the first demonstration that such events took place *within* a given contraction cycle. Two different approaches were used to determine the relationship between the degree of Ca^{2+} influx during the action potential and the strength of contraction. Using a single sucrose-gap voltage-clamp technique, injection of a constant depolarizing current into isolated segments of sheep or calf ventricular muscle produced a biphasic contracture which relaxed fully only upon repolarization back to the resting membrane potential. Although not recognized widely as such at the time, the preferential voltage-sensitivity of the second phase of contracture may represent one of the first systematic demonstrations of contractile activity resulting from an electrogenic $\text{Na}^+-\text{Ca}^{2+}$ exchange process in cardiac muscle. Following termination of maximal contracture, the resumption of rhythmic stimulation led to contractions which were potentiated to the same extent as that produced with paired pulse or high frequency stimulation. Action potentials recorded during this time displayed depressed, rather than enhanced, plateau phases and were in general prolonged. The second approach involved injection of subthreshold depolarizing or hyperpolarizing currents during the plateau phase of the action potential. Application of a 50 ms pulse of $\sim 2 \mu\text{A}$ increased the plateau height and markedly prolonged the duration of the action potential. Despite the immediate effects of the current pulse on the plateau phase, minimal effects were noted on accompanying contractions, suggesting that the additional amount of Ca^{2+} entering the cell was not available for activation of the myofilaments. Continued application of

current pulses during rhythmic stimulation potentiated successive contractions while the duration of the action potential was slightly diminished to a new steady-state level. Opposite contractile and electrophysiological effects were demonstrated following the application of 2 μ A hyperpolarizing currents. These results led the authors to conclude that the strength of contraction was governed primarily by the amount of presystolic intracellular Ca^{2+} bound to various storage sites. The data also indicated that the amount of Ca^{2+} present within this intracellular storage compartment was positively correlated with the height and duration of the action potential plateau, and negatively correlated with the intersystolic interval of preceding contractions. The data from this study also helped to explain many of the interval-dependent contractile phenomena reported to date, including post-extrasystolic potentiation (Hoffman, Bindler and Suckling, 1956), postrest potentiation (Rosen and Farah, 1955; Braveny and Kruta, 1958), frequency potentiation (Bowditch, 1871; Kruta, 1937; Teiger and Farah, 1967) as well as the positive and negative inotropic effects of activation described by Koch-Weser and Blinks (1961; 1963), and were consistent with the demonstration two years earlier by Reuter (1967) that the slow inward current observed during the plateau phase of the action potential represented Ca^{2+} entry across the sarcolemma.

Simultaneous measurements of Ca^{2+} -dependent membrane currents (henceforth referred to as I_{Ca}) and contraction were first reported by Beeler and Reuter (1970), who demonstrated that both membrane potential and Ca^{2+} entry during I_{Ca} mediated contractile force production in isolated canine ventricular muscle. Evidence for the role of intracellular stores was obtained by altering the interval between applied stimuli or by altering the holding potential to which the membrane was clamped between contractions. A range of holding potentials was identified (-70 to -50 mV) at which the membrane could be held that did not influence peak I_{Ca} in response to a constant depolarizing pulse, but which markedly depressed subsequent contractions. Interpolation of a rest period between rhythmic stimuli was found to significantly reduce the amplitude of the first postrest contraction while only slightly reducing the degree of Ca^{2+} influx across the membrane, suggesting that I_{Ca} must flow into the cell *before* appreciable contraction could be elicited (e.g., loading of an internal store). Similar results were demonstrated in isolated cat ventricular muscle

by New and Trautwein (1972) who showed that steady-state contraction was re-established over the course of 6-8 beats after a brief period of rest, and that the positive staircase took place in the absence of changes in time to peak tension or peak I_{Ca} . By calculating the net charge (Q) transfer during I_{Ca} (integrating the area enclosed by the inward current) the authors estimated net Ca^{2+} influx to be between 0.5 and 5 μM /litre fibre volume per contraction in the presence of 1.8 mM extracellular [total Ca^{2+}]. While direct activation of the myofilaments by Ca^{2+} entering the cell during the action potential could not be totally ruled out in these experiments, the results of this study were more compatible with an ability of the inward current to fill an intracellular store for later release, and it was tentatively proposed that the transient peak of the inward current appeared to be acting as a trigger for release of this Ca^{2+} from intracellular binding sites. This hypothesis was extended by Bassingthwaite and Reuter (1972) who proposed a model of EC-Coupling in which Ca^{2+} entry through voltage-gated channels served to depolarize the membrane of the lateral cisternae and elicit a regenerative release of Ca^{2+} from the SR.

The relation between I_{Ca} and contraction has also been investigated with light-sensitive Ca^{2+} channel blockers. Morad and Cleeman (1987) were able to demonstrate that rapid photoinactivation of a light-sensitive form of nifedipine produced changes in the strength of contraction which were not accompanied by similar alterations in the magnitude of Ca^{2+} entry into the cell. In frog ventricular muscle, a brief pulse of light led to an immediate increase in the plateau height and duration of the action potential which was accompanied by a similar increase in the size of contraction. Repetition of this process in cat ventricular muscle led to a prompt increase of plateau height, which subsequently diminished to an intermediary value as a new steady-state was established. Voltage-clamp studies with an identical stimulation protocol indicated that changes in action potential configuration were associated with similar directional changes of peak I_{Ca} . Despite the biphasic changes in the amount of Ca^{2+} entering the cell, recovery of contraction occurred in a monoexponential fashion over the course of 6-8 beats, consistent with the idea that the strength of contraction was governed primarily by an intermediate pool of Ca^{2+} .

The minimum Ca^{2+} influx during the flow of I_{Ca} required for direct activation of the myofilaments has been calculated in voltage-clamped internally perfused guinea-pig ventricular myocytes (London and Kreuger, 1986). It was estimated that raising intracellular [free Ca^{2+}] from pCa 7.0 to pCa 6.0 would require ~ 0.26 mM [free Ca^{2+}] to enter the cell, which would be equivalent to a sustained inward current of 15 nA for 100 ms. Although this requirement was not met in their experiments (e.g., the authors calculated a 10 μM increase of [free Ca^{2+}] per 100 ms integration time), Isenberg (1982) has estimated that an increase of intracellular [total Ca^{2+}] of 25 μM would be expected during the initial 100 ms of the Ca^{2+} current in bovine ventricular myocytes, which peaked at ~ 10 nA. Ignoring the likely possibility that clamp durations in excess of ~ 50 ms do not contribute to the transient peak of myoplasmic Ca^{2+} during contraction (Morad and Trautwein, 1968; Beeler and Reuter, 1970; Gibbons and Fozzard, 1971; Cannell, Berlin and Lederer, 1987; Beuckelmann and Wier, 1989; duBell and Houser, 1989), Fabiato (1983) has calculated that when added to the resting intracellular [free Ca^{2+}] even this unusually large amplitude current is not capable of providing enough Ca^{2+} for direct myofilament activation.

A point worth noting is that I_{Ca} is often presumed to flow across the cell surface area (e.g., the capacitance) of the cell membrane in a relatively uniform fashion (Brown and Yatani, 1986). This type of assumption is most evident for quantitative estimates of trans-sarcolemmal Ca^{2+} entry made from either whole cell voltage-clamp recordings or measurements of time-dependent shifts of extracellular [total Ca^{2+}] such as those discussed above. A potential source of error in such calculations is that while Ca^{2+} entering the cell during depolarization may have the opportunity to interact with the myofilaments to some degree at various points on the longitudinal axis of the muscle (depending on the geometry of the cell and presence of intracellular buffers), Ca^{2+} release from the SR occurs within a very limited compartment near the sarcolemma. In this respect, the tendency to under or over-estimate the contribution of either source to contraction may depend rather strongly on the discrete distribution of channels on the surface membrane, and whether or not the myofilaments could in fact be activated at sites proximal or distal to the t-tubule. The ability of certain dihydropyridine Ca^{2+} channel agonists to simultaneously mediate the gating properties

of voltage-gated Ca^{2+} channels (Hess, Lansman and Tsien, 1984; Brown, Kunze and Yatani, 1984; Thomas, Chung and Cohen, 1985) and SR function (Bose, Kobayashi, Bouchard, Hryshko, 1988*b*; Hryshko, Bouchard, Chau and Bose, 1989; Bouchard, Hryshko, Saha and Bose, 1989; Saxon and Gainullin, 1990) suggests that this may in fact be a valid concern. Furthermore, recent reports of site specific binding of both dihydropyridine compounds (Valdivia and Coronado, 1989) and ryanodine (Pessah et al, 1985; Smith et al, 1985; Inui et al, 1987; Lai et al, 1987) at the junction of the SR with the sarcolemma at the t-tubule suggests that Ca^{2+} may indeed be released into the diffusion-restricted volume t-tubule of the from the SR in response to the opening of a substantial fraction of the total number of channels present on the surface membrane. If so, then calculations of time-dependent changes of [free Ca^{2+}] necessary to elicit release from the SR may be somewhat overestimated in intact ventricular preparations compared with similar computations made for skinned cardiac cells.

Evidence from skinned cardiac fibres: Of the experimental data supporting the involvement of Ca^{2+} release from intracellular stores in the regulation of contraction, some of the most compelling evidence comes from experiments performed on skinned cardiac fibres in which the sarcolemma has been removed by microdissection. Fabiato and Fabiato (1972; 1975) first proposed the phenomenon of Ca^{2+} -induced release of Ca^{2+} in mammalian cardiac muscle as a potential link between membrane excitation and contraction. It was shown that microinjection of small amounts of bulk [free Ca^{2+}] around the skinned preparation induced a release of Ca^{2+} from storage sites within the SR, which subsequently resulted in contraction. Most of the initial studies of this nature were performed on fragments of single cardiac cells and were based, in part, on the results of previous electrophysiological studies which suggested that Ca^{2+} influx during the flow of I_{Ca} was insufficient to directly activate the myofilaments (Morad and Trautwein, 1968; Beeler and Reuter, 1970; New and Trautwein, 1972; Bassingthwaite and Reuter, 1972). The removal of the sarcolemma surrounding the myofibrils permitted direct application of varying amounts of [free Ca^{2+}] into the intracellular space, which was buffered with ethyleneglycol-bis(*B* aminoethyl ester)

N,N'-tetraacetic acid (EGTA). In the absence of electrical stimulation, cyclic contractions developed rapidly following an increase of [free Ca^{2+}] in the perfusion solution to levels which were demonstrated after destruction of the SR with detergent to be significantly less than those required to directly activate the myofibrils. Cyclic contractions induced in this manner were attributed to a regenerative release of Ca^{2+} from internal stores, in accordance with the earlier proposal by Ford and Podolsky (1972) that release of Ca^{2+} from focal regions of the SR not only initiated contraction, but also triggered a regenerative release of Ca^{2+} from adjacent regions of the membrane via electrotonic conduction. This was supported by the previous demonstration (Fabiato and Fabiato, 1972) that contractions triggered by the release of Ca^{2+} from the SR were propagated at a velocity of 50-100 $\mu\text{m}/\text{sec}$ in pluricellular fragments of cardiac cells with disrupted membranes. A second important observation made in these experiments was that the release of Ca^{2+} from the SR was graded, and did not occur in an all or none fashion as predicted by a simple regenerative process controlled primarily by a positive feedback mechanism (Costantin and Taylor, 1973). Evidence in support of this proposal came from the demonstration that the frequency of cyclic contractions could be altered by increasing the [free Ca^{2+}] trigger for release, or the interval-dependence of contractions elicited by the application of 2 mM caffeine (e.g., alterations in the level of preload of the SR with Ca^{2+}). The results of these experiments led the authors to conclude that Ca^{2+} -induced release of Ca^{2+} from the SR served to significantly amplify trans-sarcolemmal Ca^{2+} influx during the action potential which would, in turn, provide the direct stimulus for contraction. The data also provided the foundation for the postulate which would become known as the "calcium-induced release of calcium hypothesis", which holds that Ca^{2+} influx during the action potential does not directly activate the myofilaments, but rather, induces a release of Ca^{2+} from the SR which itself is responsible for contraction. Stated in another way, no trans-sarcolemmal Ca^{2+} influx of any magnitude can directly activate the myofilaments without first triggering Ca^{2+} release from the SR. Work from other laboratories at the time had indicated that rapid application of caffeine (Endo, Tanaka and Ogawa, 1970), or Ca^{2+} (Ford and Podolsky, 1970; Endo and Blinks, 1973) to skinned Ca^{2+} -loaded skeletal muscle cells also produced contractions

inferred to result from a possible release of Ca^{2+} from internal stores. However, due to the "unphysiologically high" concentration of [free Ca^{2+}] necessary to elicit the release of Ca^{2+} from intracellular site(s) (Endo and Blinks, 1973) and the growing evidence in support of a charge-coupled release mechanism (Schneider, 1970; Schneider and Chandler, 1973; Costantin and Taylor, 1973) a role for Ca^{2+} -induced release of Ca^{2+} was deemed uncertain in skeletal muscle EC-Coupling.

Subsequent experiments performed on a wide variety of preparations of isolated cardiac muscle have demonstrated convincingly that Ca^{2+} -induced release of Ca^{2+} occurs within the range of physiological [free Ca^{2+}] and [free Mg^{2+}], even after compensation for a wide variety of intracellular Ca^{2+} binding ligands (Fabiato and Fabiato, 1978; 1979; Fabiato, 1983). Experiments performed in the presence of inhibitors of mitochondrial Ca^{2+} metabolism have ruled out the contribution of Ca^{2+} released from this source to the Ca^{2+} -induced release and accumulation in the presence of physiological concentrations of Ca^{2+} and Mg^{2+} (Fabiato and Fabiato, 1975; 1979; Fabiato, 1982; see also Kitazawa, 1984). The process of Ca^{2+} -induced release of Ca^{2+} has been demonstrated in cardiac muscle obtained from most mammalian and avian species (Fabiato, 1982), although it is absent in amphibian species which possess very little or no SR. Through the use of fluorescent Ca^{2+} indicators (Fabiato and Fabiato, 1979; Fabiato, 1981; 1982; 1985a-c) it has been established that the trigger for the release of Ca^{2+} from SR stores is proportional to the *rate of change* of [free Ca^{2+}] outside the release compartment of the SR, and not simply on the absolute magnitude of this change. In this respect, it was shown that application of a given [free Ca^{2+}] could either elicit a release of Ca^{2+} from the SR or load the SR with Ca^{2+} for later release, depending on the speed of application. Other experiments indicated that a negative, rather than a positive, feedback mechanism existed between the level of [free Ca^{2+}] and the excitability of the putative release channel of the SR, e.g., an optimal [free Ca^{2+}] trigger was identified under conditions of constant intracellular Ca^{2+} loading. Microinjection of a [free Ca^{2+}] greater than that found to be optimal within a given cell reduced, rather than augmented, the amplitude of the evoked contraction, thus eliminating the possibility that the trigger Ca^{2+} was directly responsible

for contraction. Moreover, a second injection of supraoptimal [free Ca^{2+}] during the ascending phase of the Ca^{2+} transient was found to depress the amplitude of contraction due to inactivation of the release process. More recent studies utilizing photolabile forms of "caged- Ca^{2+} " have provided important evidence in support of the ability of intracellular Ca^{2+} to activate release from an intracellular compartment in intact cells (Nabauer, Ellis-Davis, Kaplan and Morad, 1989; Valdeomillos, O'Neill, Smith and Eisner, 1989; Nabauer and Morad, 1990), although some aspects of the data appear to be somewhat inconsistent with the idea that intracellular Ca^{2+} plays a major role in the inactivation of the Ca^{2+} -release mechanism (Nabauer and Morad, 1990; see also Fabiato, 1989).

The importance of these findings with respect to the control of cardiac function *in situ* is demonstrated clearly by the fact that changes in the rate and rhythm of stimulation have been reported to induce profound changes in the normal electrocardiogram (Attwell, Cohen and Eisner, 1981; Watanabe, Rauthaharju and McDonald, 1985; Nademane, Stevenson, Weiss, Frame, Antimisiaris, Suithichaiyakul and Pruitt, 1990), magnitude of trans-sarcolemmal Ca^{2+} entry during the action potential plateau (Payet, Schanne and Ruiz-Ceretti, 1981; Keung and Aronson, 1981; Watanabe, Delbridge, Bustamante and McDonald, 1983; Schouten and terKeurs, 1986; Hiraoka and Kawano, 1987) and the gating properties of the channels responsible for alterations of membrane-specific Ca^{2+} conductance (Gibbons and Fozzard, 1975; Noble and Shimoni, 1981; Hiraoka and Kawano, 1987; Escande, Coulombe, Faivre and Couraboeuf, 1986; Schouten and Morad, 1989). Although many uncertainties still exist with respect to the relative participation of Ca^{2+} release from intracellular stores and that entering the cell during the action potential in both the activation and maintenance of contraction, it is likely that these should be treated as orders of magnitude only. The sensitivity of contraction in various species to pharmacological agents which perturb the ability of the SR to sequester and retain its load of Ca^{2+} during diastole (Sutko and Willerson, 1980; Stern et al, 1983; Marban and Wier, 1985; Bers, Bridge and MacLeod, 1987; Bose, King, Hryshko and Chau, 1988; Rousseau, Smith and Meissner, 1987; Rousseau and Meissner, 1989; Kort and Lakatta, 1988b) and the frequency-dependent alterations of contraction observed in

solutions with varying ionic composition (Gibbons and Fozzard, 1971; Langer, 1979; 1982; Orchard and Lakatta, 1985; Kurihara and Sakai, 1985; Boyett, Hart, Levi and Roberts, 1987; Rich et al, 1989) strongly support the involvement of both sources in providing Ca^{2+} for myofilament activation under different stimulus conditions. Fabiato wrote in 1983, that "the most favourable appraisal of the hypothesis of Ca^{2+} -induced release of Ca^{2+} is that there was no compelling evidence against it". Mention was made of the fact that, despite the lack of such evidence, unequivocal acceptance of the hypothesis was still lacking. While scattered reports have indeed appeared that oppose certain aspects of the hypothesis (an excellent analogy may be drawn from the work of Freud), it is generally held that Ca^{2+} released from the SR in response to an action potential plays a significant role not only in the activation of contraction in most mammalian species, but also in many of the interval-dependent contractile phenomenon which have been described in the last 100 years.

1.3 Ca^{2+} Located Near the Inner Surface of the Sarcolemma

Evidence for an electrogenic Na^+ - Ca^{2+} exchange: Trans-membrane exchange of Na^+ for Ca^{2+} has been demonstrated to mediate stimulus-dependent alterations of intracellular [free Ca^{2+}] in many cell types, including nerve, cardiac muscle, smooth muscle, epithelial, and retinal photoreceptors. The need for a mechanism to extrude Ca^{2+} out of the cell in the absence of significant energy expenditure was recognized once it became clear that resting [free Ca^{2+}] in cardiac muscle was a small fraction of that required for thermodynamic equilibrium (Niedergerke, 1963) and was increased considerably during repetitive stimulation (Winegrad and Shanes, 1962; Langer and Brady, 1963). Reuter and Seitz (1968) subsequently reported on a mechanism by which Ca^{2+} was extruded from several different preparations of cardiac muscle in a manner which required a very low activation energy ($Q_{10} = 1.35$) and was greatly dependent on the concentration of Na^+ and Ca^{2+} ions in the bathing medium. By following the decay of ^{45}Ca radioactivity in rinsing solutions with varying ionic composition they were able to show that Ca^{2+} efflux depended on the ratio of $[\text{Ca}^{2+}]_o/[\text{Na}^+]_o^2$ and that the affinity of the "activation site" was greater for Ca^{2+} than for Na^+ . A second important finding in this study was that reducing $[\text{Na}^+]_o$ by equimolar substitution with lithium or sucrose resulted in a net accumulation of Ca^{2+} inside the preparation. Although the authors described an *electroneutral* exchange process on the basis of observed tracer kinetics, subsequent biochemical and electrophysiological experiments have provided strong evidence in support of an electrogenic process, with an exchange stoichiometry of approximately $3\text{Na}^+ : 1\text{Ca}^{2+}$ (Lederer and Tsien, 1979; Reeves and Hale, 1984; Kimura, Noma and Irisawa, 1986; Mechmann and Pott, 1986; Hume and Uehara, 1986a,b; Barcenaz-Ruiz, Beuckelmann and Wier, 1987; Beuckelmann and Wier, 1989; Miura and Kimura, 1989; Bridge, Spitzer and Ershler, 1990). This jump in logic did not happen all at once however, as the stoichiometry and electrogenicity of the exchanger were the subject of much speculation and debate (e.g., Reuter, 1974; Langer, 1982; Eisner and Lederer, 1985; Sheu and Blaustein, 1986).

Based primarily on results of experiments conducted on cardiac muscle (Luttgau and Niedergerke, 1958; Reuter and Seitz, 1968; Langer and Brady, 1968; Glitsch, Reuter and Scholz, 1970, see also Baker, Blaustein, Manil and Steinhardt, 1967), Reuter (1974) argued that exchange of Na^+ for Ca^{2+} across the sarcolemma was an electroneutral process. Cellular Ca^{2+} extrusion was based exclusively in this model on the electrochemical Na^+ gradient, and consequently could be described by the equilibrium equation (Eq. 1.0): $[\text{Ca}^{2+}]_i/[\text{Ca}^{2+}]_o = ([\text{Na}^+]_i/[\text{Na}^+]_o)^2$. This distribution ratio predicted that with equal affinities on either side of the membrane for Na^+ and Ca^{2+} , any perturbation of the Na^+ gradient would subsequently affect the cellular Ca^{2+} gradient. However, assuming a cellular Na^+ gradient of 1:10 this relationship predicts that with an external [free Ca^{2+}] of 1×10^{-3} M the intracellular [free Ca^{2+}] must be $\sim 1 \times 10^{-5}$ M. As it became evident that resting myoplasmic [free Ca^{2+}] was less than the predicted value by at least 2 orders of magnitude, it was recognized by Reuter that for this equation to successfully describe the experimental results either a second energy source in addition to the Na^+ gradient had to be postulated, or a third intracellular compartment close to the membrane where the resting [free Ca^{2+}] was much higher than in the bulk of the myoplasm. It is of more than passing interest that although the electrogenicity of the exchanger has been confirmed in several laboratories since that time with a wide variety of techniques, the experimental data gathered to date are consistent with the location of an incomplete barrier to diffusion close to the membranes of the sarcolemma and SR and yet in direct communication with the myofilaments, as commented on by Lederer, Niggli, and Hadley (1990).

The experiments of Benninger, Einwachter, Haas and Kern (1976) and Horackova and Vassort (1979), both employing sucrose-gap voltage-clamp techniques, demonstrated that Na^+ - Ca^{2+} exchange in frog cardiac muscle was both voltage-dependent and could generate a significant membrane potential. The results of Benninger et al. (1976) led them to conclude that their data were consistent with a voltage-dependent electroneutral carrier system. However, to adequately describe the stimulation-dependent changes of intracellular [free Ca^{2+}] inferred from tension recordings, it was necessary to postulate that the exchanger would have to operate close to

equilibrium at all potentials tested. As discussed by Sheu and Blaustein (1986), voltage-dependent electroneutrality can be maintained in the presence of large shifts in the electrochemical gradients for Na^+ and Ca^{2+} only if the rate-limiting chemical reaction were purely voltage-dependent. As will be discussed below, characterization of the kinetic properties of the exchanger has indicated that the ratio of modal activity with respect to the direction of exchange is strongly influenced by differing affinities of the exchange protein for Na^+ and Ca^{2+} on opposite sides of the membrane (Miura and Kimura, 1989). Horackova and Vassort (1979) were amongst the first to demonstrate a $[\text{Na}^+]_o$ - and voltage-dependent sarcolemmal exchange process with an exchange stoichiometry of >3 which could account for changes in both tonic tension and membrane conductance during voltage-clamping of the membrane to positive potentials.

Demonstration of the voltage-sensitivity of the exchanger has had important implications for trans-membrane ion fluxes during the action potential, as the direction in which the exchange process predominantly moves Ca^{2+} depends critically on the stoichiometry of exchange and the energy of the Na^+ electrochemical gradient (Mullins, 1979; 1981; Noble, 1984, 1986; DiFrancesco and Noble, 1985), the latter of which is significantly altered during the course of an action potential. Making a number of simplifying assumptions regarding the affinity of the exchanger for Na^+ and Ca^{2+} , Sheu and Fozzard (1982) have investigated the interrelationship between tension and the electrochemical gradients for Na^+ (\tilde{u}_{Na}) and Ca^{2+} (\tilde{u}_{Ca}) in sheep cardiac tissues. A gradual decrease of external Na^+ activity (a_{Na}^o) resulted in a reduction of intracellular Na^+ activity (a_{Na}^i) which was accompanied by an increase of both intracellular Ca^{2+} activity (a_{Ca}^i) and tension. Opposite directional changes were observed when extracellular Ca^{2+} activity (a_{Ca}^o) was reduced. Depolarization of the membrane reduced a_{Na}^i , enhanced a_{Ca}^i and resulted in the development of a contracture in the manner expected of a coupled voltage-dependent transport system with a stoichiometry of >2.5 . The necessary modification of the equilibrium equation described earlier (Eq. 1.0) to accommodate the results of Horackova and Vassort (1979), and Sheu and Fozzard (1982) produces the following relationship (Eq. 2.0): $[\text{Ca}^{2+}]_i/[\text{Ca}^{2+}]_o = ([\text{Na}^+]_i/[\text{Na}^+]_o)^n \cdot \exp^{zV_m F/RT}$. Added to equation 1.0 are conditions which describe both the voltage dependence

of the exchange process and an unknown stoichiometry, n . The electrochemical gradient for ion u , \hat{u}_{ion} , is defined by Equation 3.0 as: $\hat{u}_{ion} = RT \ln([\text{ion}]_i/[\text{ion}]_o) - zFV_m$, where V_m is membrane potential, z is the valence term, R is the gas constant ($R = 8.314 \text{ VCK}^{-1} \text{ mol}^{-1}$), T is the absolute temperature on the Kelvin scale ($T(\text{K}) = 273.16 + T(^{\circ}\text{Celsius})$), and F is the Faraday constant ($F = 9.648 \times 10^4 \text{ C mol}^{-1}$). Hence, even if the values for a_{Na}^o , a_{Na}^i , a_{Ca}^o , a_{Ca}^i are known, as in the case of Sheu and Fozzard (1982), the limitation of this equation is that it describes the exchange stoichiometry only when \hat{u}_{Na} and \hat{u}_{Ca} are in equilibrium (Axelsen and Bridge, 1985), as defined in equation 3.0. This suggests that the precise value for n for a given mode of the exchanger will depend on the interaction between \hat{u}_{Na} and \hat{u}_{Ca} (e.g., Eisner and Lederer, 1985; Sheu and Blaustein, 1986), in addition to the cooperative nature between the differing binding affinities for the exchanger on either side of the membrane for Ca^{2+} and Na^+ (Miura and Kimura, 1989). The difficulties in making such assumptions have led to a large degree of caution in attempting to calculate the apparent reversal potential for currents generated by the exchange process (E_{NaCa}) as well as the nature of voltage-dependent shifts of E_{NaCa} during the cardiac cycle. In this respect, mathematical models have helped greatly to aid the understanding of how an electrogenic Na^+ - Ca^{2+} exchange process might influence intracellular Ca^{2+} homeostasis. Models developed by Mullins (1979; 1981) and Noble (1984; 1986) have been based on values reported in the literature for non-equilibrium shifts of \hat{u}_{Na} and \hat{u}_{Ca} at different points in the action potential, and have indicated that the exchanger is a dynamic system which can alter modes of operation (e.g., in the forward direction to remove intracellular [free Ca^{2+}] when $E_m > E_{\text{NaCa}}$, or in the reverse direction to bring Ca^{2+} into the cell when $E_{\text{NaCa}} > E_m$). The predominant mode of the exchanger in such a scheme is governed by the interplay of several variables, including the values chosen for \hat{u}_{ion} (Sheu and Blaustein, 1986; Bers, 1987), degree of depolarization reached during the plateau of the action potential (Noble, 1984; 1986; Shattock and Bers, 1989), and the model used to construct the relevant equations (Eisner and Lederer, 1985; Hilgemann and Noble, 1987). As mentioned earlier, recent experiments conducted by Miura and Kimura (1989) have shown that the affinity of the exchanger for Na^+ and Ca^{2+} are not equivalent on either side of the membrane. In these

experiments, outward Na^+ - Ca^{2+} exchange current, I_{NaCa} , was activated by internal Ca^{2+} with half-maximal activation corresponding to a $K_m[\text{Ca}]_i$ of 22 nM, and enhanced by internal Na^+ with an apparent $K_m[\text{Na}]_i$ of 20.7 mM. Outward I_{NaCa} became larger as external Na^+ was reduced due to a competitive interaction between Na^+ and Ca^{2+} for the external binding site. Using different concentrations of Na^+ and Ca^{2+} in the perfusate and the dialyzing pipette, and the peak amplitude of outwardly-directed I_{NaCa} as an assay, it was determined that the apparent $K_m[\text{Ca}]_o$ (0.14 mM) exceeded the $K_m[\text{Ca}]_i$ by a factor of 6.36, while the $K_m[\text{Na}]_o$ of ~44 mM was ~2-fold greater than $K_m[\text{Na}]_i$, thus confirming the proposal of Reuter and Seitz (1968) that the affinity of the exchange process on the external face of the membrane for Ca^{2+} was greater than that for Na^+ .

As discussed above, both thermodynamic and kinetic aspects of Na^+ - Ca^{2+} exchange appear to be important determinants of the direction of trans-membrane Ca^{2+} movement. Accounting for several such considerations, Kimura, Noma and Irisawa (1986) successfully used the equation, $(n-2)E_{\text{NaCa}} = nE_{\text{Na}} - 2E_{\text{Ca}}$ (Eq. 4.0), to describe the reversal potential of a voltage and Na^+ -dependent membrane conductance recorded under their conditions with an exchange stoichiometry of $n=3$. They were able to identify a current attributed to Na^+ - Ca^{2+} exchange in internally dialyzed guinea-pig ventricular myocytes by using ionic conditions which blocked other current generating mechanisms. With internal and external Na^+ and Ca^{2+} at physiologically relevant levels, E_{NaCa} was best described by the above equation with an $n=3$, required both internal Na^+ and Ca^{2+} for operation in the outward mode, and moved ~28 pM [free Ca^{2+}]/cm²/sec at plateau potentials. In the following article in the same issue of *Nature*, Mechmann and Pott (1986) demonstrated with a similar technique that the transient rise of intracellular [free Ca^{2+}] released by the SR elicited an inward current. This current was attributed to a Na^+ - Ca^{2+} exchange mechanism based on its interdependence on membrane voltage and trans-membrane gradients for Na^+ (\hat{u}_{Na}) and Ca^{2+} (\hat{u}_{Ca}). Based on a working scheme for the exchanger proposed by Mullins (1981) and Difrancesco and Noble (1985), Barcenaz-Ruiz, Beuckelmann, and Weir (1987) described an exchange current which was proportional to the difference between Ca^{2+} influx and Ca^{2+} efflux. Under conditions in which Ca^{2+} currents and release from internal stores were suppressed,

clamping the membrane to increasingly positive potentials from a holding potential of -80 mV resulted in a graded increase in the peak and rate of rise of intracellular [free Ca^{2+}] estimated with fura 2, which were not observed when external Ca^{2+} was removed. Repolarization back to the holding potential was associated with large inward tail currents which declined in amplitude as the level of the depolarizing pulse was made more negative. The slope of the linear relation between intracellular [free Ca^{2+}] and the tail current declined exponentially as the test potential was made more positive, revealing the ability of the exchanger to alter its dominant mode and providing further evidence in support of its electrogenicity. Unidirectional transport fluxes were found to be proportional to the product of a voltage-dependent rate constant (k_{NaCa}) and the concentration of the exchanger bound on each side of the membrane, assuming a stoichiometry of $3\text{Na}^+ / 1\text{Ca}^{2+}$. The data were fitted best by the following equation (Eq. 5.0), $I_{\text{NaCa}} = k_{\text{NaCa}} [\text{Ca}^{2+} \text{ influx} - \text{Ca}^{2+} \text{ efflux}]$, which as mentioned above included a proportionality constant to account for the rate constants of ion binding during changes of membrane potential. The term Ca^{2+} influx in this equation refers to the reverse mode of the exchanger ($E_{\text{NaCa}} > E_m$), while Ca^{2+} efflux during the forward mode is proportional to the previous term $E_m > E_{\text{NaCa}}$. The authors found that this equation successfully described voltage-dependent changes of intracellular [free Ca^{2+}] observed in their own experiments, as well as the I_{NaCa} exchange currents observed by Kimura et al (1986). It is interesting that similar conclusions regarding the voltage-dependence of the exchanger (and hence net Ca^{2+} movements) have been drawn by other investigators without including a term to account for the effects of voltage on binding of ions to the exchanger *per se*. This implies both a delicate and complex balance between the various thermodynamic and voltage-dependent properties of the exchange protein that govern the direction of net Ca^{2+} flux during the course of an action potential, particularly in light of the dependence on internal and external ionic conditions of the exchanger demonstrated by Miura and Kimura (1989). I will now turn to evidence which links $\text{Na}^+ - \text{Ca}^{2+}$ exchange with the contraction of cardiac muscle, as this concept is of great importance to the interpretation of data throughout the RESULTS SECTION. To a large extent, much of the

information discussed in this portion of the text is based on the preceding arguments regarding the stoichiometry and electrogenicity of the exchanger.

Functional characteristics of an electrogenic Na^+ - Ca^{2+} exchange: Following confirmation of the electrogenicity and bimodal activity of the exchanger, a number of reports have appeared which have identified membrane currents under various experimental conditions. Quantitative interrelationships have been recently established between cell contraction and both a_{Na}^i (Cohen, Fozzard and Sheu, 1982; Eisner, Lederer and Vaughan-Jones, 1984, Boyett, Hart, Levi and Roberts, 1987; Wang, Chae, Gong and Lee, 1988) and various ionic currents attributed to the exchange process (Beucklemann and Wier, 1989; Terrar and White, 1989; Egan, Noble, Noble, Powell and Twist, 1989; Shimoni and Giles; 1989). In other settings, the process of Na^+ - Ca^{2+} exchange has been shown convincingly to be involved in the extrusion of myoplasmic Ca^{2+} during the relaxation phase of contraction (Bridge, Spitzer and Ershler, 1988; Bers and Bridge, 1989; Bridge, Smolley and Spitzer, 1990) and maintaining intracellular [free Ca^{2+}] at low resting levels (Sutko, Bers and Reeves, 1986; Bers, 1987; Bose, Kobayashi, Bouchard, Hryshko and Chau, 1988; Kort and Lakatta, 1988b).

Control of contractile events by a membrane-bound Na^+ - Ca^{2+} exchange mechanism has been assessed for the most part by manipulating either the intracellular or extracellular ionic environment or membrane potential to perturb the exchange process from equilibrium, and then relating the observed effects to normal trans-membrane ion fluxes. Under such conditions it has been shown in a number of different species that rate-dependent changes of intracellular [free Ca^{2+}] and contraction are partially related to the effects of repetitive stimulation on a_{Na}^i , in a manner similar to that previously hypothesized by Langer (1968, 1971) to involve a trans-membrane Na^+ - Ca^{2+} exchange process. As first noted by Bowditch (1871) and Woodworth (1902), cardiac muscle from most mammalian species shows a marked dependence on the frequency of rhythmic stimulation. Langer (1971) proposed the " Na^+ pump lag hypothesis" to account for this phenomenon based on the demonstration by Woodbury (1963) that increased

stimulation frequency results in a parallel increase of intracellular [total Na^+]. The increased Na^+ influx during repetitive action potentials was proposed to lead to an increase of a_{Na}^i which, in turn, was proposed to facilitate trans-sarcolemmal Ca^{2+} entry via the reverse mode of the exchanger. This increase of a_{Na}^i would be permitted by the lag in the rate of Na^+ - K^+ ATPase activity, due partially to the time course of changes in extracellular K^+ concentration (Kline and Morad, 1976; Kline, Cohen and Kupersmith, 1980). Cohen, Fozzard and Sheu (1982) have investigated the relationship between contractile force and a_{Na}^i during alterations of stimulus frequency in guinea-pig and sheep ventricular tissues. Using recessed-tip Na^+ -sensitive microelectrodes they demonstrated that an increase in the frequency of rhythmic stimulation resulted in a parallel increase of a_{Na}^i and contractile force in guinea-pig ventricular muscle. Although these measurements were not made under steady-state conditions, longer periods of stimulation were associated with greater increases of a_{Na}^i and contractile activity. a_{Na}^i was estimated in Purkinje fibres to be ~8.6, 9.6 and 10.4 mM in response to stimulation at 0.2, 1.0 and 3.0 Hz, respectively. The authors concluded that their results were consistent almost exclusively with an electrogenic Na^+ - Ca^{2+} mechanism which could influence intracellular [free Ca^{2+}] according to time- and stimulation-dependent alterations of a_{Na}^i . This experiment was extended by Wang, Chae, Gong and Lee (1988) who probed the effects of stimulation frequency on the strength of contraction and its relation to both a_{Na}^i and action potential configuration in guinea-pig ventricular muscle. In response to a graded increase in the rate of stimulation, a positive staircase of both steady-state contraction and a_{Na}^i was observed which was accompanied by a marked reduction in the plateau height and duration of the action potential. A sigmoidal relation was described between contractile force and a_{Na}^i over the range of 0.5 to 5.0 Hz, with the steepest region located within the 1-4 Hz range. Interestingly, a significant amount of hysteresis in the force- a_{Na}^i relation was noted between stimulation frequencies of 5-6 Hz. While alteration in the energy charge of the cell and [free H^+] were considered as underlying mechanisms, other investigators (Harrison and Boyett, 1990) have attributed a similar process in Na^+ -loaded guinea-pig ventricular myocytes to incomplete restitution of the action potential. Similar to the conclusions drawn by Cohen et al (1982), it was

suggested that the reduction of \hat{u}_{Na} following high frequency stimulation would facilitate Ca^{2+} entry via Na^+-Ca^{2+} exchange (Eq. 2.0) which would also result in increased buffering by the SR. Consistent with this proposal is the demonstration that intracellular stores of Ca^{2+} in numerous species are increased considerably by an increase of stimulation frequency (Gibbons and Fozzard, 1971; Kurihara and Sakai, 1985; Bouchard and Bose, 1989).

Although data from such experiments have implied a causal link between a_{Na}^i and Na^+-Ca^{2+} exchange other more direct approaches have offered information which has strengthened this notion. Brill, Fozzard, Makielski and Wasserstrom (1987) investigated the effect of prolonged depolarization on the strength of contraction and a_{Na}^i in sheep cardiac Purkinje fibres under voltage-clamp conditions with Na^+ -sensitive microelectrodes. Using a constant 200 ms depolarizing pulse to 0 mV from a holding potential of -80 mV (and hence a constant trigger for release and loading of Ca^{2+} from the SR), repolarization of the membrane to gradually increasing potentials resulted in a progressive increase of phasic twitch amplitude which occurred in the absence of changes in peak I_{Ca} . A sigmoidal relationship was demonstrated when steady-state tension was plotted against the test voltage of the post-pulse, which was not qualitatively affected by blockage of membrane Na^+ -specific or Ca^{2+} -specific conductance with tetrodotoxin (TTX) or nifedipine, or increasing the duration of the test pulse to 1800 ms (which had the effect of both inducing and amplifying the tonic component of contraction). The increase of twitch force at positive potentials was associated with a decline of a_{Na}^i by ~ 2 mM. Although the involvement of a voltage-dependent Na^+ leak could not be totally ruled out, the sigmoidal relation between twitch force and voltage was interpreted as an indication of a voltage-sensitive Na^+-Ca^{2+} exchange promoting net Ca^{2+} influx at positive potentials. Of importance to *in situ* muscle function was the fact that such changes were reported to occur within the physiological range of membrane depolarization. The greater shift of phasic tension ($\sim 400\%$ at potentials >50 mV) compared with the moderate decrease of a_{Na}^i (150%) at similar clamp potentials was a novel finding, particularly in light of the recent demonstration by Eisner et al (1984) that the relationship between phasic tension and a_{Na}^i was steep, where twitch tension is proportional to $(a_{Na}^i)^y$, and $y = 3.2$ (see also

Wang et al, 1988). This was explained on theoretical grounds to be due to the electrogenicity of the exchanger, a proposal substantiated experimentally by the voltage-sensitivity of both phases of contraction and a_{Na}^i .

In a later study, Terrar and White (1989) used a similar double pulse protocol to provide further support for the hypothesis that net Ca^{2+} entry via the reverse mode of the exchanger is thermodynamically favoured at positive membrane potentials. Rather than chopping the pulse into two distinct components (constant depolarizing with variable repolarization) two separate pulses were given: P1 of constant duration to inactivate L-type Ca^{2+} current, and the test pulse, P2, to investigate the relationship between cell shortening and various membrane currents following voltage clamp to different membrane potentials. The primary observation of this study was that following complete repolarization of P1, test-pulses to positive potentials elicited contractions in the absence of appreciable I_{Ca} . These contractions were associated with a net outward current, similar to those described by Barcenaz-Ruiz, Beuckelmann and Wier (1987) and were not affected by nifedipine or ryanodine, were markedly reduced by lowering $[\text{Ca}^{2+}]_o$, and were abolished by removal of $[\text{Ca}^{2+}]_o$ in the presence of EGTA or addition of dodecylamine to presumably inhibit sarcolemmal Na^+ - Ca^{2+} exchange. Interpolation of a single action potential resulted in a potentiation of contraction which decayed back to steady-state values in a manner similar to that observed with sequential voltage-clamp pulses. The similarities in morphology of contractions elicited with action potentials and clamps to positive potentials led Terrar and White to conclude that Ca^{2+} may indeed enter the cell through the reverse mode of Na^+ - Ca^{2+} during the latter portion of the action potential plateau as intracellular [free Ca^{2+}] is declining. Thermodynamically, this is possible because as the authors point out the membrane potential favouring Ca^{2+} influx (net outward current) becomes less positive as a_{Ca}^i decreases, assuming a_{Na}^i remains constant (e.g., the membrane does not need to become as positively charged to overcome the declining Ca^{2+} gradient). As discussed above, the degree to which Ca^{2+} could enter the cell in the reverse mode of exchange depends heavily on the relationship between E_{NaCa} and E_m . Thus, even with a sustained increase in the duration of the action potential a considerable increase of a_{Na}^i would be

required to favour the reverse mode of the exchanger, which would by virtue of the fact that it promotes Ca^{2+} entry, limit it to a very narrow range of membrane potentials.

An alternate possibility has been suggested by Boyett, Hart, Levi and Roberts (1987). On the basis of changes in developed force and a_{Na}^i which were observed during extensive alterations in the rate and rhythm of stimulation and perfusate ionic composition, a working hypothesis was proposed to explain the dependence of contraction on intracellular Ca^{2+} release and modal shifts of the exchange process. This model was one of the first which attempted to explain the degree of interaction between Ca^{2+} sequestration and release by the SR and trans-sarcolemmal Ca^{2+} entry during the flow of I_{NaCa} and I_{Ca} . It was proposed that intracellular [free Ca^{2+}] was reduced by the forward mode of the exchanger due to the continual increase of trans-sarcolemmal Ca^{2+} entry in response to repetitive stimulation. The increase of a_{Na}^i expected during repetitive stimulation (Cohen et al, 1982; Wang et al, 1988) would also oppose tension development due to its effects on intracellular pH and contractility (Eisner, Lederer and Vaughan-Jones, 1983; Bountra, Kaila and Vaughan-Jones, 1986). The depressant effects of stimulation would be opposed by the augmented integral of charge (Q) crossing the membrane per unit time associated with the Ca^{2+} current, Q_{Ca}/min ; where $Q_{\text{Ca}}/\text{min} = 60f (\int I_{\text{Ca}} * dt)$ and f is equal to the frequency of stimulation in Hz. This would have the effect of increasing intracellular [free Ca^{2+}] (Fabiato, 1985; Lee and Clusin; 1987) and loading of the SR for later release (Gibbons and Fozzard, 1971; Kurihara and Sakai, 1985; Bouchard and Bose, 1989). This will be offset to some extent by the accompanying acidification of the cell interior resulting from the rate change (Bountra, Kaila, and Vaughn-Jones, 1988), which in turn would have the effect of inhibiting the conductance and altering the gating kinetics of membrane Ca^{2+} channels (Irisawa and Sato, 1986; Kaibara, and Nakayama, 1988). Depending on the extent of frequency-dependent shortening of the action potential, Ca^{2+} efflux via the forward mode of exchange would be reduced, in combination with a possible increase of Ca^{2+} entry into the cell through the reverse mode of the exchanger in the manner described by Brill et al (1987) and Terrar and White (1989). An important aspect of this working model is that the interaction between those mechanisms facilitating and opposing net gain of intracellular [free Ca^{2+}] may help

to explain the many situations in which the strength of contraction has been reported to increase without an accompanying increase of a_{Na}^i .

Leblanc and Hume (1990) have recently added an entirely different perspective to the control of intracellular [free Ca^{2+}] by demonstrating that the exchanger may be fast enough to influence events coupling membrane excitation to contraction in cardiac muscle. This would imply that internal cation-binding sites on the exchanger have low enough binding constants to permit maximal transport velocity due to changes in both modes of exchanger which are expected to occur within the first few milliseconds of the action potential (Noble, 1984; 1985; Sheu and Fozzard, 1982; Egan et al, 1989). This requirement was satisfied by calculations (Lederer et al, 1990) which indicated that the virtually instantaneous increase of a_{Na}^i at the inner sarcolemma would reduce E_{NaCa} to an extent which would permit appreciable Ca^{2+} entry in the reverse mode of the exchanger during the first 20 ms (e.g., $E_m > E_{\text{NaCa}}$). A relatively conservative increase of a_{Na}^i from 6-8 mM during the flow of I_{Na} was estimated to produce a hyperpolarization of E_{NaCa} of ~23 mV, when $E_{\text{NaCa}} = 3E_{\text{Na}} - 2E_{\text{Ca}}$, and assuming a value of 100 nM for a_{Ca}^i . Although it is rather likely that a_{Ca}^i will in fact undergo some alteration in the first 10 ms following the upstroke of the action potential, the slower time course associated with peak I_{Ca} compared with I_{Na} (7-10 ms vs 0.5 ms) suggests that any such changes would not detract from the interpretation of the results: that is, that trans-membrane exchange of Na^+ for Ca^{2+} may indeed occur fast enough to act as, or contribute significantly towards, the trigger for intracellular Ca^{2+} release.

2.0 Alterations in the Rate and Rhythm of Stimulation:

The Interval-force Relationship

It has long been recognized that altering the conditioning interval offers a simple means of investigating the cellular mechanisms underlying contraction without undue perturbation of the normal physiological state. Bowditch (1871) was among the first to report that the interval between stimulation of isolated cardiac preparations was an important determinant of the strength of

contraction. This notion was extended by Woodworth (1902) who showed that the strength of contraction by mammalian heart muscle beating at a given frequency was the result of the stimulating effect of a rapid succession of contractions and the recuperative effect of a long pause. e.g., opposing interval-dependent factors. It was subsequently demonstrated by Kruta (1937) that these opposing factors vary with frequency in a manner such that the stimulatory action increases with each contraction and diminishes with time due to the inhibitory effect of activation. Koch-Weser and Blinks (1963) performed exhaustive studies on various preparations of isolated heart muscle and demonstrated beyond reasonable doubt that the interval between contractions was an important and direct determinant of cardiac contractility. It was also shown that factors such as diastolic fiber length and resistance to shortening did not account for the interval-force relation which was characterized as an intrinsic property of each species investigated. In one of the most frequently cited reviews on the subject (27 years ago), it was stated by Koch-Weser and Blinks (1963) that "until the nature of the factors responsible for the interval-strength relationship has been determined, it will be possible to detect changes in them only through their effects on contractility". A considerable number of studies have appeared since that time which have been performed with techniques which permit a more critical analysis of the subcellular processes responsible for contraction. Probing the interval-force relation under such conditions has generated much useful information regarding the mechanisms mediating intracellular Ca^{2+} homeostasis, which have been incorporated into several excellent reviews on the subject (Morad and Goldman, 1973; Langer, 1982; Wohlfart and Noble, 1982; Chapman, 1979; Adler, Wong, Mahler and Klassen, 1985; Gibbons, 1986; Schouten, van Deen, de Tombe and Verveen, 1987; Lewartowski and Pytkowski, 1988). The three aspects of the interval-dependence of contraction which have received the most attention in recent years are: (a) post-extrasystolic potentiation, (b) the frequency-force relation, and (c) postrest contraction. These phenomenon will be dealt with in this order as the restitution processes responsible for the "memory" of cardiac muscle to preceding contractile and electrophysiological events would be expected to become more manifest as the duration of diastole is first artificially shortened, and then lengthened (cf. Gibbons, 1986).

2.1 Post-extrasystolic Potentiation

Interpolation of a single extrasystole in the midst of a train of rhythmic stimulation produces a characteristic contractile response, where the strength of the extrasystolic contraction is positively correlated with the coupling interval separating the extrasystole from the preceding steady-state contraction, and inversely correlated with the amplitude of the following post-extrasystolic contraction. Contractile perturbations of this nature have been attributed to both the availability of Ca^{2+} within various cellular compartments (Morad and Cleeman, 1987; Cooper and Lewartowski, 1985; Wier and Yue, 1986; Bose, Hryshko, King and Chau, 1988) as well as the kinetics of Ca^{2+} movements between the different pools (Fabiato, 1985*b*; Hilgemann and Noble, 1987). Wier and Yue (1986) have shown that the recovery of the extrasystolic contraction and de-potentiation of the post-extrasystolic contraction occur with monoexponential time courses which are virtually superimposable, similar to those previously reported to occur in preparations of intact ventricle (Yue, Burkhoff, Franz, Hunter and Sagawa, 1985). In addition, both processes were found to be strongly correlated with time-dependent shifts in the velocity of contraction (dP/dt) and intracellular [free Ca^{2+}], thus ruling out stimulus-related shifts of myofilament Ca^{2+} sensitivity. Extrasystolic contractions are associated in general with action potentials displaying either potentiated or prolonged plateau phases, the extent and direction of which are dependent on the species and area of the heart investigated. The plateau of the action potential has been reported to be potentiated in certain ventricular (canine: Hiraoka and Sano, 1976; Wier, 1980; Tseng, 1988; cat: Morad and Cleeman, 1987; rabbit: Hiraoka and Kawano, 1987) and atrial (rabbit: Hilgemann, 1986*b*) preparations, but not others (guinea-pig ventricle: Cooper and Lewartowski, 1985), suggesting that cellular Ca^{2+} influx is enhanced during the extrasystolic contraction for the majority of tissues studied. This proposal is supported by the demonstration that the extent and direction of extracellular Ca^{2+} depletion signals (Morad and Cleeman, 1987; Hilgemann, 1986*b*) and kinetics of D600-sensitive Ca^{2+} current (Hiraoka and Kawano, 1987; Tseng, 1988) are highly

sensitive to small perturbations of the rate and rhythm of stimulation in a manner which is negatively related to the strength of accompanying contractions.

A variety of experimental approaches have provided data in support of the conclusion that the decrease of contractile force which accompanies extrasystolic stimulation reflects the fundamental properties of an intracellular pool of Ca^{2+} , as opposed to Ca^{2+} entry across the sarcolemma during the action potential. Indeed, if the transient peak of the Ca^{2+} current were to provide the majority of Ca^{2+} for myofilament activation, then the bulk of evidence would predict that the strength of the extrasystolic contraction would surpass that of preceding steady-state contractions. In fact, this can only be demonstrated once the Ca^{2+} channels present in the release site of the SR are converted into a subconducting state following application of ryanodine (Bose, Hryshko, King and Chau, 1988). Morad and Cleeman (1987) have shown that the plateau phase of action potentials recorded from cat ventricular muscle were significantly elevated during depressed extrasystolic contractions. Depletions of extracellular [free Ca^{2+}] assessed with antipyrilazo III were greater for extrasystolic contractions than for either the preceding steady-state contraction or the following post-extrasystolic contraction, leading the authors to conclude that trans-sarcolemmal Ca^{2+} entry was enhanced during the extrasystole. The importance of intracellular stores of Ca^{2+} was implied by the fact that similar observations were made with respect to stimulus-dependent excursions of extracellular [free Ca^{2+}] in strips of frog ventricle in the absence of post-extrasystolic potentiation. A similar relation between Ca^{2+} influx and force of contraction during single extrasystolic and post-extrasystolic contractions has been reported in rabbit atrial muscle (Hilgemann, 1986b). Large depletions of extracellular [free Ca^{2+}] were recorded during premature contractions which were markedly increased along with the degree of post-extrasystolic potentiation following a graded reduction of the basic cycle length preceding the ES/PES pair. The demonstration that maximal depletion signals were terminated well before the membrane repolarized to -20 mV implied that peak I_{Ca} was enhanced during the extrasystole. This was later confirmed by Tseng (1988) in experiments performed on isolated guinea-pig and canine ventricular myocytes who found that I_{Ca} peak restituted fully by 50 ms in cells depolarized at various coupling

intervals from a holding potential of -80 mV. For clamp intervals between 50 and 5000 ms, peak I_{Ca} and both the fast and slow components of current inactivation were potentiated in a bell-shaped fashion beyond the values associated with steady-state stimulation, thus helping to explain the "supranormal" action potentials recorded from multicellular preparations. Potentiation of I_{Ca} in this fashion has been described previously in isolated guinea-pig (Lee, 1987; 1990; Fedida, Noble and Spindler, 1988; Garney, Charnet, Pye and Chargeot, 1989) and amphibian ventricular cells (Noble and Shimoni, 1981; Schouten and Morad, 1990), and appears to be related to small changes of both intracellular [free Ca^{2+}] and membrane voltage. This proposal is supported by the marked reduction of current overshoot in the experiments of Tseng (1988; see also Fedida et al, 1988) by replacement of Ca^{2+} as the charge carrier by Ba^{2+} , reduction of extracellular Ca^{2+} , increasing the concentration of EGTA or 1,2-bis(2-aminophenoxy)ethane *N,N,N',N'*-tetraacetic acid (BAPTA) in the dialyzing pipette, or administration of D600, caffeine or ryanodine, with a role for Ca^{2+} -mediated de-phosphorylation of a channel subunit was also suggested on the basis of the sensitivity of I_{Ca} -overshoot to reduction of the holding potential to -30 mV or altering the duration or polarity of the test pulse.

Cooper and Lewartowski (1985) have investigated the cellular mechanisms underlying post-extrasystolic potentiation in Langendorff-perfused guinea-pig ventricles. The degree of post-extrasystolic potentiation was related to the contractile state of the preparation by injecting the extrasystolic stimulus at different points during the recovery of contractile force following a brief period of rest. The main observation in this study was that the amount of potentiation observed after interpolation of an extrasystole at a given coupling interval was invariably proportional to the size of preceding steady-state contractions at any time point during the course of contractile recovery. This relationship remained unaltered in response to raising or lowering extracellular [total Ca^{2+}] and was abolished completely following introduction of 10 mM caffeine into the preparation. Similar results were obtained when trains of paired-pulse stimulation were imposed at different points in the recovery process. The data led the authors to conclude that post-extrasystolic potentiation was a direct function of Ca^{2+} entry into the myoplasm during the flow of I_{Ca} .

independent of how much Ca^{2+} was available for release from intracellular stores. Of interest is the observation that action potentials recorded during extrasystolic contractions displayed depressed, rather than elevated, plateau phases. This may have been related to the high concentration of extracellular K^+ employed in these experiments to suppress nodal automaticity (7.40 mM), which in addition to its predicted effects on both time-dependent (e.g. I_{to} and I_{K}) and time-independent (e.g., I_{K1}) sarcolemmal potassium conductance, would also be expected to affect the time course and extent of mechanical restitution (Lipsius, Gibbons and Fozzard, 1982).

This experiment was extended by Bose, Hryshko, King and Chau (1988a) who probed the relative contribution of Ca^{2+} entry into the cell during the action potential and Ca^{2+} released from the SR towards post-extrasystolic potentiation in canine ventricular muscle. This was done through the use of ryanodine to impair quantitative release of Ca^{2+} from intracellular stores and by comparing the interval-dependence of post-extrasystolic potentiation with potentiated contractions resulting from transient bursts of high-frequency stimulation or resumption of stimulation after variable periods of rest (e.g., postrest-potentiation). Interpolation of a single extrasystole at gradually increasing coupling intervals resulted in a monoexponential recovery of the extrasystolic contraction and decay of the post-extrasystolic contraction in a manner similar to that described for ferret ventricle by Wier and Yue (1986). Administration of 30 nM ryanodine produced striking effects on the recovery of the extrasystolic contraction, which became larger than the preceding steady-state contraction at coupling intervals < 300 ms. While the degree of post-extrasystolic potentiation remained unaltered at this concentration of ryanodine it was blocked completely at concentrations > 1 μM . The sensitivity of various limbs of the interval-force relation to potentiation or inhibition by ryanodine was inversely related to the length of the diastolic interval preceding contraction, and as such could be described by a rank order of: postrest contraction >> post-extrasystolic contraction > extrasystolic contraction > contractions elicited with high-frequency stimulation. Contractions which were strongly dependent on Ca^{2+} release from the SR, such as those elicited after a period of rest, were thus preferentially depressed by ryanodine. This is consistent with the ability of this compound in cardiac muscle to convert Ca^{2+} -release channels

embedded in the membrane of the SR to an open subconductance state (Rousseau, Smith and Meissner, 1987) without altering the ability of the SR to buffer intracellular [free Ca^{2+}] (Bers, Bridge and MacLeod, 1987; Bouchard, Hryshko, Saha and Bose, 1989; Bers, Bridge and Spitzer, 1990). The contribution of intracellular stores of Ca^{2+} to the amount of post-extrasystolic potentiation was further investigated by interpolating an extrasystole after a single postrest contraction had been elicited in response to a graded increase in the duration of rest. In contrast to the experiments of Cooper and Lewartowski described above, the contribution of trans-sarcolemmal Ca^{2+} influx to the amount of post-extrasystolic potentiation was quantified in these experiments by correcting for the degree of contractile restitution during the course of rest. This was done by normalizing the amplitude of the post-extrasystolic contraction following subtraction of the second postrest contraction which would have occurred in the absence of an extrasystolic stimulus, and hence for the "residual potentiation" remaining after the first postrest beat. As the period of rest was extended from 10 to 1200 sec postrest contraction was augmented in a bell-shaped fashion, peaking at ~60 sec. Despite the continual repriming of the release pool during the course of rest, the degree of post-extrasystolic potentiation began to decline from the moment rhythmic stimulation was terminated, suggesting an independence of potentiation from SR Ca^{2+} stores. However, comparison of the time-dependent decay of the corrected post-extrasystolic potentiation and the second postrest contraction in the absence of extrasystolic stimulation indicates that the degree of residual potentiation also begins to fall off from the moment rhythmic stimulation is terminated, which would be expected to strongly influence the amount of Ca^{2+} available for release during post-extrasystolic contraction.

An important role for extracellular Ca^{2+} entry during the extrasystolic contraction is reinforced by the fact that the amplitude of corrected post-extrasystolic contractions remained relatively stable at rest intervals > 100 sec (~100% of steady-state contraction) whereas the second postrest contraction decayed exponentially during this time. As discussed by the authors, this result suggests that Ca^{2+} entry during the extrasystole is a requisite for potentiation of the following contraction. A second important finding was the marked effect of ryanodine on the magnitude of

extrasystolic contraction. As discussed above, this compound converts the release channel complex of the SR to a subconducting state, and as such effectively short-circuits both the recirculation process and the inactivated transitional state of these channels. Due to the fact that peak Ca^{2+} entry into the cell (and hence the trigger for release of Ca^{2+} from internal stores) is enhanced during the extrasystole (Morad and Cleeman, 1987; Hilgemann, 1986b; Tseng, 1988) it seems reasonable to suggest, based on the actions of ryanodine, that the depressed amplitude of extrasystolic contractions reflects the degree of refractoriness of the release channel complex. This proposal is consistent with the demonstration in skinned cardiac fibres (Fabiato, 1985b) that both extrasystolic and post-extrasystolic contractile phenomena can be reproduced by appropriate timing of simulated [free Ca^{2+}] transients. If this assumption is correct, then it is likely that the rise of intracellular [free Ca^{2+}] during the post-extrasystolic contraction is a reflection of the balance between Ca^{2+} available for release from the SR prior to injection of the premature stimulus and the amount of Ca^{2+} entry during depolarization. As discussed by Hilgemann and Noble (1987) cellular Ca^{2+} uptake prevails during premature stimulation due to the inactivated state of the SR Ca^{2+} channels, which can be overcome by ryanodine. Following removal of activation, presumably with first-order kinetics (Wier and Yue, 1986), the augmented pool of Ca^{2+} is recirculated back to the release compartment where it is then available for release into the myofilaments.

2.2 Frequency-force Relationship

An important physiological characteristic of cardiac muscle is its inherent ability to modify the strength of contraction following an increase in the rate of rhythmic stimulation. With the exception of several rodent species, this results in a positive staircase of steady-state contraction. For the most part, this is accomplished by an increase in the maximal rate of force development (dP/dt) with either no change or a slight decrease of the time required to attain peak force (Kaufmann and Fleckenstein, 1965; Sumner, Braveny and Kruta, 1967; Langer and Brady, 1968; London and Krueger, 1986; Lewartowski and Pytkowski, 1988). The demonstration by Langer (1965) that

cellular Ca^{2+} uptake in canine ventricular muscle was enhanced following an increase in stimulation frequency supported the widely held hypothesis that an increase of intracellular [free Ca^{2+}] would facilitate the rate of active state development. This idea was strengthened following the description of a Ca^{2+} -carrying inward current during the plateau of the action potential by Reuter in 1967. The correlation of frequency-dependent Ca^{2+} exchange in cardiac muscle with Na^+ and K^+ kinetics (Langer and Brady, 1966; 1968; Langer, 1967; Reuter and Seitz, 1968) resulted in the development of the "Na⁺ pump lag" hypothesis to account for the positive staircase phenomenon, as discussed above. However, the quantitative dissociation of Ca^{2+} entry during I_{Ca} and peak contraction during different patterns of stimulation (Wood, Hepner and Weidmann, 1969; Antoni, Jacob and Kaufmann, 1969) and other electrophysiological evidence which suggested that Ca^{2+} entry during depolarization did so into a confined space near the inner surface of the sarcolemma (Basingthwaite and Reuter, 1972) led to the proposal by Allen, Jewell and Wood (1976) that interval-dependent contractions were generated by a release of Ca^{2+} from labile stores within the SR. Although release from intracellular stores has been implicated strongly in the control of contraction the degree to which the frequency-dependence of contraction is related to rate-dependent alterations of the cellular processes governing release from intracellular stores is unknown. This situation is made more complex by the growing body of evidence in support of a $[\text{Na}^+]_o$ -dependent pool of Ca^{2+} localized within a functional cell compartment in which diffusion of small ions is restricted (Lederer, Niggli and Hadley, 1990), and which is very likely in direct communication with the SR. In addition, this compartment appears to have exchange kinetics rapid enough to affect the time course of single excitations (Mitchell, Powell, Terrar, Twist and Spindler, 1985; Miura and Kimura, 1989; Terrar and White, 1989; Leblanc and Hume, 1990; Gruver, Katz and Messineo, 1990) and hence, by virtue of its close apposition to the cell membrane, would also be expected to be strongly influenced by net Ca^{2+} movement during the action potential. If these assumptions are reasonably correct, the amount of Ca^{2+} delivered to the myofilaments during contraction would be directly related to the *degree* of interaction between Ca^{2+} entering during the time-averaged flow of I_{Ca} , that released from the SR in response to an action potential

and the fraction of time spent by the $\text{Na}^+ - \text{Ca}^{2+}$ exchange in reverse mode, which will depend on the theoretical quantity $E_m - E_{\text{NaCa}}$.

A sudden increase in the rate of rhythmic stimulation produces a biphasic contractile response in ventricular preparations from most mammalian species, where the immediate effect is seen as a decrease in the strength of contraction, similar to the effect of interpolating an extrasystole, which is then followed by a graded increase of contraction to a new steady-state level (e.g., "positive staircase" or "treppe"). Similar to the restitution and de-potentialization of contraction following injection of a single extrasystolic stimuli (Wier and Yue, 1986) both the inhibitory and facilitatory components of the contractile staircase develop with exponential time courses. Figure 1 is taken from the work of Blinks and Koch-Weser (1961) and illustrates several important characteristics of the two opposing restitution processes in a strip of isolated cat atrium. Experiments performed on a diverse range of species led to the development of a descriptive model in which the strength of contraction at a given conditioning interval could be fit uniformly by the relation: $P_t = RC + [\int \text{PIEA} - \int \text{NIEA}]$ (Eq. 6.0), where P is the strength of contraction at interval t , RC is the rested state contraction, $\int \text{PIEA}$ is the cumulated positive inotropic effect of activation, and $\int \text{NIEA}$ is the cumulated negative inotropic effect of activation. The term rested state contraction was used to describe contractions which were preceded by period of rest long enough such that the strength of contraction becomes independent of the previous stimulation pattern. Hence, individual rested state contractions represent an intrinsic contractile property of each preparation. This is shown in the lower panel of Fig. 1, which illustrates the independence of the first postrest contraction from alterations in the rate of stimulation and steady-state contraction amplitude preceding the period of rest. The "act of contraction" was interpreted in this model to result in a change of the contractile state, or predisposition, of the muscle in such a way as to oppose developed tension of subsequent beats. This process was referred to as the negative inotropic effect of activation, which can be seen clearly as an immediate decay of contractile strength following an increase in the rate of stimulation (upper panel) or resumption of stimulation after a period of rest (lower panel). An important property of the NIEA is its capacity to cumulate if repetitive contractions follow one

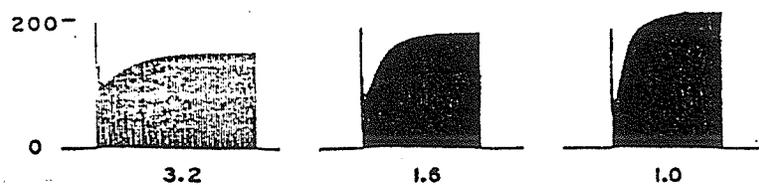
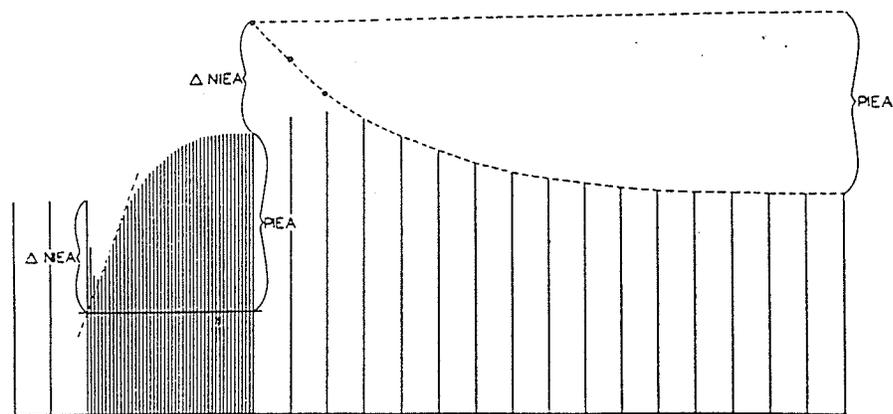


Figure 1. Schematic representation taken from Blinks and Koch-Weser (1961) of the interval-force relation for mammalian cardiac muscle. *A)* Cumulative nature of the positive and negative inotropic effects of activation associated with a transient increase in the rate of stimulation. *B)* Tension recordings made from a guinea-pig left atrial preparation. Stimulation was resumed at a constant frequency, with 3.2, 1.6 and 1.0-sec between beats, after rest intervals of sufficient duration (~5-min) to produce rested-state contractions (38°C).

another with short enough diastolic intervals (Blinks and Koch-Weser, 1961; Koch-Weser and Blinks, 1963). Later experiments have indicated that this process may be related to both incomplete mechanical restitution (Ragnarsdottir et al, 1982; Orchard and Lakatta, 1985; Schouten et al, 1987) and/or incomplete recovery of Ca^{2+} channels embedded in the membrane of the SR (Fabiato, 1983; 1985b). This effect is most easily seen in rat ventricular muscle where an abrupt increase in the rate of stimulation results in an exponential decline in the strength of contraction to a new steady-state level (e.g., "negative staircase") in the absence of the second rising phase of developed force (Kelly and Hoffman, 1960; Forester and Mainwood, 1974; Orchard and Lakatta, 1985). In addition to the NIEA component, each contraction was proposed to possess a relatively small positive inotropic effect which decays with slow monoexponential kinetics. Thus, the strength of contraction at any one frequency of stimulation will be determined by the intrinsic ability of a given preparation to produce a significant contraction after a long period of rest (e.g., rat vs guinea-pig), plus the opposing forces of the cumulative NIEA and PIEA (Blinks and Koch-Weser, 1961; Koch-Weser and Blinks, 1963).

Although much information has been gained since that time regarding the source of Ca^{2+} for postrest contractions (Allen, Jewell and Wood, 1976; Reiter, Seibel, Karema, 1978; Ragnarsdottir et al, 1982; Bers, 1985; Bridge, 1986; Hilgemann, 1986a; Bers, Bridge and MacLeod, 1987; Bose et al, 1988) and the opposing inotropic forces of mechanical restitution (Morad and Goldman, 1973; Wolfart and Noble, 1983; Chapman, 1979; Pytkowski and Lewartowski, 1988; see also Morad and Cleeman, 1987; Fabiato, 1985b; Schouten, deTomb, and Verveen, 1987; Bose et al, 1988) it is surprising that relatively little is known about the cellular mechanisms responsible for the staircase phenomenon. Most reports of this nature have focused on α_{Na}^i -related inotropic mechanisms (Cohen et al, 1982; Brill et al, 1987; Boyett et al, 1987; Wang et al, 1988) and contribution of Ca^{2+} from this source toward the development of the positive staircase seems fairly certain. However, few studies have appeared with the specific aim of analyzing the contribution of Ca^{2+} release from intracellular stores to the staircase phenomenon and most information regarding this matter has been gained indirectly (Gibbons and Fozzard, 1971; Endoh and Iijima, 1981; Kurihara and Sakai,

1985; Fabiato, 1985c; Bose et al, 1988b; Kort and Lakatta, 1988b; Bouchard and Bose, 1989). As discussed above, a minimum of three pools of Ca^{2+} are available from which Ca^{2+} may be drawn for contraction, and it is quite likely that each is profoundly affected by alterations of membrane voltage and diastolic interval. Even ignoring the possibility that significant movement of Ca^{2+} occurs between the compartments within a cycle of contraction (Chapman, 1979; Morad and Cleeman, 1987; Schouten et al, 1987; Hilgemann and Noble, 1987) the total store of Ca^{2+} within individual compartments would be expected to undergo significant perturbation following an increase in the rate of rhythmic stimulation.

Ionic currents underlying the plateau phase of the action potential: Characteristic changes in the action potential and underlying currents that result from altered stimulation frequency are for the most part consistent with an increase of time-averaged Ca^{2+} entry into the intracellular space and a reduction of cellular Ca^{2+} efflux. With the notable exception of rabbit atrial and ventricular preparations (Hilgemann, 1986a; Ruiz-Petrich and Leblanc, 1989), most preparations of mammalian myocardium display abbreviated action potentials following an increase in the frequency of steady-state stimulation. As discussed by Boyett and Jewell (1980) this appears to reflect primarily the rate-dependence of ionic currents which are responsible for the plateau phase of the action potential, as little change in the rate of terminal repolarization is often observed. The refractory period for threshold stimulation is also affected by the rate of stimulation. Previous studies have indicated that the period of time required for restitution of the action potential is significantly reduced in isolated ventricular muscle (Moore et al, 1965) and the *in situ* heart (Janse et al, 1969). An inverse relationship has often been described between the force of contraction and speed of membrane repolarization (Boyett and Jewell, 1980), although this relationship appears to break down when the rhythm of stimulation is perturbed and the amplitude of accompanying contractions potentiated (Greenspan, Edmands and Frisch, 1967; Boyett and Jewell, 1978; King and Bose, 1984; Schouten and terKeurs, 1986; Hilgemann, 1986a; Hryshko, Bouchard, Chau and Bose, 1989).

Transient outward current: There is abundant evidence suggesting that rate-dependent changes of the ionic currents underlying the plateau phase of the action potential are related to perturbations of the level of intracellular [free Ca^{2+}] that accompany this intervention. Changes in the duration of the early plateau appear to be due primarily to the opposing actions of a transient outward current carried by K^+ ions (I_{to}) and Ca^{2+} entry into the cell during I_{Ca} . A rate-dependent decrease in the amplitude of I_{to} has been recorded from many different cardiac tissues (Reuter, 1968; Peper and Trautwein, 1968; Gibbons and Fozzard, 1975; Hiraoka and Hiraoka, 1975; Payett, Schanne and Ruiz-Ceretti, 1981). This reduction of I_{to} peak occurs in part due to incomplete recovery at faster rates of stimulation and is opposed by the increase of intracellular [free Ca^{2+}] which occurs in most mammalian and amphibian species. As discussed by Boyett and Jewell (1980) time constants for current inactivation range from 450-1600 ms, although later work by Tseng (1989) has suggested that the transient outward current is comprised of two separate currents, each with its own set of voltage-dependent inactivation variables. Thus, only very slow rates of stimulation (< 1.0 Hz) would permit complete recovery of a significant population of the channels responsible for generating I_{to} . This proposal is consistent with the observation that premature action potentials, which for the most part display potentiated plateau phases, are associated with a reduction of I_{to} peak (Hiraoka and Kawano, 1987; Tseng, 1988).

With respect to the positive frequency-force relation, a number of reports have indicated that inactivation of the transient outward current is also sensitive to small changes of intracellular [free Ca^{2+}]. The observation that the peak amplitude and time course of recovery of I_{to} and contraction co-varied with premature stimulation or application of cardiac glycosides led Siegelbaum, Tsien and Kass (1977) to propose that I_{to} was a " Ca^{2+} -activated" current. As mentioned above, evidence has been presented to indicate that I_{to} is an amalgam of two separate outward currents in canine ventricular myocytes (Tseng, 1989). A 4-aminopyridine sensitive transient outward current was identified in these experiments which decayed with a single exponential time course in a voltage dependent fashion. A second and smaller current component displaying a slower time course was

designated I_{to2} and was augmented by raising extracellular [total Ca^{2+}] and inhibited following the application of caffeine or ryanodine to the perfusate, although the presence of two distinct current components seems to depend on the species investigated (e.g., Gibbons and Fozzard, 1975). That the rate-related decrease of I_{to} in canine ventricular myocytes (Tseng et al, 1988; Tseng, 1989) may be related to altered levels of intracellular [free Ca^{2+}] under physiological conditions, is supported by the positive slope of the staircase response for contraction which accompanies a similar intervention in intact canine ventricular muscle (Endoh et al, 1982; Bose et al, 1988a; Bouchard and Bose, 1989).

The participation of I_{to} to repolarization in isolated human atrium has been studied by Shibata, Drury, Refsum, Aldrete and Giles (1989) with a combination of conventional microelectrode and voltage-clamp techniques. A "robust" transient outward current was identified in these experiments with an activation threshold of ~ -50 mV and a half-activation point of $+1$ mV. Both the steady-state component and the transient peak of the outward current were greatly suppressed by administration of 4-aminopyridine (0.5 mM). An important feature of this current was that its reactivation displayed both time- and voltage-dependence, suggesting a role for I_{to} in frequency-induced alterations of the plateau phase of the action potential. To this end, a graded increase of stimulation frequency from 0.25-1.18 Hz had the effect of shortening the overall duration of the action potential, while abbreviating the duration of the plateau phase and abolishing the notch, or "hump", of rapid repolarization. Recordings of the frequency-dependence of I_{to} indicated that both the transient and steady-state current components were depressed in response to an increase in the rate of stimulation (0.2-3.75 Hz). Similar changes in action potential configuration and current kinetics could be mimicked by application of 4-aminopyridine. It is worth noting that this compound also had the effect of potentiating steady-state contraction to equal degrees in response to stimulation at frequencies ranging from 0.2-3.75 Hz, which the authors suggested may have been due to reduced overlap between I_{to} and I_{Ca} at various plateau potentials. Although not obtained from ventricular tissue, this result is encouraging as it represents one of the few investigations of the sort of electrophysiological and mechanical changes one might expect in the

range of physiological heart rates known to occur during the course of moderate to vigorous daily activity in humans. Hilgemann (1986a) has also shown a similar effect of 4-aminopyridine on steady-state contraction and action potential configuration in rabbit atrium. A brief period of rest in this tissue results in a potentiation of contraction which is accompanied by action potentials with a shortened plateau phase which is then followed by a prominent slowing of terminal repolarization, which Giles and Shimoni (1989) later attributed to an electrogenic $\text{Na}^+ - \text{Ca}^{2+}$ current. Addition of 4-aminopyridine in these experiments had a preferential inotropic effect on steady-state contraction and markedly enhanced both the plateau voltage and duration. That the period of rest was without effect on action potential configuration suggests that inhibition of I_{to} in rabbit atrium exerts its inotropic effects primarily by altering the conductance of the membrane to Ca^{2+} , although saturation of an intracellular pool can not be ruled out on the basis of these results. This would tentatively imply that the findings of Leblanc and Hume (1990) regarding the participation of I_{NaCa} in initiating contraction may extend to other species in that the transient outward current may make more of an indirect contribution to raising peak intracellular [free Ca^{2+}] during a twitch than previously appreciated. This follows the rapid interaction between E_{NaCa} and E_{m} in the first 5-25 ms following the upstroke of the action potential (Mullins, 1981; Difrancesco and Noble, 1985; Bers, 1987; Hilgemann and Noble, 1987; Egan et al, 1989).

Slow inward current: Although a fair amount of information has been gathered with respect to the contribution of slow inward currents to plateau potentials, the picture is far less clear with respect to the contribution of Ca^{2+} entry during I_{Ca} towards the inotropic effect of high-frequency stimulation. When the field was so thoroughly reviewed by Boyett and Jewell in 1980, few investigations had been undertaken to isolate the various ionic currents responsible for the action potential plateau, and to determine their relative importance during alterations in the rate of stimulation. The characterisation of many such ionic currents since that time has been made possible largely by the refinement of the patch-clamp technique (Hamill, Marty, Sackmann, Neher and Sigworth, 1981) and the description of methods facilitating the " Ca^{2+} tolerance" of freshly

dispersed cardiac myocytes (Powell and Twist, 1976; Isenberg and Klockner, 1982; Bihler, Ho and Sawh, 1984; Mitra and Morad, 1985). Many investigators, using a combination of voltage-clamp and optical techniques, have examined the effects of stimulation frequency on I_{Ca} kinetics and the results in general have been equivocal. Under a wide range of experimental conditions, the peak of the Ca^{2+} current has been described in response to an increase in the rate of stimulation to be both depressed (Reuter, 1979; Gibbons and Fozzard, 1975; Simurda et al, 1976, Mitchell et al, 1985; Mitra and Morad, 1986; Schouten and Morad, 1989) and potentiated (Noble and Shimoni, 1981; Mitra and Morad, 1986; Schouten and Morad, 1989), while other authors have reported an insensitivity of I_{Ca} peak to drive rate (Mitchell et al, 1985; London and Kreuger, 1986). In this respect it is important to note that only in a few of the studies listed above were simultaneous recordings made of both electrophysiological and contractile activity. This is due primarily to the difficulty in maintaining microelectrode impalements in the presence of significant cell shortening or contraction, a problem which may be overcome by newer microelectrode designs which favour long-term impalements in vigorously contracting preparations (Fedida, Sethi, Mulder and terKeurs, 1989). Most earlier studies performed on multicellular ventricular preparations have demonstrated a depressant effect of stimulation on peak I_{Ca} , which coincided with the effects observed on the height of the action potential plateau (Reuter, 1979; Gibbons and Fozzard, 1975; Simurda et al, 1976). The positive inotropic effect of high-frequency stimulation on peak I_{Ca} in this setting has generally been explained by assuming that time-averaged Ca^{2+} influx, expressed as Q_{Ca}/min , is greater for a given period of time than during similar intervals preceding the frequency increase (Morad and Goldman, 1973; Allen, Jewell and Wood, 1976; Fabiato, 1985c; Bridge, 1986) which was supported by the observation that cumulative extracellular [total Ca^{2+}] depletion signals (Bers, 1983; Hilgemann, 1986a) and the amount of Ca^{2+} estimated to reside within the SR (Gibbons and Fozzard, 1971; Kurihara and Sakai, 1985; Bouchard and Bose, 1989) were markedly enhanced following an increase in the rate of stimulation.

Later studies, however, have indicated that the sensitivity of the Ca^{2+} current to changes in the rate of stimulation depends strongly on both the resting membrane potential preceding stimulation

(Mitra and Morad, 1986; Schouten and Morad, 1989) and the duration of membrane depolarization during stimulation (Mitchell et al, 1985). These investigations were prompted to a large extent by the demonstration by Noble and Shimoni (1981) of a positive I_{Ca} -frequency relation in thin bundles of frog ventricular muscle. The authors interpreted this result as evidence for a "facilitation" mechanism whereby small amounts of intracellular [free Ca^{2+}] could potentiate the current during successive beats. This was supported by the demonstration that the amplitude of peak I_{Ca} and concomitant contractions recovered in an identical fashion after a period of rest, and were potentiated to the same extent following an increase of clamp frequency. Fedida, Noble and Spindler (1988a) have described a similar process in guinea-pig ventricular myocytes during conditions which moderately enhanced myoplasmic Ca^{2+} , whereas the opposite results were obtained when intracellular [free Ca^{2+}] was raised to pathologically high levels. Thus, it appears that similar to the optimal conditions under which Ca^{2+} release from the SR can be triggered by trans-sarcolemmal Ca^{2+} entry (Fabiato, 1983; 1985b), there is also a bell-shaped relationship between the conductance of membrane Ca^{2+} channels and intracellular [free Ca^{2+}] (Lee and Tsien, 1982; Lee, Marban and Tsien, 1985; Mitchell et al, 1985; 1987; Fedida, Noble and Spindler, 1988b; Nabauer, Cleeman and Morad, 1988; Gurney et al, 1989).

In addition to possible modulation by the level of intracellular [free Ca^{2+}], the conductance of the membrane to Ca^{2+} during different patterns of stimulation appears also to be strongly dependent on the holding potential used to study it. In one of the few early reports of a positive I_{Ca} -frequency relation (frog ventricle; Noble and Shimoni, 1981), the holding potential was set at -80 mV, rather than the -50 to -40 mV typically employed to investigate the gating and inactivation kinetics of I_{Ca} . Schouten and Morad (1989) have re-examined the effect of stimulation frequency on frog ventricular myocytes clamped at various holding potentials in the presence and absence of 3'5' cyclic adenosine monophosphate (cAMP). From a holding potential of -90 mV they were unable to show any effect of stimulation frequency on peak I_{Ca} in the 0.1 to 1.0 Hz range. Conversely, reduction of the holding potential to -40 mV between depolarizing pulses led to a negative I_{Ca} -frequency relation, which was partially antagonized by inclusion of 25 μ M cAMP in the

microelectrode filling solution. Increasing the frequency of clamping from a holding potential of -90 mV in the presence of cAMP resulted in a 20-50% increase of peak I_{Ca} . The voltage threshold for conversion of the "staircase" was -60 mV, which was close to the activation threshold of I_{Ca} in that tissue. Although the contribution of a significant steady-state Ca^{2+} conductance, or leak, could not be excluded in this experiment the authors concluded that this effect did not depend on the presence of intracellular Ca^{2+} ions, as substitution of Ba^{2+} as the charge carrier did not alter the positive I_{Ca} -frequency relationship after compensation for the negative shift of the $I-V$ relation induced by Ba^{2+} (see also Noble and Shimoni, 1981). The results of this study suggest that the holding potential plays an important role in the modulation of I_{Ca} . A similar relationship has been demonstrated in mammalian ventricle by Mitra and Morad (1986). Voltage-clamp experiments conducted on guinea-pig ventricular myocytes revealed two populations of Ca^{2+} channels (Bean, 1985) which could be distinguished by dissimilarities in their activation threshold, inactivation kinetics and pharmacological sensitivities to organic and non-organic Ca^{2+} channel blockers. From a holding potential of -100 mV a "low threshold" (T-type) current was identified which activated at -60 mV, displayed relatively fast inactivation kinetics, and was almost completely inactivated at potentials > -40 mV. A second slowly inactivating "high threshold" (L-type) current was demonstrated having an excitation threshold of -30 mV. While an increase of steady-state stimulation frequency depressed currents elicited by depolarization to a test potential of -30 mV from a holding potential of -100 mV, currents elicited by depolarization to +10 mV were markedly enhanced. Similar conclusions were drawn by Lee (1990) who found that frequency-potentiated currents in guinea-pig ventricular cells were carried through L-type channels and not by T-type channels (based on their differential voltage-sensitivity).

If, as suggested by Schouten and Morad (1989), internal Ca^{2+} is not a requisite for current potentiation what type of voltage-dependent mechanism might be responsible? Cellular metabolism of cAMP was suggested on the basis of their observation that the effects of stimulation frequency could be qualitatively mimicked by theophylline (0.5 mM). It was argued that cAMP is a well known regulator of Ca^{2+} channel availability (Tsien et al, 1986; Trautwein et al, 1990), and that

depolarization in some as yet unknown fashion could result in an inhibition of phosphodiesterase activity. This mechanism may not predominate in contracting tissue however, as an inverse relationship has been shown to exist between the level of intracellular [free Ca^{2+}] and cellular content of cAMP at different frequencies of stimulation and external Ca^{2+} concentrations (Endoh, Brodde, Rennhardt and Schumann, 1976), which would be partially masked by the conditions used in the above experiments. The applicability of data obtained from either study to *in situ* cardiac function is made more difficult by the fact that contractions were suppressed in each case with high concentrations of EGTA within the dialyzing pipette solution. This would be expected to considerably reduce the degree of negative feedback by increased concentrations of myoplasmic Ca^{2+} as well as any contribution made by the Ca^{2+} -sensitive component of I_{to} , and may be related to the insensitivity of I_{Ca} to stimulation frequency in internally-perfused freely contracting guinea-pig ventricular cells held at -40 mV (London and Krueger, 1986). Thus, despite the fact that many ventricular preparations held at "physiological" holding potentials do show a positive I_{Ca} -frequency relation, this result can not be easily reconciled with the characteristic changes of the plateau phase of the action potential or the transient outward current which occur when the rate of stimulation is enhanced in intact multicellular preparations. This difference yet remains to be resolved.

Rate-dependent alterations of intracellular Ca^{2+} loading: Early evidence in support of the involvement of an intracellular compartment of Ca^{2+} in the frequency-force relation came from the studies of Wood, Hepner and Weidmann (1969) and Antoni, Jacob and Kaufmann (1969). In these experiments an increase in the rate of stimulation or number of stimulations in a train preceding a fixed period of rest resulted in the potentiation of postrest contraction with little change or a reduction of the plateau voltage of the accompanying action potential. As Ca^{2+} was known to enter the cell during the transient plateau phase (Reuter, 1967) this result implied the contribution of Ca^{2+} from an intermediate source, e.g., within the intracellular space. As discussed by Gibbons (1986), the period of rest used in such experiments is generally long enough to exceed the

restitution processes involved in generating steady-state contractions but short enough to avoid the development of rested state contractions. Using this type of stimulation protocol, Allen, Jewell and Wood (1976) have demonstrated a rate dependent increase in the amplitude of postrest contractions recorded from isolated cat papillary muscles at various test intervals. In addition to facilitating peak contraction amplitude, the increase of stimulation frequency also dramatically prolonged the time required for the development of the rested state, thus excluding a direct relation between trans-sarcolemmal Ca^{2+} entry and the strength of postrest contractions. Similar qualitative results have been obtained regarding the restitution of postrest contraction and the strength of preceding steady-state contractions in canine (Endoh and Iijima, 1982; Bose et al, 1988a), guinea-pig (Fedida et al, 1988) and human ventricular preparations (Quagebuer, Schouten and terKeurs, 1986; Morgan, Erny, Allen, Grossman and Gwathmey, 1990).

In an attempt to gain an understanding of the source of Ca^{2+} for contractions elicited at different rates of stimulation, different experimental approaches have been utilized to determine the relationship between ionic shifts related to the rate of stimulation and associated contractile activity. A strong relationship has been demonstrated between cellular ^{45}Ca uptake and the strength of contraction in canine ventricular muscle upon an increase in the frequency of stimulation (Langer and Brady, 1963; Langer, 1965). Using double-barreled Ca^{2+} -sensitive microelectrodes, Lado, Sheu and Fozzard (1982) have also shown a rate-dependent increase of a_{Ca}^i in sheep cardiac Purkinje fibres. Similar conclusions were drawn by Hilgemann (1986b) following the demonstration that the magnitude of extracellular [total Ca^{2+}] depletion signals were markedly enhanced following an increase in the rate of stimulation. Experiments conducted on ventricular myocytes isolated from rat and rabbit hearts (Rich, Langer and Klassen, 1987) suggest that a significant fraction of Ca^{2+} entering the cell during stimulation is sequestered into a rapidly exchangeable compartment localized near the inner surface of the membrane which remains in rapid equilibrium with the extracellular space, while the remaining Ca^{2+} is buffered primarily by the SR. Although the precise ratio of Ca^{2+} taken up by either compartment in response to stimulation at a given frequency is a matter for speculation (e.g., Lewartowski and Pytkowski, 1988), it seems

reasonable to assume that the amount of Ca^{2+} sequestered into the SR for later release is enhanced in response to an increase of stimulation frequency (Gibbons and Fozzard, 1971; Kurihara and Sakai, 1985; Bouchard and Bose, 1989). This proposal is supported by the demonstration that an increase of intracellular [free Ca^{2+}] serves to stimulate the ATPase-phospholamban system of the SR (Tada and Katz, 1982), as well as the rate at which this Ca^{2+} can be pumped (Froehlich, Lakatta, Beard, Spurgeon, Weisfeldt and Gerstenblith, 1978). Similar conclusions have been drawn as to both the existence and availability of intracellular Ca^{2+} stores and the strength of contractions which occur in response to variations in the rate and rhythm of stimulation using X-ray probe microanalysis to investigate the elemental distribution of various cellular compartments. In these experiments, the most profound alterations of cellular Ca^{2+} content accompanying changes in the pattern of stimulation were found in the lateral cisternae of the SR in multicellular preparations of guinea-pig (Wendt-Gallitelli, 1985; Wendt-Gallitelli and Jacob, 1985; see also Isenberg and Wendt-Gallitelli, 1987) and rabbit (Tormey and Walsh, 1989) ventricular muscle.

Other, more indirect, evidence supporting the involvement of intracellular release of Ca^{2+} includes the demonstration in rabbit papillary muscles that the frequency-force relation has a bell-shaped dependence on extracellular [total Ca^{2+}] (Singal, Gupta and Prasad, 1985). The slope of the positive staircase peaked at ~ 3.0 mM extracellular [total Ca^{2+}], following which elevation of bath Ca^{2+} exerted an inhibitory effect on the slope of the frequency relation, leading eventually to a rate-dependent depression of steady-state contraction at extracellular [total Ca^{2+}] > 7.5 mM. This result is consistent with the rate-dependent saturation of an intracellular compartment with Ca^{2+} (Allen et al, 1985, Kort and Lakatta, 1988a,b) and is supported by the observation that a gradual increase of resting tension developed as the stimulation frequency was enhanced only in those experiments in which external Ca^{2+} was significantly elevated and the slope of the staircase response was inhibited. In a similar experiment, Kort and Lakatta (1988b) investigated the relationship between frequency-dependent contractions and spontaneous SR Ca^{2+} release in rabbit ventricular muscle. Unlike " Ca^{2+} -induced release of Ca^{2+} ", which is defined as Ca^{2+} release

induced by a rapid change of intracellular [free Ca^{2+}] at the outer surface of the SR, "spontaneous release of Ca^{2+} " refers to oscillations of accumulation and release of Ca^{2+} by the SR resulting in cyclic contractions which occur when the steady-state intracellular [free Ca^{2+}] is above a critical threshold level (Fabiato and Fabiato, 1972; 1975; 1978). In the experiments of Kort and Lakatta (1988a), the average frequency of spontaneous release measured during the course of a one minute rest interval rose in parallel with serial increases of extracellular [total Ca^{2+}] up to 20 mM. In the presence of 20 mM external Ca^{2+} a negative frequency-force relationship was demonstrated, although the amplitude of contractions elicited at a basal frequency of 0.1 Hz had a linear dependence on extracellular [total Ca^{2+}]. In addition to inhibiting the staircase response, raising external Ca^{2+} markedly enhanced the average frequency of spontaneous release by as much as by 500%. This effect could be overcome by the addition of ryanodine to inhibit Ca^{2+} storage in the SR, which also restored the positive contractile staircase. The authors concluded that the depression of the staircase response in the presence of high concentrations of extracellular Ca^{2+} was the result of Ca^{2+} overloading of the SR and the resulting increase in the frequency of spontaneous SR Ca^{2+} release.

Perhaps the strongest evidence in support of a physiologically relevant increase of SR Ca^{2+} uptake and release into the myofilament comes from studies employing rapid cooling contractures to estimate the content of Ca^{2+} present within pooled compartments of the SR. This technique was originally described by Sakai in 1964 in conjunction with caffeine administration as an assay for intracellular Ca^{2+} loading in skeletal muscle, and subsequently extended to cardiac muscle by Kurihara and Sakai in 1985. In contrast to skeletal muscle preparations, contractures elicited by rapidly lowering perfusate temperature from $\sim 35^{\circ}\text{C}$ to $0\text{-}2^{\circ}\text{C}$ (<1 sec) in cardiac muscle do not require pretreatment with caffeine, thus leaving the mechanisms mediating the EC-Coupling cascade relatively unperturbed during the process of cooling. Initial studies performed to determine the validity of cooling contractures as representative of intracellular Ca^{2+} loading have provided strong evidence in support of the SR as the source of Ca^{2+} activating contractures, including: the demonstration that they are (a) profoundly affected by pharmacological

manipulations which alter the availability and release of Ca^{2+} from the SR, (b) remain unaffected by experimental manipulations designed to alter Ca^{2+} influx across the sarcolemma during cooling, and (c) have a species dependence similar to that shown for postrest contraction (Bers, 1985), spontaneous diastolic Ca^{2+} release from stores within the SR (Stern, Capogrossi and Lakatta, 1988), and dependence of contraction on Ca^{2+} -induced release of Ca^{2+} from the SR (Fabiato, 1982; 1983). Since that time this technique has enjoyed wide use as a reliable measure of intracellular Ca^{2+} loading in intact tissues under various physiological (Bridge, 1986; Bers, 1989; Bers and Bridge, 1989; Hryshko, Stiffel and Bers, 1989; Bouchard and Bose, 1989; Bers, Bridge and Spitzer, 1989), pharmacological (Bers, Bridge and MacLeod, 1987; Hryshko, Kobayashi and Bose, 1989; Bouchard, Hryshko, Saha and Bose, 1989) and pathophysiological conditions (Bers and Bridge, 1988; Komai and Rusy, 1989). Taking into consideration the large drop in sensitivity of the myofilaments to Ca^{2+} during cooling (Harrison and Bers, 1988; 1989), comparison of quantitative differences of intracellular Ca^{2+} transients associated with cooling and regular stimulation (Bers, Bridge and Spitzer, 1989) suggests that rapid cooling of cardiac muscle induces the release of the majority of Ca^{2+} present within the SR. Montgomery, MacLeod and Williams (1990) have shown that following reconstitution into planar lipid bilayers, the 96 pS Ca^{2+} -release channel of the SR is converted during cooling to 12°C into a subconducting state (56 pS). Lifetime analysis of channel properties indicated that cooling also enhanced the open probability of the channel and reduced the frequency of closing. If the assumption is correct that a similar process occurs during cooling to 0-2°C, then the observations of Montgomery and co-workers may provide a possible mechanism by which cooling induces the development of contracture in the absence of significant membrane depolarization (Kurihara and Sakai, 1985; Bridge, 1986).

Kurihara and Sakai (1985) have shown in guinea-pig ventricular muscle that increasing the frequency of stimulation results in a parallel increase in the strength of steady-state contraction and rapid cooling contracture in the absence of significant changes in the plateau phase of the action potential. The relative increase of contraction and cooling contracture in this experiment was similar to that noted when extracellular $[\text{total Ca}^{2+}]$ was raised or the number of beats at a fixed

frequency prior to cooling was increased. Similar observations have been made in canine ventricular muscle (Bouchard and Bose, 1989). In this study the rate-dependent increase of steady-state cooling contracture amplitude was observed to be proportional to the slope of the positive staircase of steady-state contraction and rate of recirculation of Ca^{2+} between functional compartments of the SR, both of which were affected equally by raising or lowering extracellular $[\text{total Ca}^{2+}]$. Gibbons and Fozzard (1971) used a similar perfusion technique to estimate the size of intracellular Ca^{2+} stores in sheep atrial trabeculae. Instead of rapidly cooling the preparation, however, the ionic composition of the perfusion medium was switched to one containing 108 mM K^+ and 50 mM Na^+ . This had the effect of producing a biphasic contracture, where the first rapid phase of contracture development was proportional to the rate of stimulation and extracellular $[\text{total Ca}^{2+}]$. The authors concluded that the amplitude of the first rapid phase of contracture reflected an intracellular pool of Ca^{2+} which was normally released in response to membrane depolarization during an action potential (e.g., Wood et al, 1969). The second, slower, phase of tension rise was found to be generally insensitive to conditions which altered the first rapid phase of contracture. Instead, this delayed component of contracture had a greater dependence on the level of depolarization reached during the course of contracture, similar to the delayed peak observed in the voltage-clamp studies of Wood, Hepner and Weidmann (1969).

Evidence obtained with the different approaches described above are consistent with the interpretation that frequency-dependent alterations of net sarcolemmal Ca^{2+} transport influences the extent of Ca^{2+} replenishment and release from the intracellular stores. It is likely that part of this Ca^{2+} is derived from the increase of time-averaged Ca^{2+} influx during I_{Ca} as well as shifts of the dominant mode of the $\text{Na}^+-\text{Ca}^{2+}$ process due to possible rate-dependent changes of the theoretical quantity $E_m - E_{\text{NaCa}}$. The fact that the positive frequency-force relation can be abolished or reversed following application of various organic or inorganic Ca^{2+} channel blockers (Braveny, Juggi and Mohan, 1985), and either lowering extracellular $[\text{total Na}^+]$ (Hilgemann, 1986a) or agents which inhibit the Na^+ pump (Singal and Prasad, 1979) support the involvement of both

pathways, although the strength of this conclusion is tempered significantly by the known use-dependence of such test agents.

2.3 Postrest Contraction

Restitution of contraction following variable periods of mechanical rest has been used in several laboratories to gain insight into the control of cardiac contractility. Studies of this nature have demonstrated clearly that there is a continuous recovery (or restitution) process which takes place between stimulated contractions and is renewed after each stimulus (Kruta and Braveny, 1961; Koch-Weser and Blinks, 1963; Gibbons, 1986). A schematic representation of this concept is illustrated in Fig. 2, which has been derived by plotting the strength of contractions elicited in response to test intervals extending from premature stimulation (upper panel) to rested state contractions (lower panel). The exponential time course of contractile restitution in either direction indicates that steady-state contractions do not reflect a "steady-state" of the cellular processes responsible for the delivery of Ca^{2+} to the myofilaments. As demonstrated for both voltage-dependent (Lipsius, Gibbons and Fozzard, 1982) and time-dependent (Schouten, vanDeen, deTomb and Verveen, 1987) restitution, the strength of contraction at a given interval following stimulation at a constant frequency appears to reflect the integrated result of a number of basic cellular processes, and as such can be used as an important determinant of cardiac contractility. The precise shape of restitution curves describing the time-dependence of postrest contraction varies widely with each tissue type investigated, having the steepest upward slope in those species possessing extensively developed sarcotubular networks (e.g., rat ventricle) and the greatest negative slope in species with little or no SR (rabbit ventricle and frog). In the case of rat or canine ventricular muscle (Endoh and Iijima, 1981; Ragnarsdottir, Wohlfart and Johannson, 1982; Bers, 1985; Bose, Hryshko, King and Chau, 1988; Bouchard and Bose, 1989) contraction can be potentiated by rest intervals as short as 2 sec following termination of rhythmic stimulation and can remain potentiated well after 10 min of rest (depending on the experimental conditions). Similar

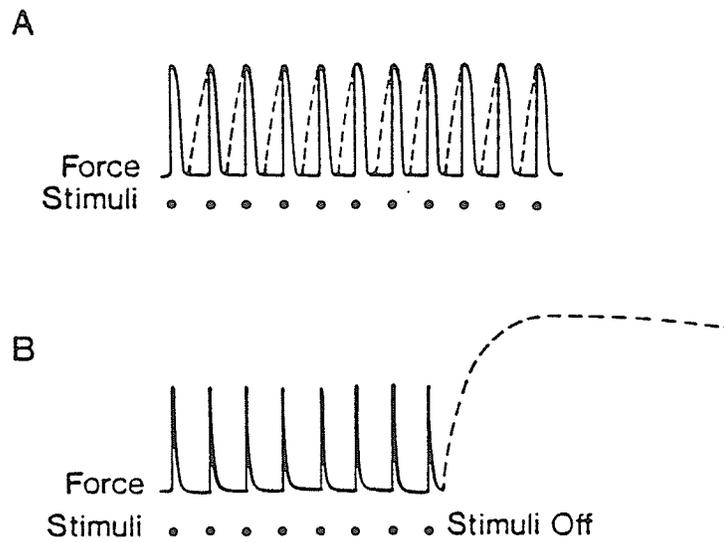


Figure 2. Schematic representation taken from Gibbons (1986) of the restitution of contraction in mammalian ventricular muscle. Dashed lines were obtained by plotting the amplitude of extrasystolic (*Panel A*) and postrest contraction (*Panel B*) in relation to preceding steady-state contractions.

results have been shown in rabbit atrium (Hilgemann, 1986a) and sheep Purkinje fibres (Gibbons and Fozzard, 1975; Boyett et al, 1987). In most species which display a strong positive frequency-force relation, resumption of stimulation after even a very brief rest interval (<2 sec) results in "rest-decay" whereby the amplitude of the first postrest contraction is smaller than that of the preceding steady-state contraction. This has been shown in rabbit ventricle (Bers, 1985; Kort and Lakatta, 1988b) guinea-pig atrial and ventricular tissues (Pytkowski, 1988; Beyer, Hergeroder and Ravens, 1988; Johannson and Asgrimsson, 1989) and is most prominent in tissues lacking significant intracellular stores of Ca^{2+} (e.g., frog ventricle: Noble and Shimoni, 1981; Bers, 1985).

In general, postrest contractions are highly sensitive to pharmacological manipulations which alter intracellular Ca^{2+} stores, rather than those which perturb the entry of Ca^{2+} during contraction. For example, it has been shown that the amplitude of the first postrest beat and ensuing recovery of steady-state contraction are preferentially blocked by agents which alter the ability of the SR to sequester and release Ca^{2+} , such as ryanodine, caffeine or strontium (Bers, 1985; Boyett et al, 1987; Kort and Lakatta, 1988b; Bose et al, 1988; Pytkowski, 1988; Beyers et al, 1988; Bouchard et al, 1989; Hryshko et al, 1989a,c), as opposed to those which alter steady-state Ca^{2+} influx such as cobalt and lanthanum (Bers, 1985), or various organic and inorganic Ca^{2+} channels blockers (Saha, Hryshko, Bouchard, Chau and Bose, 1989). This compares favourably with the observation that rapid cooling contracture decay is accelerated in a similar fashion by almost all of the interventions mentioned above which perturb SR function and provides a reasonable explanation for the time-dependent decay of postrest contraction.

Calcium influx during postrest contractions estimated with Ca^{2+} -sensitive electrodes (Bers, 1985; Bers and MacLeod, 1986) or tetramethylmurexide (Hilgemann, 1986b) has been found to be depressed compared with preceding steady-state contractions. This is consistent with observations made on numerous tissue types that I_{Ca} peak is depressed during the first postrest excitation in ventricular myocytes voltage-clamped from physiological holding potentials (Bers and Hess, 1984; Houser and duBell, 1988; Hryshko and Bers, 1988; duBell and Houser, 1989). Action potentials recorded during the recovery of contraction display depressed plateau voltages in most

cardiac tissues, including rat (Schouten and terKeurs, 1986; Johannsson and Asgrimsson, 1989), dog (Endoh and Iijima, 1981; Hryshko, Bouchard, Chau and Bose, 1989) and rabbit (Bers, 1985; Hilgemann, 1986a), although not others (guinea-pig: Beyer, Hergeroder and Ravens, 1988; Johannsson and Asgrimsson, 1989). Changes in the duration of the action potential appear to be closely related to the degree of inotropy associated with the accompanying contraction; prolonged in tissues with strong postrest contractions, e.g., rat ventricle, canine ventricle, rabbit atrium and sheep Purkinje tissues, and depressed in species with poor postrest contractions such as rabbit, guinea-pig and frog ventricle. As discussed above, changes in the rate of repolarization during postrest stimulation are strongly influenced by the species-specific sensitivity of the transient outward in various tissues to both rate changes and the level of myoplasmic Ca^{2+} (Peper and Trautwein, 1968; Gibbons and Fozzard, 1975; Hilgemann, 1986a; Hiroaka and Kawano, 1987; Tseng, 1988; Shibata et al, 1989), and shifts in modal activity of the Na^+ - Ca^{2+} exchanger during the course of the Ca^{2+} transient (Schouten and terKeurs, 1986; Hilgemann, 1986a; Hilgemann and Noble, 1987; Egan, Noble, Noble, Powell and Spindler, 1989; Giles and Shimoni, 1989a).

For a given level of intracellular loading, there appear to be a minimum of three factors which together determine the amount of force that can be developed during contractions elicited in response to postrest stimulation. These are (a) time-dependent inactivation of the Ca^{2+} -release channel complex, (b) time-dependent recirculation of Ca^{2+} between functional pools of the SR, and (c) the rate of Ca^{2+} leak from the release compartment. Each of these processes will be addressed separately in the following sections.

Time-dependent inactivation of the Ca^{2+} -release channel: Evidence has been presented by Fabiato (1983, 1985b) that Ca^{2+} -induced release of Ca^{2+} from the SR occurs via membrane channels with both Ca^{2+} - and time-dependent inactivation kinetics. To this end, application of 5 mM caffeine to skinned canine Purkinje fibres following various delays after a Ca^{2+} transient triggered by Ca^{2+} -induced release of Ca^{2+} led to a situation where no test interval could be demonstrated following which caffeine could not elicit a subsequent Ca^{2+} transient (including any

time during a transient elicited by Ca^{2+} -induced release of Ca^{2+}). The restitution of Ca^{2+} transients elicited by caffeine occurred in an exponential fashion; with a rapid phase taking 750 ms to complete, following which a slower phase of Ca^{2+} accumulation was observed which reached a peak at ~ 16 sec. After determining the bulk solution [free Ca^{2+}] which triggered a Ca^{2+} transient equivalent to that induced by caffeine ($\text{pCa} = 5.85$), the experiments were repeated to determine the restitution of Ca^{2+} -induced release of Ca^{2+} . In contrast to the caffeine experiments, no Ca^{2+} -induced release of Ca^{2+} could be elicited with delays < 750 ms (approximately the time required to reprime the release site to 66.6% of its maximal capacity), following which the amplitude of Ca^{2+} -induced release of Ca^{2+} increased rapidly to the point where the magnitude of the Ca^{2+} transient was no longer significantly different from that induced by caffeine (~ 3.5 sec). From this point onwards, the amount of Ca^{2+} released from the SR was dependent solely on the reaccumulation of Ca^{2+} within the release pool prior to stimulation. An important set of observations during these experiments was that the duration of the absolute refractory period could not be overcome by increasing the pCa trigger between 6.60 and 5.60, and that a reduction of the bath temperature from 22°C to 12°C increased both the absolute and relative refractory periods by a factor of 4. It was concluded, that if the Q_{10} of ~ 4 is applicable to the 22°C to $35\text{-}37^\circ\text{C}$ temperature range then the absolute refractory period would not impinge on *in situ* cardiac function, even at the maximal heart rate. This can be illustrated by the fact that extrasystolic contractions can be elicited with a delay following normal contraction of as short as 250 ms, after which the amplitude of the extrasystolic contraction recovers with an exponential time course similar to that of the parallel increase of caffeine-induced and Ca^{2+} -induced release of Ca^{2+} (Yue, Burkhoff, Franz, Hunter and Sagawa, 1985; Wier and Yue, 1986). Data will be presented in SECTION 3 of the RESULTS which suggests that although the absolute mechanical refractory period of intact ventricular preparations to premature stimulation (< 200 ms) may be related more to restitution of the action potential, the relative refractory period of Ca^{2+} -induced release of Ca^{2+} can be demonstrated (200-600 ms), and may be overcome partially by increasing the amount of Ca^{2+} reaccumulation into the release compartment.

Time-dependent recirculation of Ca^{2+} : Schouten, vanDeen, deTombe and Verveen (1987) have derived a mathematical model to describe contractile force as a function of stimulus interval in mammalian ventricular muscle (cf. Koch-Weser and Blinks, 1963). Peak force in this analysis was assumed to be proportional to the amount of Ca^{2+} released from the release compartment of the SR. Six independent parameters were necessary to reproduce the interval-force relation, assuming a recirculating fraction, $r = 0.65$. Although data from cooling experiments performed in our laboratory suggest that this may be an overestimate of this value at stimulation frequencies < 1.0 Hz, simulations of post-extrasystolic potentiation, frequency-potentiation, and postrest-potentiation could be constructed which fit the experimental data reasonably well by including three intracellular compartments, and assuming $[\text{Ca}^{2+}]_o$ to be < 1.0 mM. An uptake compartment was described from which Ca^{2+} is transported in a unidirectional fashion to a release compartment. The rate of Ca^{2+} transport was assumed in this model to be proportional to the amount of Ca^{2+} present within the uptake compartment at a given moment in time. This assumption is supported by the observation that exponential recovery of extrasystolic contraction and de-potentiation of post-extrasystolic contraction is affected in a linear fashion by an increase in the rate of steady-state stimulation or extracellular $[\text{total Ca}^{2+}]$ (Wood, Hepner and Weidmann, 1969; Wier and Yue, 1986; Bose, Hryshko, King and Chau, 1988; Johansson and Agrimsson, 1989; see also Orchard and Lakatta, 1985; Allen, Jewell and Wood, 1976). An important component of this model was the inclusion of a third rapidly exchanging compartment which was capable of transporting Ca^{2+} to and from the release compartment in a *bidirectional* fashion, similar to that proposed earlier by Ragnarsdottir, Wohlfart and Johannsson (1982) and later modified by Rich, Langer and Klassen (1987) and Shattock and Bers (1989). The utility of this model is that it helps to explain the wide spectrum of postrest responses seen in various tissues in relation to the functional characteristics of the SR, and not simply by its ability to store and release Ca^{2+} .

Complementary information has been gained from ultrastructural studies, evidence from which suggests that the SR of mammalian ventricular muscle may be composed of more than one

structurally distinct compartment, although not all authors agree with this interpretation (Hunter, Haworth and Berkoff, 1981; Lewartowski and Pytkowski, 1988; Wendt-Gallitelli and Isenberg, 1989; for review, see Sommer and Jennings, 1986). A combination of biochemical and immunoelectron microscopical studies have revealed an anastomosing network of sarcotubules surrounding the myofibrils which contain a uniform distribution of Ca^{2+} -ATPase (Jorgensen, Sheu, Daly and McLennan, 1982) and its regulatory protein phospholamban (Jorgensen and Jones, 1987). Extending from this network in an area confined mainly to the I-band region is the corbular SR (Jorgensen, Broderick, Somlyo and Somlyo, 1988) which contains the Ca^{2+} binding protein calsequestrin. The junctional SR also contains large concentrations of calsequestrin and is apposed closely to the sarcolemma at the transverse tubule, to which it is physically connected by junctional proteins referred to as "foot proteins" (Somlyo, 1979; Franzini-Armstrong, 1980; Meissner, 1986). Based on the relative content of the three compartments in resting papillary muscles obtained from rat hearts (junctional-SR > corbular-SR >> network-SR), Jorgensen et al (1988) have suggested that enough Ca^{2+} was present in the first two compartments to support 50-90% contraction assuming the entire contents of each were released in response to an action potential. The estimated increase of 247 μM [total Ca^{2+}]/litre cell water is rather likely to be significantly overestimated with respect to physiological contraction, as the preparations were equilibrated in 2.0 mM extracellular [total Ca^{2+}] and then rested for 10 min prior to flash-freezing. This conclusion is supported by a number of related observations including the demonstration that the SR of rat ventricle is (a) the most extensively developed of all mammalian, avian and amphibian species studied to date (Sommer and Jennings, 1986; see also Fabiato, 1982; 1983; Bers, 1985; Stern et al, 1988), (b) may actually gain small amounts of Ca^{2+} during the course of quiescence (Ragnarsdottir, Wohlfart and Johannsson, 1982; Lukas and Bose, 1986; Kort and Lakatta, 1988a; Shattock and Bers, 1989), and (c) other data obtained with a number of methodological approaches which indicate that intracellular stores in the rat are saturated at extracellular [total Ca^{2+}] equal to or greater than ~ 2.0 mM (Fabiato, 1981; Schouten and terKeurs, 1986; Kentish, terKeurs, Ricciardi, Bucx and Noble, 1986; Kennedy, Akera and Brody, 1987; Capogrossi, Stern

and Lakatta, 1988). Although the points considered above temper somewhat the conclusions reached from electron probe microanalysis studies regarding the amount of Ca^{2+} present within focal regions of the SR, the data do provide a rough estimate of the buffering capacity of the reticulum for Ca^{2+} and, to a certain extent, offer some qualitative information regarding the relative volume of SR attributed to functional "release" and/or "uptake" sites, and hence the degree to which contraction may be governed by rate constants for Ca^{2+} transport between these compartments during the normal diastolic interval.

In contrast to most mammalian and amphibian species, ventricular muscle from the rat (Kelly and Hoffman, 1960; Forester and Mainwood, 1974), mouse (Temma and Akera, 1986), and hamster (Howlett and Gordon, 1987) exhibit a negative staircase of steady-state contraction in response to a graded increase in the rate of stimulation. Although the possibility does exist that this may reflect tissue hypoxia in thicker preparations (Schouten and terKeurs, 1986; Gulch and Ebrecht, 1986), this is unlikely due to the demonstration of this phenomenon in freshly dispersed ventricular myocytes (Mitchell, Powel, Terrar and Twist, 1985; Haworth, Griffin, Saleh, Goknur and Berkoff, 1987; Capogrossi, Stern and Lakatta, 1988). It has been proposed from modelling studies on the rate of mechanical restitution in the rat (Capogrossi, Stern and Lakatta, 1988; Stern, Capogrossi, Spurgeon and Lakatta, 1988) that the average Ca^{2+} release (% maximal) can be increased at a given extracellular [total Ca^{2+}] by lowering the rate of stimulation, or alternatively, at a given point during the restitution process by raising extracellular Ca^{2+} to an optimal concentration (<2.0 mM). Opposite results were obtained by Schouten et al (1987) who calculated that an increase of priming frequency in the presence of 0.8 mM extracellular Ca^{2+} would lead to an increase of force development at test intervals > 1 sec. However, this apparent difference is reconciled by earlier reports (Forrester and Mainwood, 1974; Orchard and Lakatta, 1985) of a positive frequency-force relation in rat ventricular muscle bathed in extracellular [total Ca^{2+}] <1.0 mM. That the time constant for Ca^{2+} transport between functional pools may be involved in limiting the strength of steady-state contraction is suggested by the fact that although postrest contractions at longer test intervals (>30 sec) are not significantly altered by raising or lowering

the rate of preceding stimulation (Bouchard and Bose, 1989), contractions elicited at coupling intervals between 2-10 sec are depressed in a frequency-dependent manner, the extent to which is irrespective of the extracellular Ca^{2+} employed (see Fig. 52).

Time-dependent leak of Ca^{2+} from intracellular stores: The third aspect of SR function which plays an important role in mediating the amount of Ca^{2+} released in response to postrest stimulation is the poorly-defined process of Ca^{2+} "leak" from the SR, eventually into the extracellular space. Although relatively little direct information is available regarding the cellular mechanism(s) underlying this process, a large amount of indirect evidence supports the involvement of the Na^+ - Ca^{2+} exchange process. One of the characteristic features of the postrest response in all species studied to date is that if sufficient time is allowed to pass during the course of rest, contraction amplitude decays to the point where rested state contractions develop (Kruta and Braveny, 1961; Blinks and Koch-Weser, 1961; Koch-Weser and Blinks, 1963; Allen, Jewell and Wood, 1976). This decay has been correlated with a net loss of cellular Ca^{2+} content measured by electron probe X-ray microanalysis (Wendt-Gallitelli, 1985; Wendt-Gallitelli and Jacob, 1985; Walsh and Tormay, 1989) and the decay of radioactive Ca^{2+} (Janczewski and Lewartowski, 1986; Pytkowski, 1988; Lewartowski and Pytkowski, 1988), and is seen in intact ventricular preparations as a time-dependent repletion of net extracellular [total Ca^{2+}] (Hilgemann, Delay and Langer, 1983; Hilgemann, 1986a; 1986b; Bers and MacLeod, 1986; Bers, Bridge and MacLeod, 1987).

Experiments performed with the rapid cooling technique have strongly implicated the SR as the source of this Ca^{2+} . In an elegant series of experiments, Bridge (1986) was able to demonstrate that postrest decay of cooling contracture amplitude in rabbit ventricular muscle was almost identical in its time course with the rest-decay of contraction reported earlier for rabbit ventricular muscle by Bers (1985). Further experiments showed that the loss of cellular Ca^{2+} occurred with a similar rate constant ($t_{1/2} = 90$ sec) to the leak of Ca^{2+} from saponin-treated guinea-pig ventricular fibres reported previously by Kitazawa (1984), and was paralleled in experiments performed under identical conditions by the loss of total intracellular Ca^{2+} estimated with atomic absorption

spectroscopy. With the exception of rat ventricular muscle, which appears to gain tissue Ca^{2+} during quiescence (Kort and Lakatta, 1988b; Shattock and Bers, 1989), time-dependent loss of Ca^{2+} from the SR has been reported in ventricular preparations from almost all species investigated with the rapid cooling technique (guinea-pig: Kurihara and Sakai, 1985; Bers, 1989; Bers, Bridge and Spitzer, 1989; rabbit: Bridge, 1986; Bers, Bridge and MacLeod, 1987; Hryshko, Stiffel and Bers, 1989; canine: Hryshko, Kobayashi and Bose, 1989; Bouchard and Bose, 1989).

One of the first systematic investigations of the cellular mechanisms responsible for the intracellular Ca^{2+} leak was reported by Sutko, Bers and Reeves (1986), who investigated the effects of interventions known to affect the trans-membrane exchange of Na^+ for Ca^{2+} on steady-state and postrest contractions. A preferential effect on postrest contraction was demonstrated following an increase of extracellular $[\text{total Ca}^{2+}]$, reduction of external Na^+ while maintaining a constant $[\text{Ca}^{2+}]_o/[\text{Na}^+]_o^2$ ratio, and inhibition of the Na^+ pump by reducing external K^+ or by acetylstrophanthidin. These effects were not due to a general increase in contractility, as parallel effects on postrest and steady-state contractions were obtained in response to treatment with isoproterenol, norepinephrine and histamine, while inhibition of SR function with ryanodine preferentially inhibited postrest contraction. The authors concluded that the maintenance of postrest contraction following reduction of the cellular Na^+ gradient was due to the inhibition of $[\text{Na}^+]_o$ -dependent diastolic Ca^{2+} efflux. A second conclusion was that the Na^+ - Ca^{2+} exchange process, through its ability to modulate the total intracellular Ca^{2+} load, was also responsible for determining the amount of Ca^{2+} available for release in response to rhythmic stimulation. To this end, the recovery of steady-state contraction during postrest recovery has been associated with parallel shifts of both a_{Na}^i (Boyett et al, 1987; Shattock and Bers, 1989) and cumulative extracellular $[\text{total Ca}^{2+}]$ depletion (Bers, 1985; Hilgemann, 1986a). Using Ca^{2+} -sensitive microelectrode to track changes of extracellular $[\text{total Ca}^{2+}]$, Bers and MacLeod (1986) demonstrated that the repletion of Ca^{2+} in the diffusible extracellular space during the course of rest was strongly inhibited by reducing external Na^+ to 70 mM or by acetylstrophanthidin. In addition to its effects on $[\text{total Ca}^{2+}]$ excursions during rest, low- Na^+ perfusion also greatly

decreased the extent and delayed the onset of the depletion signal following resumption of stimulation after rest, leading the authors to suggest that the extent of tissue Ca^{2+} was related to the electrochemical Na^+ gradient. Similar results were obtained by Hilgemann (1986b) using tetraethylmurexide to monitor extracellular [total Ca^{2+}] during rest in rabbit atrial muscle.

Rest-decay of rapid cooling contractures in guinea-pig ventricular myocytes has been studied by Bers, Bridge and Spitzer (1989) with the intracellular fluorescent Ca^{2+} indicator indo-1. The time-dependent reduction in the magnitude of rapid cooling contracture and intracellular Ca^{2+} transients was found to be almost completely prevented by removal of $[\text{Na}^+]_o$ during the course of rest. The lack of rest-decay prompted the authors to conclude that the ability of the Na^+ - Ca^{2+} exchanger to extrude Ca^{2+} from resting cells was much greater than that of the sarcolemmal Ca^{2+} -ATPase, due to the fact that the gradient for cellular Ca^{2+} extrusion during cooling from 22°C to $0\text{-}1^\circ\text{C}$ was greatly reduced (Na^+ -free, Ca^{2+} -free medium). Perfusion with low- Na^+ solutions was also found to inhibit the progressive decay of serial cooling contractures, thus revealing the reduced ability of the cells to extrude myoplasmic Ca^{2+} during rewarming (and hence increased uptake by the SR Ca^{2+} -ATPase). Similar results have been obtained with the paired-cooling protocol in rabbit ventricular myocytes by Hryshko, Stiffel and Bers (1989).

It is unknown whether the diastolic leak of intracellular Ca^{2+} occurs directly through a $[\text{Na}^+]_o$ -dependent mechanism located at the junction of the sarcolemma and SR at the transverse tubule, or if the exchanger simply mediates the extrusion of Ca^{2+} from the Ca^{2+} -release channel complex in a closed intracellular compartment similar to that hypothesized by Lederer, Niggli and Hadley (1990). Recent experiments conducted with purified enantiomers and the racemic form of the dihydropyridine BAY K 8644 may help shed some light on this issue. The original observation that BAY K 8644 had a negative inotropic effect on postrest contraction was made by Bose, Kobayashi, Hryshko and Chau in 1986, and appeared to contrast with the positive inotropic effect of this compound on steady-state contraction (Thomas, Chung and Cohen, 1985; Thomas, Gross, Pfitzer and Ruegg, 1985) and membrane Ca^{2+} conductance (Hess, Lansman and Tsien, 1984; Brown, Kunze and Yatani, 1984). It was subsequently shown that BAY K 8644 accelerated the loss of Ca^{2+}

from the SR during diastole, which manifested as a striking acceleration in the rate of decay of postrest contraction and postrest cooling contracture (Hryshko, Kobayashi and Bose, 1989; Bouchard, Hryshko, Saha and Bose, 1989). A reduction of trans-sarcolemmal Ca^{2+} entry was ruled out as an effector of this process, because action potentials displayed elevated and prolonged plateau voltages during postrest contractions (Hryshko, Bouchard, Chau and Bose, 1989; Saha, Hryshko, Bouchard, Chau and Bose, 1989). An increase in the average frequency or statistical probability of asynchronous release of Ca^{2+} from intracellular stores during diastole was also discounted, as segmenting the power spectrum of scattered light intensity fluctuations in the presence of BAY K 8644 showed that discrete fluctuations of myofilament oscillation in the 0.3 to 10 Hz range were uniformly reduced (Bose, Kobayashi, Bouchard, Hryshko, 1988; Hryshko, Kobayashi and Bose, 1989). Pharmacological studies comparing the effect of BAY K 8644 on postrest contraction with those elicited by caffeine or ryanodine indicated that the combination of electrophysiological and mechanical effects produced by BAY K 8644 were relatively compatible with those of ryanodine (Bouchard, Hryshko, Saha and Bose, 1989), an effect that was found to be preferential to the (-) enantiomer (agonist form) of BAY K 8644 (Saha, Hryshko, Bouchard, Chau and Bose, 1989). Somewhat similar results were obtained in rat ventricular muscle by Saxon and Gainullin (1990) who found that the full depressant effect of BAY K 8644 on postrest contraction required prior depolarization of the preparation by raising the external K^+ to 10 mM. The effect was reversed following administration of 5 mM manganese, or depolarization of the membrane by 30 mV following an increase of external K^+ to a total concentration of 20 mM.

The results of this type of experiment may inevitably provide important information regarding the molecular constituents of the macroscopic junctional membrane/ Ca^{2+} -release channel complex. At minimum, the data support the existence of a physical contact between the SR and dihydropyridine-sensitive Ca^{2+} channel (Cannell, Berlin and Lederer, 1987; Cohen and Lederer, 1988; Bean and Rios, 1989), not unlike that shown to control intracellular Ca^{2+} release in skeletal muscle (Schneider, 1970; Schneider and Chandler, 1973; Rios and Pizarro, 1989). The partial protection of the BAY K 8644-induced depressant effect by lowering $[\text{Na}^+]_o$ (Hryshko, 1987) also

suggests that Ca^{2+} -induced release of Ca^{2+} may indeed occur within an intracellular compartment of limited volume which also contains a dense population of Na^+ - Ca^{2+} exchange proteins (Leblanc and Hume, 1990). This proposal is consistent with the protective effect of low- Na^+ perfusion on rest-decay, and the fact that a sufficient reduction of $[\text{Na}^+]_o$ results in reverse Na^+ - Ca^{2+} exchange activity measured by ramp currents (Miura and Kimura, 1989). A similar interpretation has been used to explain the time-dependent depletion of extracellular $[\text{total Ca}^{2+}]$ (Hilgemann, 1986b) or the production of substantial cooling contractures (Kurihara and Sakai, 1985) in the absence of electrical stimulation. If the assumption of Schouten et al (1987) regarding the proportionality of the rate of recirculation to the amount of Ca^{2+} present within the uptake compartment is correct, then it is possible that the preservation of postrest contraction takes place in the absence of concomitant changes in the rate of this leak. As will be shown in the RESULTS SECTION, this conclusion may be supported by the demonstration that lowering $[\text{Na}^+]_o$ significantly increases both the rate and extent of mechanical restitution while leaving unaffected the rate-dependence of peak postrest contraction amplitude.

A second possible mechanism for cellular Ca^{2+} extrusion may be the Ca^{2+} -permeable channels recently described by Coulombe, Lefevre, Baro and Coraboeuf (1989). These channels can be recorded in cell-attached, excised inside-out and excised outside-out membrane patches. They show little or no permeability to Na^+ or K^+ , and are open at negative membrane potentials. Of importance with respect to the data of Bose et al (1986; 1988), Hryshko et al (1989a,b) and Saxon and Gainullin (1990) was the finding in these experiments that application of racemic BAY K 8644 significantly enhanced the probability of opening of the channel, the degree to which was enhanced by clamping the cells to more *negative* membrane potentials. However, due to the erratic behaviour of these channels with respect to their opening and closing events, it is for further investigation to resolve whether they might have a physiological role in cellular Ca^{2+} homeostasis. As discussed by Bridge (1986), a time-dependent "leak" of Ca^{2+} would provide an important mechanism to avoid overloading of labile intracellular stores with Ca^{2+} . Prolonged periods of excessive accumulation of Ca^{2+} within the SR has been shown under various experimental

conditions to inhibit developed tension under steady-state conditions, followed inevitably by cell death if left unabated (Ishide, Watanabe and Takishima, 1984; Fabiato, 1983; Allen, Eisner, Pirolo and Smith, 1985; Wier, Cannell, Berlin, Marban, Lederer, 1987; Watanabe, Ishide, Takishima, 1987; Stern, Capogrossi, Spurgeon and Lakatta, 1988; Bers and Bridge, 1988; Bose, Kobayashi, Bouchard and Hryshko, 1988; terKeurs, Schouten, Bucx, Mulder, deTombe, 1988).

3.0 Hypotheses Relating to the Control of Contraction in Mammalian Ventricular Muscle

3.1 Introduction

The foregoing discussion has introduced several key mechanisms that are thought to mediate the rise of intracellular Ca^{2+} during the contraction of mammalian cardiac muscle. Of these, the main focus of the text was on the role of intracellular stores of Ca^{2+} , namely the sarcoplasmic reticulum, in governing contraction on a beat to beat basis. The specific aim of experiments conducted for this Thesis was to quantitate the contribution of Ca^{2+} release from the SR toward the development of contractions elicited in response to perturbations of the rate and rhythm of stimulation. To this end, different patterns of electrical stimulation have been used to reveal, or unmask, different sources of Ca^{2+} available to activate the myofilaments. This will be particularly evident in the experiments which focus on the role of the Na^+ - Ca^{2+} exchange process in mediating contraction in canine ventricular muscle. In addition to altering the ionic composition and temperature of the bathing media, species differences in cardiac ultrastructure have also been used as a tool to study the cellular mechanisms involved in EC-Coupling. This was done in an attempt to avoid numerous pitfalls usually associated with the use of specific pharmacological agents in this setting, although both approaches have been taken in many of the experimental blocks.

The experiments have been broken up into four sections, which represent a series of hypotheses that were put forward to test certain aspects of a model of EC-Coupling for cardiac muscle. The model is of a descriptive nature in that it was based originally on experimental data, and was first proposed by King and Bose in 1984 to account for the mechanism of biphasic contractions in strontium-treated canine ventricular muscle. Two fundamental assumptions of this model were (a) that a fixed, but labile, store of Ca^{2+} was located within the SR, the contents of which was susceptible to changes in the preceding pattern of electrical stimulation, and (b) that

the force of contraction was controlled primarily by Ca^{2+} released from the SR in response to an action potential. These specific assumptions were supported by the available literature and have been made frequently in reviews of the field (Morad and Goldman, 1973; Wolfhart and Noble, 1982; Chapman, 1983; Adler et al, 1985; Gibbons, 1986; Schouten et al, 1987; Lewartowski and Pytkowski, 1988). Later experiments conducted on canine ventricular muscle in this laboratory demonstrated clearly the need to assume more than one intracellular compartment for Ca^{2+} storage and release (Hryshko, 1987) and also provided evidence in support of a direct structural link between dihydropyridine-sensitive Ca^{2+} channels present within the sarcolemma and the release site of the SR (Bose et al, 1986; 1988b; Hryshko and Bose, 1988; Hryshko et al, 1989a,b). The model of excitation-contraction coupling that best fit the experimental data to this point was a "recirculation" type model, similar to that originally proposed by Morad and Goldman (1973) and Wohlfart and Noble (1982).

The principal approach adopted for the experiments presented here has been to describe the quantitative relationship between Ca^{2+} availability within the SR and the trigger for release during different patterns of electrical stimulation and the force of contraction. The amount of Ca^{2+} present within pooled compartments of the SR and that fraction of Ca^{2+} located within the release compartment specifically were estimated independently by the response of various muscle preparations to rapid lowering (<1 sec) of the perfusate temperature from 37°C to 0-2°C (e.g., rapid cooling contracture; Kurihara and Sakai, 1985; Bridge, 1986; Bers, Bridge and MacLeod, 1987) and postrest electrical stimulation at constant coupling intervals. Time constants for unidirectional Ca^{2+} transport within the cell and recovery from inactivation of Ca^{2+} channels associated with the release compartment of the SR were estimated with various stimulation protocols. We have also used the whole-cell variant of the patch-clamp technique (Hamill et al, 1981) in guinea-pig ventricular myocytes in conjunction with parallel mechanical studies on multicellular preparations in an attempt to relate the entry of Ca^{2+} into the cell through voltage-gated channels with accompanying contractions elicited in response to variation of the conditioning interval and perfusate ionic conditions. However due to the preliminary nature of this

work and due to necessary temporal and spatial constraints, data from these experiments has not been described.

3.2 Description of the Model

The working hypothesis described here is a composite model based on past experimental findings by various investigators within the laboratory (King, 1982; King and Bose, 1984; Hryshko, 1987; Bose et al, 1988a,b; Hryshko et al, 1989a) as well as earlier models of EC-Coupling (Morad and Goldman, 1973; Wohlfart and Noble, 1982; Chapman, 1983; Fabiato, 1983; Morad and Cleeman, 1987) and intracellular Ca^{2+} movements within the mammalian myocardium (Koch-Weser and Blinks, 1963; Allen et al, 1976; Adler, et al, 1985; Gibbons, 1986; Schouten et al, 1987; Boyett et al, 1987; Lewartowski and Pytkowski, 1988). The model has been used both to make predictions regarding the cellular mechanisms which might mediate the strength of contraction under different experimental conditions, as well as to fit a number of experimental observations which were not readily explained by other models, or working hypotheses, found in the literature at the time it was constructed.

Contraction is assumed to reflect the rise of intracellular Ca^{2+} following the upstroke of an action potential from a resting level of near 100 nM to a transient peak of $\sim 1\text{-}1.5 \mu\text{M}$ (Allen and Blinks, 1978; Weir, 1980; Weir et al, 1986; Cannell et al, 1987; Beuckelmann and Weir, 1988; Callaewert et al, 1989). This transient increase of intracellular [free Ca^{2+}] appears to reflect primarily a graded release from internal stores localised specifically within the SR (Fabiato and Fabiato, 1976; 1978; Fabiato, 1983; 1985a-c), stimulated by the influx of Ca^{2+} itself across the sarcolemma through voltage-gated channels (Fabiato, 1983; Fabiato, 1985b,c; Morad and Cleeman, 1987; Callaewert et al, 1989; Nabauer et al, 1989) or possibly the reverse mode of the Na^+ - Ca^{2+} exchange (Leblanc and Hume, 1990). In addition to acting as a trigger for intracellular Ca^{2+} release, trans-membrane Ca^{2+} entry (or inhibited efflux) during the flow of either I_{Ca} or I_{NaCa} also serves to replenish the SR with Ca^{2+} for later release. As discussed in the INTRODUCTION, Ca^{2+} entry during the action

potential is unlikely to be responsible for direct activation of the myofilaments, except in those contractions associated with greatly prolonged action potentials (Morad and Trautwein, 1968; Brill et al, 1986; Boyett et al, 1987; Terrar and White, 1989) or in species with poorly developed or functioning SR. Repolarization of the action potential is associated with the termination of contraction and net cellular Ca^{2+} loss into both the extracellular space (Bers, 1983; Hilgemann, 1986a,b) and SR (Bers and Bridge, 1989) which occurs through the activity of the forward mode of the Na^+ - Ca^{2+} exchange process and a combination of the sarcolemmal and SR ATP-dependent Ca^{2+} pumps.

The volume of Ca^{2+} taken up into the sarcotubular network of the SR following contraction is subsequently made available for release in a time-dependent fashion, either through a time-dependent translocation from an "uptake" site to a "release" site during diastole (Morad and Goldman, 1973; Wohlfart and Noble, 1982; Schouten et al, 1987), or a slow recovery of the SR Ca^{2+} -release channels (Fabiato, 1985b). This process of mechanical restitution occurs at a rate proportional to the amount of Ca^{2+} present within the uptake compartment (Schouten et al, 1987), which can be described by the time-dependent decrease of postrest cooling contractures (Bridge, 1986) or decay in the amplitude of recirculating fraction of Ca^{2+} available for release in response to postrest stimulation (Ragnarsdottir et al, 1982; Bers, 1985; Bose et al, 1988; Hryshko, 1987). This inotropic effect of Ca^{2+} recirculation is opposed by a continual "leak" of Ca^{2+} , also from the release compartment of the SR, eventually out into the extracellular space (Hilgemann et al, 1984; Bers and MacLeod, 1986; Hilgemann, 1986a,b; Bers et al, 1987). The mechanisms responsible for extruding this Ca^{2+} out of the cell are identical to those which remove it during relaxation. Thus, with a constant initial intracellular load of Ca^{2+} the model predicts that the amount of Ca^{2+} available for release is a function of the rate of Ca^{2+} transport between functional pools during diastole, the rate of Ca^{2+} loss from the SR, and the recovery from inactivation of the process of Ca^{2+} -induced release of Ca^{2+} . While not all aspects of this working hypothesis are universally accepted, most components can be found within several frequently cited reviews or papers,

particularly those dealing with EC-Coupling in species that are strongly dependent on intracellular Ca^{2+} stores for contraction.

3.3 Hypotheses Related to the Control of Cardiac Contraction

Section 1: The first section of the Thesis deals with the effects of pharmacological agents which perturb trans-sarcolammel Ca^{2+} entry and SR Ca^{2+} sequestration and release on the contractile properties of canine ventricular muscle. These experiments were designed specifically to probe the mechanism by which the Ca^{2+} channel agonist BAY K 8644 impairs postrest contraction, which are supported in canine ventricular muscle almost entirely by Ca^{2+} release from intracellular stores. A second, and perhaps more important, reason for conducting these experiments was to gain insight into the physiological control of intracellular Ca^{2+} release by dihydropyridine-sensitive Ca^{2+} channels, and to get a better understanding of the physical separation between the often described leak of Ca^{2+} from the SR and site at which Ca^{2+} is sequestered back into the sarcotubular network. This was addressed by comparing the action of BAY K 8644 to ryanodine and caffeine, two other pharmacological probes with differential specificity to either the junctional membrane- Ca^{2+} -release channel complex (Rousseau and Meissner, 1987; Rousseau et al, 1989) or the ability of the SR to sequester myoplasmic Ca^{2+} (Weber and Herz, 1968; Fuchs, 1969). Past experiments in our laboratory (Bose et al, 1986; 1988b; Hryshko and Bose, 1988; Hryshko et al, 1989a,b) indicated that BAY K 8644 accelerated the diastolic loss of Ca^{2+} from the SR without affecting the myofilaments in transit. From this and other data it was concluded that BAY K 8644 possessed the unique ability as a sarcolemmal Ca^{2+} channel agonist to modulate the release of Ca^{2+} from intracellular stores, independent of its known ability to increase sarcolemmal Ca^{2+} conductance.

The purpose of the experiments in SECTION 1 was to probe the specific mechanism by which BAY K 8644 altered the function of the SR. It was hypothesized that if this effect was specific to the junctional membrane- Ca^{2+} -release channel complex, then similar mechanical and

electrophysiological effects would be seen with ryanodine or caffeine, as both of these agents have been shown to alter the gating properties of the Ca^{2+} -release channels after incorporation into planar lipid bilayers. The ability of the SR to take up and release Ca^{2+} in the presence of the three test agents was estimated with a combination of steady-state and postrest contractions and the response of the muscle to rapid cooling. Quantitative changes in the entry of Ca^{2+} into the muscle during depolarization of the preparation and kinetics of Ca^{2+} release from the SR associated with single excitations were assessed indirectly by simultaneously recording trans-membrane action potentials and accompanying contractions (cf. Hryshko, 1987; Hryshko et al, 1989a).

Section 2: In this section, the mechanism of the atypical negative frequency-force relationship of rat ventricular muscle was investigated and compared with the positive force staircase in canine ventricular muscles of comparable diameter. Of all the mammalian species studied to date, the negative staircase has only been observed in ventricular preparations from the rat (Kelly and Hoffman, 1960; Forester and Mainwood, 1974), hamster (Howlett et al, 1989) and mouse (Temma and Akera, 1986). At the time these experiments were conducted the prevailing explanations for the negative staircase were that the decreased strength of contraction was a result of (a) an accompanying decrease of intracellular Ca^{2+} loading and subsequent release into the myofilaments (Fabiato, 1985c; Mitchell et al, 1985), (b) a manifestation of intracellular Ca^{2+} -overload due to saturation of the SR with Ca^{2+} (Forester and Mainwood, 1974; Mitchell et al, 1985), or (c) due to ischemia in the core cells of the preparation induced by high-frequency stimulation (Schouten and terKeurs, 1986; Gulch and Ebrecht, 1986).

The purpose of these experiments was to address separately each of these hypotheses by relating changes in twitch amplitude at different rates of rhythmic stimulation in similar preparations of rat and canine ventricle with the amount of Ca^{2+} available for release from the SR under steady-state conditions. The amount of Ca^{2+} present within the SR was estimated with rapid cooling contractures and postrest stimulation. To determine the possible role of intracellular Ca^{2+} -overload, these experiments were conducted both before and after raising the $[\text{Ca}^{2+}]_o$ from 1.25

to 2.5 mM. In addition to probing for frequency-induced alterations in the amount of Ca^{2+} located within intracellular stores, a second facet of this study was to see if exceeding the "critical diameter" of 0.2 mm suggested by Schouten and terKeurs (1986) produced a qualitative shift in the slope of the frequency-force relation in muscles obtained from either species investigated.

Section 3: The experiments in SECTION 3 were conducted to test the notion that frequency-dependent changes in the force of steady state contraction in canine ventricle occurs concomitant with, but not entirely a result of, changes in SR Ca^{2+} loading *per se*. A second direction that was also pursued during this work was the possibility that a small fraction of myoplasmic Ca^{2+} during contraction could be made available to the myofilaments independent of intracellular Ca^{2+} availability or release. These experiments were stimulated in part by the results of calculations described in SECTION 2 of the Thesis pertaining to the interval-force relation and also by recent experimental links established between myoplasmic Ca^{2+} levels and cell contraction with rate-dependent alterations of α_{Na}^i (Cohen et al, 1982; Brill et al, 1986; Boyett et al, 1987; Wang et al, 1988), and various time, voltage and stimulation-dependent ionic currents attributed to an electrogenic Na^+ - Ca^{2+} exchange process (Mitchell et al, 1986; Kimura et al, 1986; Mechmann and Pott, 1986; Barcenaz-Ruiz et al, 1987; Beuckelmann et al, 1989; Egan et al, 1989; Shimoni and Giles, 1989; Terrar and White, 1989).

The possibility that frequency-dependent alterations in either contractile strength or the amount of Ca^{2+} available for release from the SR were related to sarcolemmal Na^+ - Ca^{2+} exchange was tested by repeating various stimulation protocols after reducing the trans-membrane driving force for the exchange process. Fifty percent reduction of $[\text{Na}^+]_o$ by equimolar substitution of NaCl with LiCl was chosen over more complete substitution in these experiments to leave the exchange process functioning with enough residual activity to permit shifts in modal activity to occur during the change in E_{NaCa} accompanying the action potential, and to avoid complications associated with intracellular Ca^{2+} -overloading. The specific aim of these experiments was to test the hypothesis that a $[\text{Na}^+]$ -dependent process is responsible for a significant proportion of elevated

myoplasmic Ca^{2+} during high frequency stimulation. This was tested by comparing rate-dependent changes in the strength of contraction with steady-state cooling contracture and postrest contraction measurements both before and after lowering the bathing $[\text{Na}^+]$ from 140 to 70 mM or following the addition of drugs known to interfere with normal functioning of the SR. Once the effect of $[\text{Na}^+]_o$ -withdrawal was established on the frequency-dependence of contraction, other stimulation protocols were carried out in solutions of varying ionic composition for the purpose of further delimiting the manner in which the Na^+ - Ca^{2+} exchange process helps govern contraction.

Section 4: In this final section of the Thesis, the utility of our experimental model of EC-Coupling in rat ventricular muscle was tested to determine if SR Ca^{2+} homeostasis was perturbed in a model of diabetic cardiomyopathy which has been often associated with defects of SR Ca^{2+} availability (Fein et al, 1980; 1983, Nordin et al, 1985). Data available in the literature prior to beginning these experiments suggested that the decreased contractility of diabetic cardiac tissues was consistent with either chronic Ca^{2+} -overload (Fein et al, 1983; Nordin et al, 1985), or a depletion of Ca^{2+} pools located within the SR (Lopaschuk et al, 1983; Penpargkul et al, 1983; Bergh et al, 1988; Horackova and Murphy, 1988; Tani and Neely, 1988).

Rats were made diabetic with intravenous injection of streptozotocin and studied after 4 and 8 weeks of diabetes. Possible defects of SR Ca^{2+} handling associated with the disease process were determined by the response of muscles to changes in the rate and rhythm of stimulation and rapid cooling. The hypothesis was that if intracellular Ca^{2+} content and release was perturbed in diabetes, then an accompanying alteration of steady-state cooling contracture and postrest contraction should be measurable. This was further tested in these experiments by altering the $[\text{Ca}^{2+}]_o$ in a such a way so as to enhance any existing predisposition of the preparation to a state of intracellular Ca^{2+} -overload.

**EFFECTS OF CAFFEINE AND RYANODINE ON DEPRESSION OF POSTREST
TENSION DEVELOPMENT PRODUCED BY BAY K 8644 IN CANINE VENTRICULAR MUSCLE**

SECTION 1

SUMMARY

Postrest contraction amplitude has proven to be a useful qualitative indicator of sarcoplasmic reticulum Ca^{2+} release in isolated preparations of mammalian ventricular tissue. The phenomenon of "postrest-potential" in canine ventricular muscle is converted to "postrest-depression" by the racemic form of the Ca^{2+} channel agonist BAY K 8644 as well as other chemically diverse agents such as caffeine and ryanodine. Rapid cooling contractures and postrest contraction amplitude were used as independent measures for both sarcoplasmic reticulum Ca^{2+} content and release. Simultaneous recordings of transmembrane action potentials and accompanying contractions were obtained to determine the association between electrophysiological and mechanical events in the presence of the various test agents. The present study was designed to elucidate the mechanism by which BAY K 8644, caffeine and ryanodine alter force production after variable periods of rest. BAY K 8644 (1 μM) increased steady state contraction in response to a constant train of stimulation, caused rest-depression after 2 and 8 min rest, prolonged action potential duration and increased action potential plateau amplitude. Augmented steady state tension was not accompanied by any change in time to peak tension or rapid cooling contracture amplitude. However, the post-rest rapid cooling contracture was greatly diminished compared to that observed prior to BAY K 8644 treatment. Caffeine (3 and 5 mM) caused rest-depression with an increase in steady state contraction amplitude. Along with this there was a slight decrease in action potential duration and plateau amplitude and an increase in time to peak tension. The rapid cooling contractures were virtually abolished at all conditioning intervals. The effect of caffeine on twitch tension and cooling contracture is consistent with the ability of this compound to inhibit Ca^{2+} accumulation by the sarcoplasmic reticulum. A combination of BAY K 8644 and caffeine caused significantly less rest-depression than that seen with BAY K 8644 alone. The augmented twitch tension was accompanied by a long time to peak tension and action potential duration. However there was no increase in the amplitude of the rapid cooling contracture, either after a regular train of stimulation or after rest, compared to that seen

after BAY K 8644. Ryanodine (10 nM), produced rest-depression, reduced steady state twitch tension and augmented the rest-depression produced by BAY K 8644. The steady state rapid cooling contracture remained unchanged when both agents were present simultaneously, while the post-rest rapid cooling contracture was significantly depressed compared to that observed with BAY K 8644 alone. BAY K 8644 and ryanodine appear to have similar actions with respect to promoting diastolic loss of Ca^{2+} from the sarcoplasmic reticulum. Although caffeine also decreases post-rest potentiation, it antagonises rest-depression caused by BAY K 8644. The data from these experiments suggest that this reversal is a result of depressed intracellular Ca^{2+} buffering and enhanced myofilament sensitivity produced by caffeine in the presence of increased transmembrane Ca^{2+} influx promoted by BAY K 8644.

INTRODUCTION

Previous studies on the effect of the racemic dihydropyridine compound BAY K 8644 on ionic currents in voltage-clamped ventricular cells have indicated that this compound enhances peak Ca^{2+} entry during the Ca^{2+} current (I_{Ca}) by increasing the mean open time and the probability of opening of voltage-gated Ca^{2+} channels (Hess et al, 1984; Brown et al, 1984; Thomas et al, 1985). Consistent with its effects on peak I_{Ca} , application of BAY K 8644 to diverse preparations of isolated ventricular muscle also augments peak isometric tension development in response to rhythmic electrical stimulation (Thomas et al, 1985a; Bose et al, 1987; Hryshko & Bose, 1988). In view of its effects on steady-state contraction and membrane Ca^{2+} conductance it is thus rather surprising that BAY K 8644 also dramatically impairs the ability of ventricular muscle to develop tension after a period of rest (Hryshko & Bose, 1988; Hryshko et al, 1989a; Saha et al, 1989).

Experiments performed on isolated ventricular muscle from several mammalian species have shown that the strength of contractions elicited after brief periods of rest (postrest contraction) can be transiently increased upon resumption of rhythmic stimulation. The degree to which such contractions are potentiated depends both on the experimental conditions and species investigated. This potentiation is usually biphasic in that a maximum point on the restitution curve is observed following some characteristic period of rest, after which postrest contraction amplitude declines (Koch-Weser & Blinks, 1963; Morad and Goldman, 1973; Ragnarsdottir et al, 1982; Schouten et al, 1987). Measurements of time-dependent shifts of extracellular Ca^{2+} concentration and sarcoplasmic reticulum (SR) Ca^{2+} content during the course of rest (Bers, 1983; Hilgemann, 1986) have indicated that the SR appears to be the primary source of Ca^{2+} for postrest contractions in several species, including rat, guinea-pig, rabbit, and dog (Allen et al, 1976; King & Bose, 1984; Bers, 1985; 1989; Kurihara & Sakai, 1985; Bridge, 1986; Bose et al, 1988a). In contrast to the phenomenon of postrest potentiation observed with short rest intervals, extended periods of mechanical quiescence lead inevitably to depressed postrest contractions in most mammalian

species (Koch-Weser & Blinks, 1961; 1963). In atrial preparations (Thomas et al, 1985a), even after prolonged rest, there is a substantial residual "rested state" contraction, believed to be due to release of Ca^{2+} from the SR. This is in contrast to the "rested state" contraction in ventricular muscle from selected species which has been postulated to be a result of transsarcolemmal Ca^{2+} influx and is characterized by a slower onset of a very small contraction (Reiter et al, 1978). The inhibition of postrest force production of the type seen in the presence of BAY K 8644 is similar to that observed following long rest intervals in control muscles, suggesting that this agent might enhance ongoing diastolic Ca^{2+} loss from the SR and that this would in turn lead to a reduction in the availability of activator Ca^{2+} for release upon resumption of stimulation.

The methylxanthine caffeine and neutral plant alkaloid ryanodine are used commonly to study excitation-contraction coupling in mammalian cardiac muscle due to their differential ability to either inhibit Ca^{2+} release from the SR or its uptake following the termination of contraction (Weber & Herz, 1968; Blinks et al, 1972; Fabiato, 1981; Bers et al, 1987). The objective of the present study was to design experiments which would make use of the known properties of these compounds to reveal the mechanism by which BAY K 8644 affects postrest force production. To achieve this, BAY K 8644 was tested alone and in combination with either caffeine or ryanodine to determine if they had a similar mechanism of action on postrest contractions. It was reasoned that this should make the combined effect of BAY K 8644 and any of the other agents on rest potentiation additive. Rapid cooling contractures were also used to estimate global loading of the SR with Ca^{2+} . Simultaneous recordings of transmembrane action potentials and twitch contractions were obtained to determine the relationship between electrical and mechanical events within a given contraction. Preliminary data have been presented to the Federation of American Societies for Experimental Biology (Bouchard et al, 1988).

MATERIALS AND METHODS

Experimental Preparation

Mongrel dogs of either sex weighing 8-12 kg were anaesthetized with sodium pentobarbital (30 mg/kg i.v.). Hearts were rapidly excised through a lateral thoracotomy and the coronary vasculature flushed with 50 ml cold (4°C) Kreb's-Henseleit (KH) solution. KH solution had the following composition in mM: NaCl, 118; KCl, 4.7; CaCl, 2.5; MgSO₄, 1.2; KH₂PO₄, 1.4; NaHCO₃, 25 and glucose, 11. Thin, free running right ventricular trabeculae (0.2-0.5 mm diameter) were tied using 8-0 silk (Matuda) and dissected free from the wall of the heart.

Tension Measurements

Following dissection from the heart, the muscles were transferred to a 2 ml horizontal recirculating bath containing oxygenated (95% O₂/5% CO₂) KH solution maintained at a pH of 7.4 and a temperature of 37°C. Muscles were field stimulated by pulses delivered by a computer-driven stimulus sequencer (Boyechko & Bose, 1982) at a frequency of 0.5 Hz and an amplitude 75-100% above threshold voltage, and equilibrated for 45-60 minutes after which they were stretched to an optimal length for maximal isometric force production (L_{max}). Isometric contractions were recorded with a force transducer (Grass FT03C). Periods of rest ranging from 30-240 seconds were randomly imposed on the otherwise constant (steady-state) stimulus pattern.

Rapid Cooling Contracture

The muscle chamber formed part of a recirculation apparatus with a single bath and two reservoirs connected in parallel with the bath. This system allowed very rapid (<1 sec) switching of bathing solutions. Rapid cooling contractures were elicited by cooling the preparation from 37°C to 0-2°C by switching the solution entering the muscle chamber through a parallel by-pass system. Identical rest periods to those used in the twitch contraction experiments were examined, where the rapid cooling contractures were measured in the place of a regularly driven beat (e.g., steady-

state rapid cooling contracture) or after variable periods of rest (postrest rapid cooling contracture).

Electrophysiological Measurements

For simultaneous recordings of transmembrane action potentials and contractions, one end of the trabecula was fixed with stainless steel insect pins to a silicone rubber base in the bath and the other end was connected to an isometric force transducer. Glass micropipettes were pulled with a Brown & Flaming micropipette puller (Model P-77; Sutter Instruments Co.) and filled with 3M KCl (tip impedance 8-25 megaohms) to record transmembrane action potentials from the surface cells of the trabeculae. Electrodes were connected to a high input impedance amplifier (Neuroprobe 1600; Transidyne General Corp.). A 50 mV calibration signal and 0 mV absolute voltage level were given prior to each electrical stimulus. These signals, together with recordings of action potential and accompanying contractions were monitored during experiments with a 4 channel Hewlett-Packard storage oscilloscope and recorded on a thermal chart recorder (Model 8500; Astro Med Inc.) and a Zenith VHS recorder (Model VP-2220; Zenith Corp.) coupled to an analog to digital converter and PCM adapter (Model VR-10; Instrutec Corp.) for later analysis.

Drug Preparation

BAY K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) was a generous gift from Dr. A. Scriabine (Miles Institute of Preclinical Research) and was dissolved in 100% ethanol. The final concentration of ethanol in the solution did not exceed 0.1% and was usually 0.01%. In the presence of 0.01% there was no decrease in steady-state contractions while with 0.1% there was a 25% decrease in the strength of isometric contraction in response to rhythmic stimulation at 0.5 Hz. No decrease in rest-potential was observed with this entire range of concentrations. Caffeine and ryanodine were obtained from Sigma Chemicals and were dissolved in double-distilled deionized water. All chemicals for physiological solutions were reagent grade. BAY K 8644 was used at a concentration of 1 μ M. Although rest depression could be demonstrated with much lower concentrations (e.g., 0.01 μ M), the onset and stabilization of the

effect were much slower. The same was true for ryanodine which was effective in concentrations as low as 1 nM. The concentration range of caffeine chosen (3-5 mM) was similar to the one shown by us previously to impair SR-mediated contractions (King & Bose, 1984; Hryshko et al, 1989a). A further increase in the concentration of caffeine to 10 mM produced greater effects on contraction, but the tissue response deteriorated rapidly.

Statistics

Statistical significance was determined with analysis of variance followed by Duncan's new multiple range test for multiple comparisons and Student's paired t-test for self-controlled experiments (Steel & Torrie, 1960). The level of significance chosen for all experiments was $P < 0.05$. Data points have been expressed throughout this study as the mean \pm S.E.M.

RESULTS

Rest Potentiation

Figure 3 depicts the contractile response of isolated canine right ventricular muscle to 2 and 8 minutes rest periods. The control muscle (panel A) shows potentiation of postrest contraction after both rest intervals. Addition of the positive inotropes BAY K 8644 (1 μ M, panel B) and caffeine (3 mM, panel C) as well as the negative inotrope ryanodine (10 nM, panel D) resulted in depression of postrest contraction. The amplitude of postrest contractions was found consistently to be smaller after 8 minutes rest compared to those observed after rest for 2 minutes in all preparations studied.

Figures 4 and 5 illustrate the effect of combining BAY K 8644 with caffeine or ryanodine on tension developed after 8 minutes of rest. As may have been expected if these compounds acted by a common functional mechanism, further addition of ryanodine resulted in an increase in the depression of postrest contraction compared to that observed with BAY K 8644 alone. In contrast, the addition of caffeine to the BAY K 8644-treated preparation reduced the postrest depression. As can be seen in the pooled data provided in Fig. 5, both combinations of treatments produced a slight but statistically insignificant depression of tension development in response to a rhythmic train of stimulation.

Rapid Cooling Contractures

Rapid cooling contractures were elicited to estimate the functional capacity of the SR to store Ca^{2+} during various phases of stimulation (Kurihara & Sakai, 1985; Bridge, 1986) in the presence of the three test agents. Figure 6 illustrates the typical response of canine ventricular muscle to rapid cooling. Panel A shows the progressive decrease in the amplitude of cooling contractures following a progressive increase in the duration of rest prior to cooling. In the presence of BAY K 8644 (1 μ M; Panel B) the time-dependent decay of postrest cooling contracture amplitude was significantly accelerated. On the other hand BAY K 8644 slightly increased the

amplitude of rapid cooling contractures elicited in place of a regularly driven beat. This may be a reflection of an inability of the SR to retain Ca^{2+} during rest without an impairment of the Ca^{2+} uptake process. Caffeine (5 mM; Panel C) also decreased the amplitude of the rapid cooling contracture after the various durations of rest. Unlike BAY K 8644, however, caffeine markedly reduced the amplitude of rapid cooling contractures immediately following a train of rhythmic stimulation. Ryanodine (10 nM; Panel D) resembled BAY K 8644 in inhibiting post-rest rapid cooling contractures without inhibiting cooling contractures elicited in place of a regularly driven beat. However, unlike BAY K 8644, ryanodine had a negative, rather than a positive, inotropic effect on steady-state contraction amplitude.

The effect of combining BAY K 8644 with either caffeine or ryanodine on rapid cooling contracture is shown in Fig. 7. Responses of control muscles and those after treatment with BAY K 8644 are shown for reference (Panel A & B). On further addition of caffeine (5 mM) to the BAY K 8644-treated muscle rapid cooling contractures were abolished immediately following a train of stimuli or after rest (Panel C). Despite this, tension development in response to rhythmic stimulation remained unaltered. The maintenance of tension development in response to rhythmic stimulation, in spite of the absence of rapid cooling contracture in the place of a regularly driven beat, may reflect the dependence of steady-state force generation on transsarcolemmal Ca^{2+} influx in the presence of caffeine. Ryanodine (10 nM) further decreased the postrest rapid cooling contracture following 2 and 8 minutes rest, which was already reduced by BAY K 8644. It should be noted that unlike caffeine, ryanodine did not decrease the cooling contracture immediately following a train of stimuli. The results of the rapid cooling experiments indicate that the spectrum of action of ryanodine on the contractile properties of canine ventricle resembles that of BAY K 8644 more so than that of caffeine. Pooled data from 6 experiments are shown in Fig. 8.

Electrophysiological Studies

Transmembrane action potentials and accompanying contractions were obtained to determine if the apparent transsarcolemmal Ca^{2+} entry associated with membrane depolarization due to caffeine was antagonizing the effect of BAY K 8644 in decreasing postrest twitch tension. Many studies have indicated that time to peak tension associated with isometric contraction can be influenced by both the degree and rate of transsarcolemmal Ca^{2+} entry as well as Ca^{2+} uptake and release from the SR. Thus, an increase in strength of SR-mediated postrest contraction in the presence of any of the three test agents could be brought about by either enhanced transsarcolemmal Ca^{2+} entry or a reduction of Ca^{2+} uptake by the SR.

The effects of BAY K 8644 (1 μM) alone and in combination with caffeine (3 mM) or ryanodine (10 nM) on the transmembrane action potential and accompanying contraction are illustrated in Figs 9 and 10, respectively. The recordings in each Figure were made from a single preparation. Data from 7 such experiments are summarized in Figs. 11 and 12. Figure 11 represents the pooled data showing the effect of BAY K 8644, caffeine and ryanodine on the action potential duration at 50% repolarization during contractions obtained in response to rhythmic stimulation or after two and eight minutes rest.

As demonstrated in earlier experiments (Hryshko et al, 1989a,b; Saha et al, 1989) administration of BAY K 8644 was found to significantly prolong the action potential duration in response to steady-state and postrest stimulation (see Figs. 8 and 10) as well as elevating the height of the action potential plateau. Similar effects on the time course of membrane depolarization were observed when caffeine was combined with BAY K 8644 (Figs. 9 and 11). The addition of ryanodine to the BAY K 8644-treated muscle produced a dramatic increase in action potential duration during all test beats (Figs. 10 and 11). Thus, the combination of either caffeine or ryanodine with BAY K 8644 was demonstrated to have similar, albeit quantitatively different, effects on transmembrane action potential duration. Presumably, these interventions would have similar effects on transsarcolemmal Ca^{2+} influx associated with the prolonged action potentials, which

would help to explain the partial protection of BAY K 8644-induced postrest depression by caffeine but not the contractile response to the combination of BAY K 8644 and ryanodine.

Table 4 illustrates the effect of the three test agents on time to peak tension and relaxation time for contractions elicited in response to steady-state stimulation at 0.5 Hz and after 2 minutes rest. Addition of BAY K 8644 had no effect on time to peak tension at any of the conditioning intervals studied. However, a small but statistically significant prolongation of the terminal portion of relaxation was observed in the presence of BAY K 8644 (e.g., the final 10%). In comparison, both caffeine and ryanodine significantly prolonged the time to peak tension as well as relaxation time throughout the contraction, ryanodine having a much greater effect than caffeine.

Pooled data from 7 experiments showing the effect of combining BAY K 8644 with caffeine or ryanodine on time to peak tension measurements obtained during steady-state contractions and those elicited after 2 and 8 minutes rest are illustrated in Fig. 12. A single dose of BAY K 8644 had no significant effect on the time to peak tension at any conditioning interval investigated. In contrast, the combination of either caffeine or ryanodine with BAY K 8644 significantly prolonged the time course of contraction during continuous stimulation or after rest, and thus the effect of combining the drugs was greater than the change produced by either agent alone. In this regard, it is important to stress that the combination of either caffeine or ryanodine with BAY K 8644 produced similar directional changes in action potential duration and time to peak tension but only caffeine antagonized the BAY K 8644-induced postrest depression. Furthermore, these data also suggest that transsarcolemmal Ca^{2+} influx alone may not be the primary mechanism responsible for the ability of caffeine to antagonize BAY K 8644-induced postrest depression.

DISCUSSION

The new observation of this study is that although BAY K 8644, caffeine and ryanodine convert postrest potentiation in canine ventricular trabeculae to postrest depression, there are several fundamental differences in the spectrum of actions of these three agents. The conversion of rest-potentiation to rest-depression by BAY K 8644, first described by us (Bose et al, 1987; Hryshko & Bose, 1988; Hryshko et al, 1989a; Bouchard et al, 1988), appears to reflect a previously unrecognized ability of this dihydropyridine compound to modulate SR function entirely independent of its effects on membrane Ca^{2+} conductance.

Considerable experimental evidence suggests that redistribution of Ca^{2+} within the SR during diastole may be responsible for the potentiation of contraction amplitude following a period of rest (Koch-Weser & Blinks, 1961; 1963; King & Bose, 1984; Bers, 1985; Bers et al, 1987; Hilgemann, 1986a; 1986b; Bridge, 1986; Schouten et al, 1987). From similar experiments it has also been suggested that there may be a continuous but slow loss of Ca^{2+} from the SR during diastole. The latter, if allowed to continue for an extended period of time results ultimately in a decrease of postrest contraction amplitude (Koch-Weser & Blinks, 1961; 1963; Bose et al, 1988a). In the present study, the addition of BAY K 8644 to the perfusate caused rest-depression which is similar to that seen normally with prolonged rest in canine ventricle. This was opposite to its contrasting action of enhancing the amplitude of steady-state contraction. Based on a consideration of several models of excitation-contraction coupling in mammalian cardiac muscle proposed by various investigators (Koch-Weser & Blinks, 1963; Wood et al., 1969; Morad & Goldman, 1973; Wohlfart & Noble, 1982; Schouten et al, 1987; Bose et al., 1988a), depression of postrest contraction due to BAY K 8644 might reflect one of two processes: (a) an acceleration of ongoing diastolic Ca^{2+} loss from the SR passing through the myoplasm so as to influence the contractile apparatus, or (b) a pathway by which Ca^{2+} is lost from this organelle which bypasses the contractile apparatus. Experiments involving rapid cooling of cardiac muscle to measure sarcoplasmic reticulum Ca^{2+} content as well as others using caffeine or ryanodine as

pharmacological tools were done to obtain some insight into the ability of BAY K 8644 to perturb intracellular Ca^{2+} handling by the SR.

BAY K 8644

We have shown previously that a racemic mixture of the dihydropyridine compound BAY K 8644 prolongs action potential duration both during steady stimulation as well as after rest (Bose et al, 1987; Hryshko et al, 1989a; Saha et al, 1989). This is consistent with the known ability of BAY K 8644 to increase transsarcolemmal Ca^{2+} entry. The increase in amplitude of the steady-state contraction can be explained on this basis. Of particular interest is the unusual effect of this compound not only to decrease postrest potentiation but to convert it to postrest depression. This is likely a result of a decrease in the amount of Ca^{2+} released from the SR following the resumption of stimulation, which itself could be due either to increased refractoriness of the Ca^{2+} release mechanism or simply to a depletion of the release pool. Despite this the ability of the SR to take up Ca^{2+} from the myoplasm in the presence of BAY K 8644 remained unaffected, as shown by the lack of change of the time to peak tension seen in this study and others (Thomas et al, 1985a). Further evidence in support of the maintained ability of the SR to pump Ca^{2+} in the presence of BAY K 8644 is the increase in amplitude of rapid cooling contracture following a sustained train of stimuli, indicating the increase in global SR Ca^{2+} availability. However, the rapid decrease in the amplitude of rapid cooling contractures after the initiation of rest indicates that the loss of Ca^{2+} from the SR is accelerated by BAY K 8644. Previous investigations have failed to demonstrate any effect of this agent on sarcoplasmic reticular function in skinned cardiac muscle fibers or on Ca^{2+} transport in sarcoplasmic reticulum vesicles from skeletal muscle (Thomas et al, 1985b; Zorzato et al, 1985). One factor which may contribute to this unusual effect of BAY K 8644 on postrest contraction, as opposed to contractions resulting from continuous stimulation, may be the requirement for a functional junction between the t-tubule and SR which is retained only in the intact ventricular preparation. We have found that the inhibitory effect of BAY K 8644 on postrest

contraction is not seen in atrial preparations, which are poor in t-tubules (Sommer & Johnson, 1979).

Postrest force production has been shown previously to be strongly influenced by compounds which affect Ca^{2+} handling by the SR (Bers, 1983; 1985; 1987; Bers et al, 1987; Hilgemann 1986a,b). In such experiments, a gradual increase in the content of Ca^{2+} in the extracellular space of cardiac muscle during diastole was found to be facilitated by the administration of ryanodine and was accompanied by a decrease in postrest tension development. Thus, it appears that postrest contraction in most mammalian species is governed to a greater extent by intracellular Ca^{2+} release as opposed to transsarcolemmal Ca^{2+} entry during muscle activation. It should be noted that while postrest rapid cooling contracture amplitude decreases continuously with increasing rest durations, postrest contractions evoked by electrical stimulation are biphasic in their pattern of restitution over the course of 5-240 seconds. This finding is consistent with the notion that rapid cooling contractures better represent total SR Ca^{2+} content while electrically evoked postrest contractions better represent the Ca^{2+} pool located in the release site of the SR. This explanation, however, is incomplete as it raises the question of the route which this Ca^{2+} presumably takes in moving from the SR, eventually to the extracellular space.

We have hypothesized that the apparent "leak" of Ca^{2+} from the SR takes place close to the t-tubule-lateral cisternal junctions. This proposal is based on data obtained from experiments performed on both skinned fibers and vesicles prepared from SR membranes (Thomas et al, 1985b; Zorzato et al, 1985), and those in which the effect of BAY K 8644 on asynchronous diastolic sarcomere motion was assessed and measurements of time to peak tension of contractions (Bose et al, 1987; present study). Scattered light intensity fluctuation of coherent light due to wavelike myofilament motion is believed to be caused by spontaneous oscillatory Ca^{2+} release from the SR of ventricular muscle (Lappe & Lakatta, 1980; Lakatta & Lappe, 1981). Since these oscillations are measured during diastole, their depression after the addition of BAY K 8644 (Bose et al, 1987;1988b) suggests that Ca^{2+} present in the myoplasm during diastole is reduced by BAY K

8644. This could result from decreased release from the lateral cisternae, probably due to a decrease in the volume of Ca^{2+} present within the release compartment. Ryanodine, which is known to increase Ca^{2+} leak from the SR (Bers et al, 1987) also reduces scattered light intensity fluctuation (Sutko et al., 1986b; Kobayashi & Bose, unpublished observations). The time to peak tension associated with contractions produced by continuous electrical stimulation or after variable periods of rest has been consistently reported to be unaffected by treatment with BAY K 8644 (Thomas et al, 1985a; Hryshko et al, 1989a). This suggests that prolongation of the duration of active state (due to alterations of SR Ca^{2+} uptake and release) do not contribute to the positive inotropy produced by this agent. Failure to slow relaxation rate throughout the twitch, as opposed to slowing of the final 10% of the relaxation phase, suggests that the ability of the SR to sequester Ca^{2+} is also not markedly impaired by BAY K 8644, if at all. Diastolic leak of Ca^{2+} from the SR might be expected to result in a uniform slowing of relaxation, as occurs with caffeine or ryanodine alone. As this did not occur in the presence of BAY K 8644, it may be concluded that the pathway taken by Ca^{2+} after it leaves the SR during diastole does not include primarily the myofilaments, and this release may indeed be from regions of the SR closer to the sarcolemma. In this regard it should be noted that low concentrations of external Na^+ partially reverses depression of postrest force production by agents or procedures which cause rest-depression, including BAY K 8644 (Sutko et al, 1986a; Bose et al, 1987). This suggests that the sarcolemmal Na^+ - Ca^{2+} exchange process may be mediating the apparent diastolic loss of Ca^{2+} in these muscles, including those treated with BAY K 8644.

BAY K 8644 plus Caffeine

In most mammalian species studied, the effect of administration of caffeine on excitation-contraction coupling in heart muscle appears to have two distinct phases. That this appears to be universal among species with very different excitation-contraction coupling cascades likely reflects the diversity of actions of this compound within the cardiac cell. The initial response to caffeine is an enhancement of Ca^{2+} -induced release of Ca^{2+} from the SR as measured by increased force

production, increased scattered light intensity fluctuation and ^{45}Ca flux (Fabiato, 1981; Stern et al, 1985; Sutko et al, 1986b; Rasmussen et al, 1987). The second action of caffeine is to gradually deplete intracellular Ca^{2+} pool(s), and in turn, quantitative release from the SR in response to an action potential. The small positive inotropy observed with this agent is likely a reflection of a number of intracellular processes which favour myofilament activation, including inhibition of sarcoplasmic reticulum Ca^{2+} ATPase (Weber & Herz, 1968), enhanced myofilament Ca^{2+} sensitivity (McClellan & Winegrad, 1978) and inhibition of phosphodiesterase (Butcher & Sutherland, 1962), the latter of which may also enhance peak I_{Ca} through its effects on cAMP-dependent protein kinase.

Caffeine has been previously shown to inhibit postrest tension development (Bers, 1983). The interaction between caffeine and BAY K 8644 was tested to gain insight into the depressant action of BAY K 8644 on postrest contraction. It was initially hypothesized, that if these agents utilized a similar mechanism to inhibit postrest contraction, then the effect of combining them should be additive. This effect was not observed. Instead, caffeine was found to partially reverse the depressant effect of BAY K 8644 on postrest contraction. Moreover, rapid cooling contractures observed under the influence of both drugs retained the characteristics of that observed with caffeine alone, which suggests that Ca^{2+} stores within the SR were severely depleted during continuous stimulation under the influence of both inotropes, even though the postrest contraction was less depressed. Thus, it seems unlikely that the caffeine-induced protection against the rest-depression induced by BAY K 8644 was due to enhanced Ca^{2+} release from the SR. This explanation is supported by the absence of significant cooling contractures when evoked immediately after a train of regular stimuli or after periods of rest, or data obtained in previous studies where other, indirect, measures of intracellular Ca^{2+} movements were found to be depressed by caffeine administration (Sutko et al, 1986b; Rasmussen et al, 1987; Bers, 1987).

In addition to the possibility of enhanced transsarcolemmal Ca^{2+} entry associated with the effect of caffeine to prolong action potential duration, increased myofilament activation during the postrest beat may be a likely explanation for the caffeine-induced antagonism of the effect of

BAY K 8644 on postrest tension development. It could be argued that enhanced activation of contraction may be related to either the prolongation of action potential duration and a possible increase in Ca^{2+} current or time to peak tension under these conditions. However, as discussed below, similar electrophysiological changes were found with BAY K 8644 alone and in combination with ryanodine, both of which resulted in greater rest-depression. Furthermore, both caffeine and ryanodine prolong time to peak tension when present alone or in combination with BAY K 8644 to a similar extent. Despite the similarity in their effect on the twitch profile, antagonism of BAY K 8644-induced rest-depression was observed only with the combination of BAY K 8644 and caffeine. These data do not support the hypothesis that enhanced transsarcolemmal Ca^{2+} influx alone is responsible for the reduction of BAY K 8644-induced rest-depression by caffeine. It appears that despite the characteristic rest-depression produced by either caffeine or BAY K 8644 alone the decreased depression when the two are combined may result from the augmented Ca^{2+} current due to BAY K 8644, and reduced sequestration of myoplasmic Ca^{2+} by the SR as well as sensitization of the contractile apparatus in the presence of caffeine.

BAY K 8644 plus Ryanodine

Ryanodine is a negative inotrope, which binds specifically to the Ca^{2+} release channel localized on the terminal cisternae of the SR (Inui et al, 1987). Its binding is modulated by caffeine (Pessah et al, 1986), although in this study the two were never present simultaneously. This alkaloid decreases ventricular contractility during rhythmic stimulation and after rest by decreasing the content of Ca^{2+} present within the SR (Sutko and Willerson, 1980; Bers et al, 1987), increasing the passage of Ca^{2+} from the intracellular stores to the extracellular space (Hilgemann et al, 1984; Bers et al, 1987; Hilgemann, 1986a; 1986b), decreasing diastolic Ca^{2+} -mediated myofilament motions (Sutko et al, 1986b), while only slightly reducing ^{45}Ca uptake (Rasmussen et al, 1987).

Addition of ryanodine in the presence of BAY K 8644 further decreased the amplitude of postrest contraction and cooling contractures. The data are in agreement with the prediction that the two drugs share a similar mechanism of action. This is supported by the lack of change in rapid

cooling contracture amplitude immediately following a train of rhythmic stimulation and the further increase in action potential duration observed under the influence of both agents. Thus, although the amount of Ca^{2+} released from the SR is impaired by ryanodine (Hilgemann, 1986a; 1986b; Bers et al, 1987; Rousseau et al, 1987), the ability of this organelle to sequester myoplasmic free Ca^{2+} appears to remain unaffected. One effect of ryanodine which does not parallel that of BAY K 8644, however, is the increase in time to peak tension (162.5%) and marked increase in relaxation time (277.5%). Other effects such as those observed on the action potential duration under the influence of both drugs differ significantly from the response seen with BAY K 8644. This suggests that even in the face of a possible large increase in the duration and peak of transsarcolemmal Ca^{2+} entry, the storage pool within the release site of the SR is unable to retain its load of Ca^{2+} over the course of time, similar to that observed with BAY K 8644 alone. If one assumes that Ca^{2+} pumping by the SR is not impaired by the combination of BAY K 8644 and ryanodine (on the basis of rapid cooling contractures not being impaired during steady stimulation), then the significant increase in the time taken for both peak tension development and relaxation with this treatment indicates that Ca^{2+} may be passing through the myofilaments on its way out from the SR during the process of "leak". Caffeine which causes an intermediate amount of prolongation in time to peak contraction and relaxation time than ryanodine may be acting by a different mechanism, as suggested earlier. However it would be impossible to rule out increased leak of Ca^{2+} from the SR under its influence. Studies on Ca^{2+} release channels incorporated into planar lipid bilayers indicate that they are converted to an open high conductance state by caffeine (Rousseau & Meissner, 1989). Ryanodine in contrast keeps the channels open in a low conductance state (Rousseau et al, 1987), which may explain a longer duration of Ca^{2+} leak leading to a greater prolongation of tension development and recovery. The fact that a similar extent of change is not seen with only BAY K 8644 may be because, in this case, the leakage of Ca^{2+} may be occurring from a very limited site from the SR.

In summary, the results of the experiments where BAY K 8644 was co-administered with either caffeine or ryanodine suggest that the latter two drugs do not share a similar mechanism of action in depressing postrest tension development in canine ventricular muscle. Of the two agents tested in combination with BAY K 8644, ryanodine produced contractile and electrophysiological changes which were somewhat similar to those observed with BAY K 8644 alone. It is likely that the depression of postrest contraction by BAY K 8644 is indicative of an acceleration of ongoing diastolic Ca^{2+} loss from the SR into the extracellular space, without influencing the myofilaments significantly during transit. This apparently leaves less activator Ca^{2+} available for release upon resumption of stimulation.

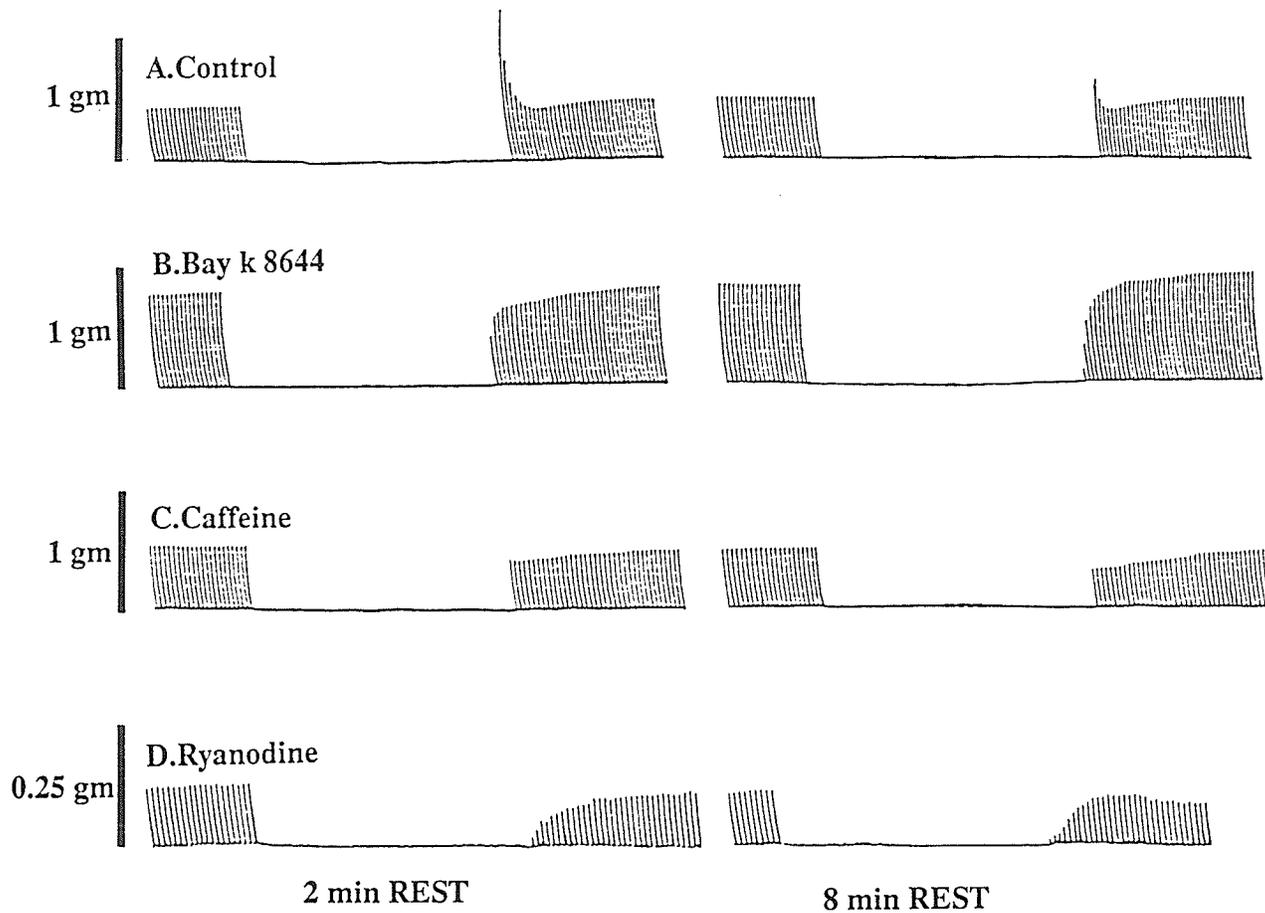


Figure 3. Typical response of canine ventricular trabeculae to varying periods of rest in an untreated muscle (Panel A) and after treatment with 1 μ M BAY K 8644 (Panel B), 5 mM caffeine (Panel C) and 10 nM ryanodine (Panel D). Rest durations of 2 (left) and 8 (right) minutes were imposed after a continuous train of steady-state stimulation at a frequency of 0.5 Hz.

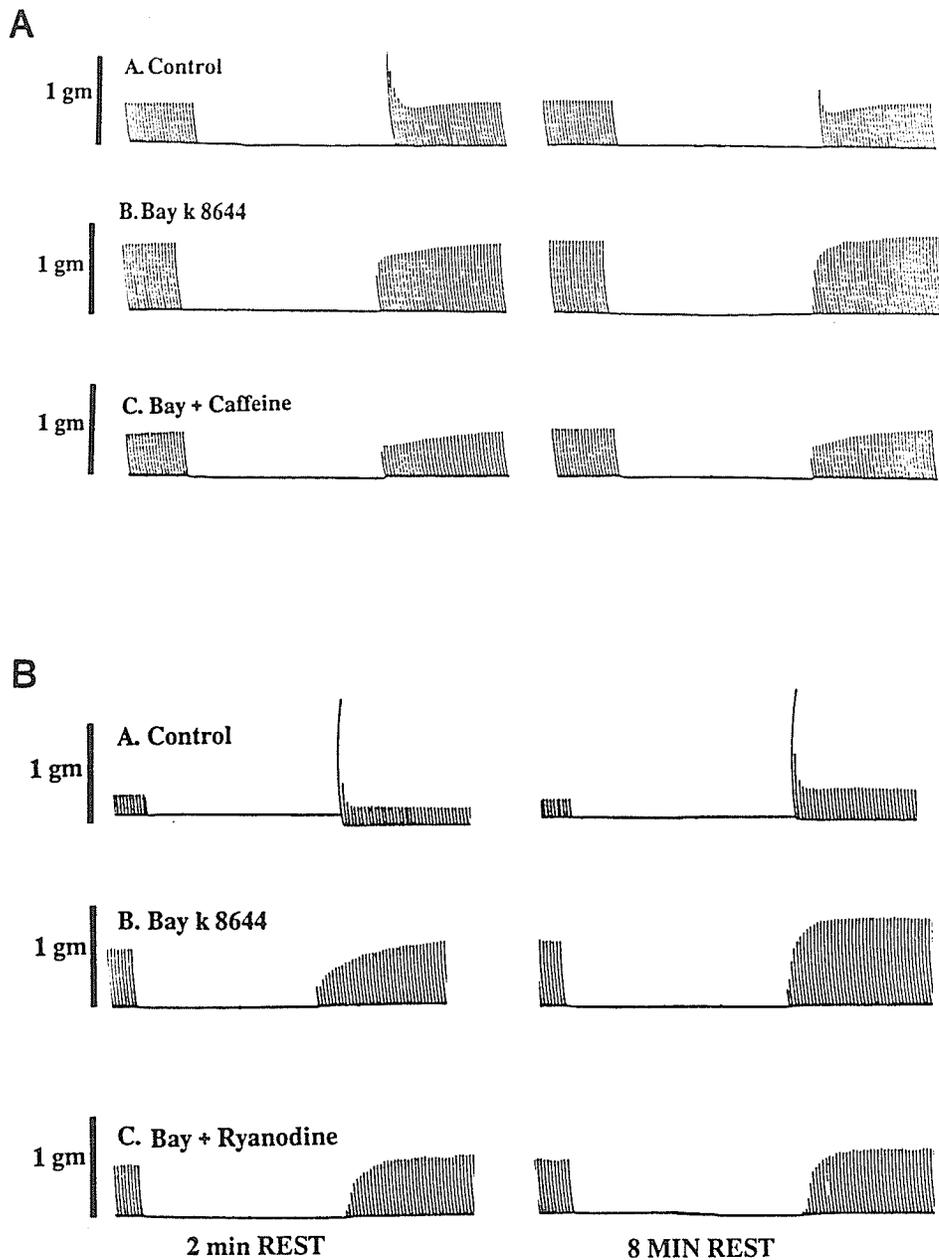


Figure 4. Representative contractile response of canine ventricular muscle to application of BAY K 8644 ($1 \mu\text{M}$) alone or in combination with 5 mM caffeine (Panel A) or 10 nM ryanodine (Panel B). Postrest contractions were elicited after either 2 or 8 minutes rest.

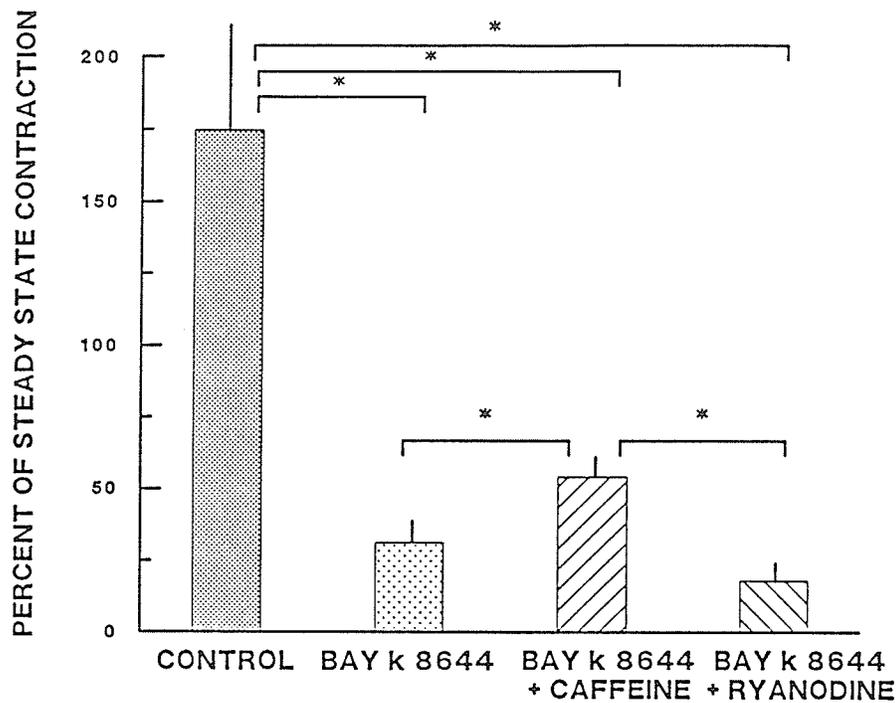


Figure 5. Pooled data from 6 experiments showing the effect of combining caffeine (5 mM) or ryanodine (10 nM) with BAY K 8644 (1 μ M) on tension development of the first postrest beat after 8 minutes rest. Results are expressed as a percentage of steady-state tension of the contraction preceding the test interval. Statistical significance of observed differences were analyzed with an analysis of variance followed by Duncan's new multiple range test for multiple comparisons. * denotes $P < 0.05$.

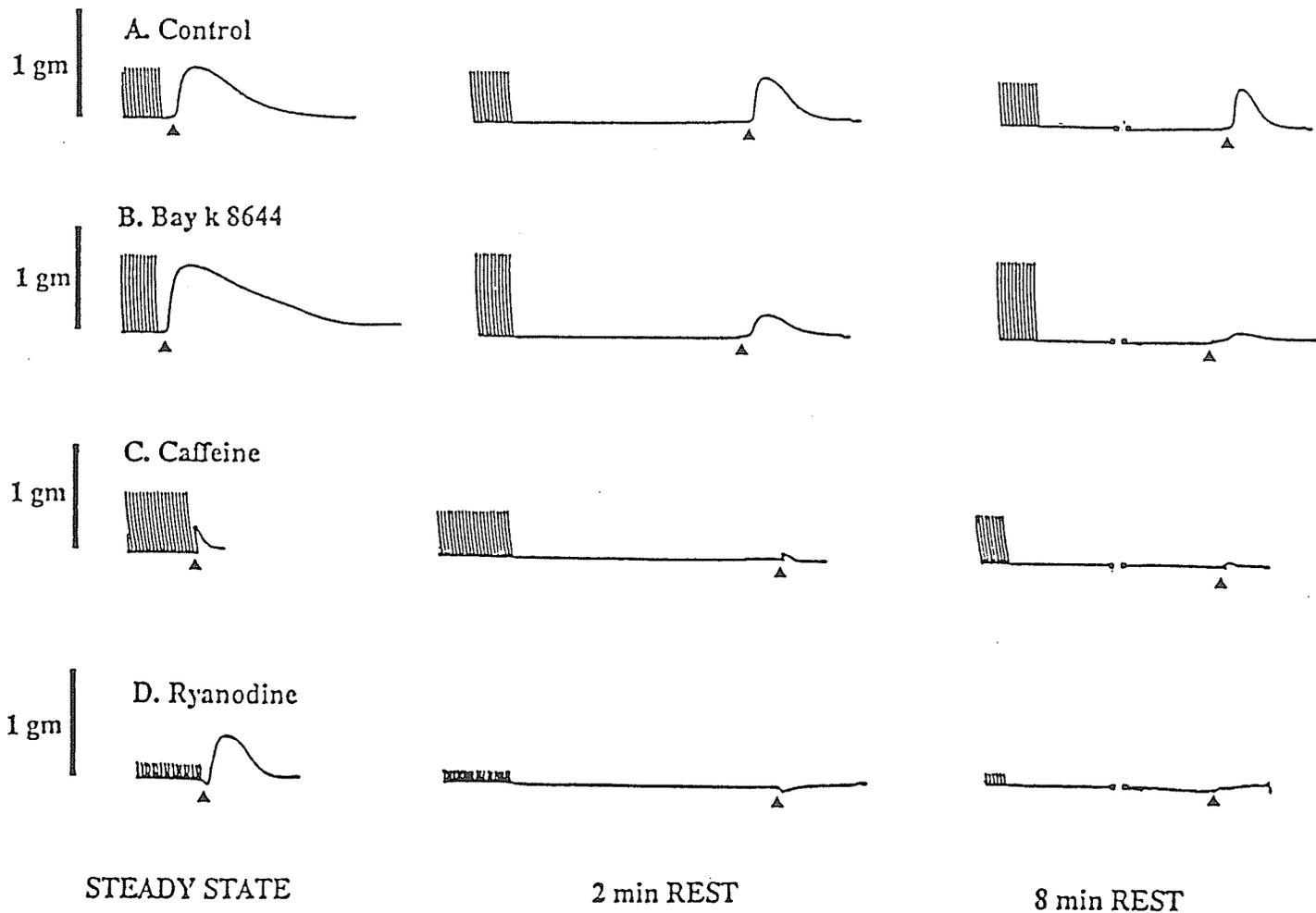


Figure 6. Typical responses of canine ventricular trabeculae to a rapid change (<1 sec) in temperature from 37°C to $0\text{-}2^{\circ}\text{C}$. Rapid cooling contractures were obtained in the place of a regularly driven beat (left), or after 2 (center) and 8 (right) minutes rest. The moment of solution change is indicated by the arrow under the individual recordings. Panel A represents the control trace and Panels B-D after administration of BAY K 8644 ($1\ \mu\text{M}$), caffeine ($5\ \text{mM}$), and ryanodine ($10\ \text{nM}$). The stimulus frequency was $0.5\ \text{Hz}$ for all rapid cooling contracture experiments.

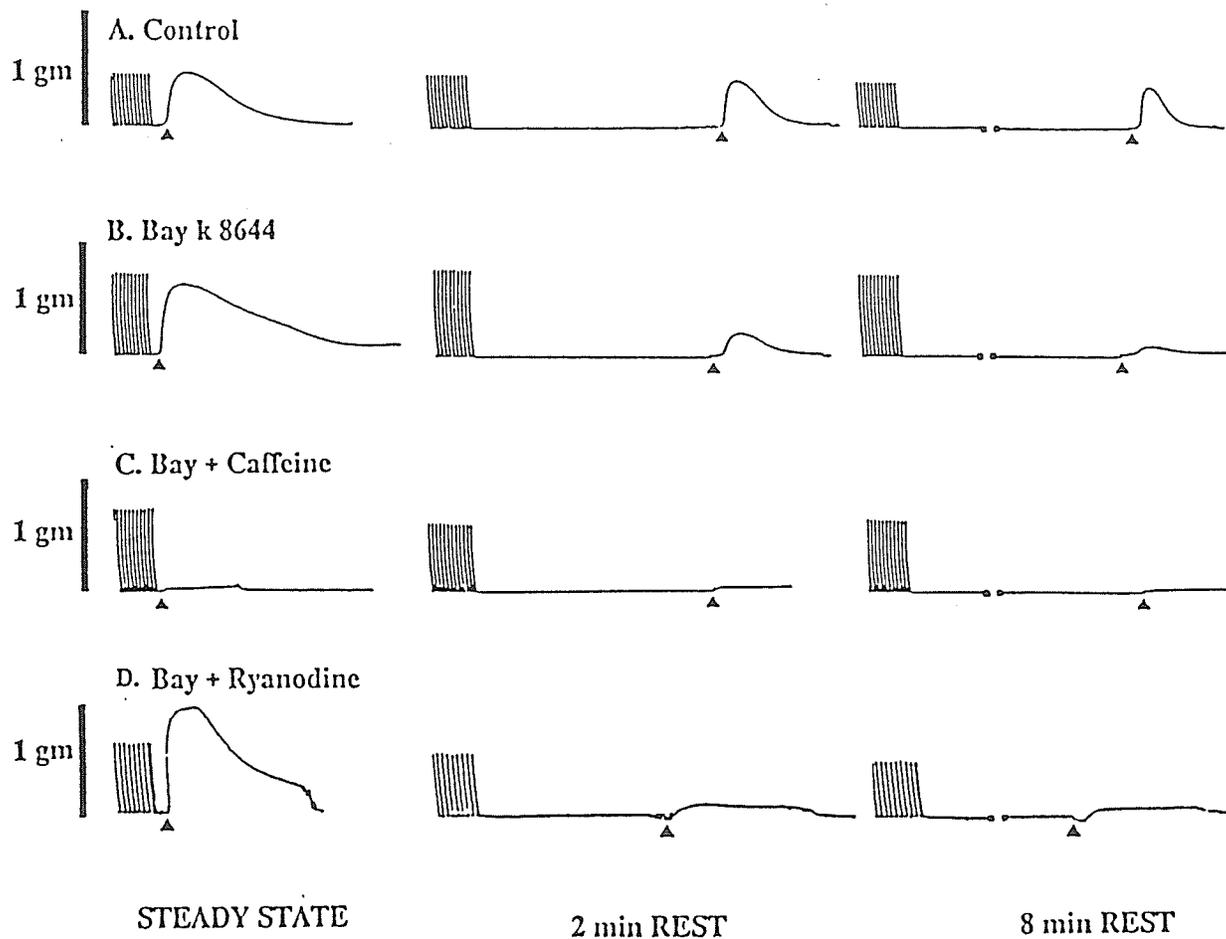


Figure 7. Effect of combining BAY K 8644 (1 μ M) with caffeine (5 mM) or ryanodine (10 nM) on rapid cooling contracture amplitude. The protocol was similar to that described in Figure 3.

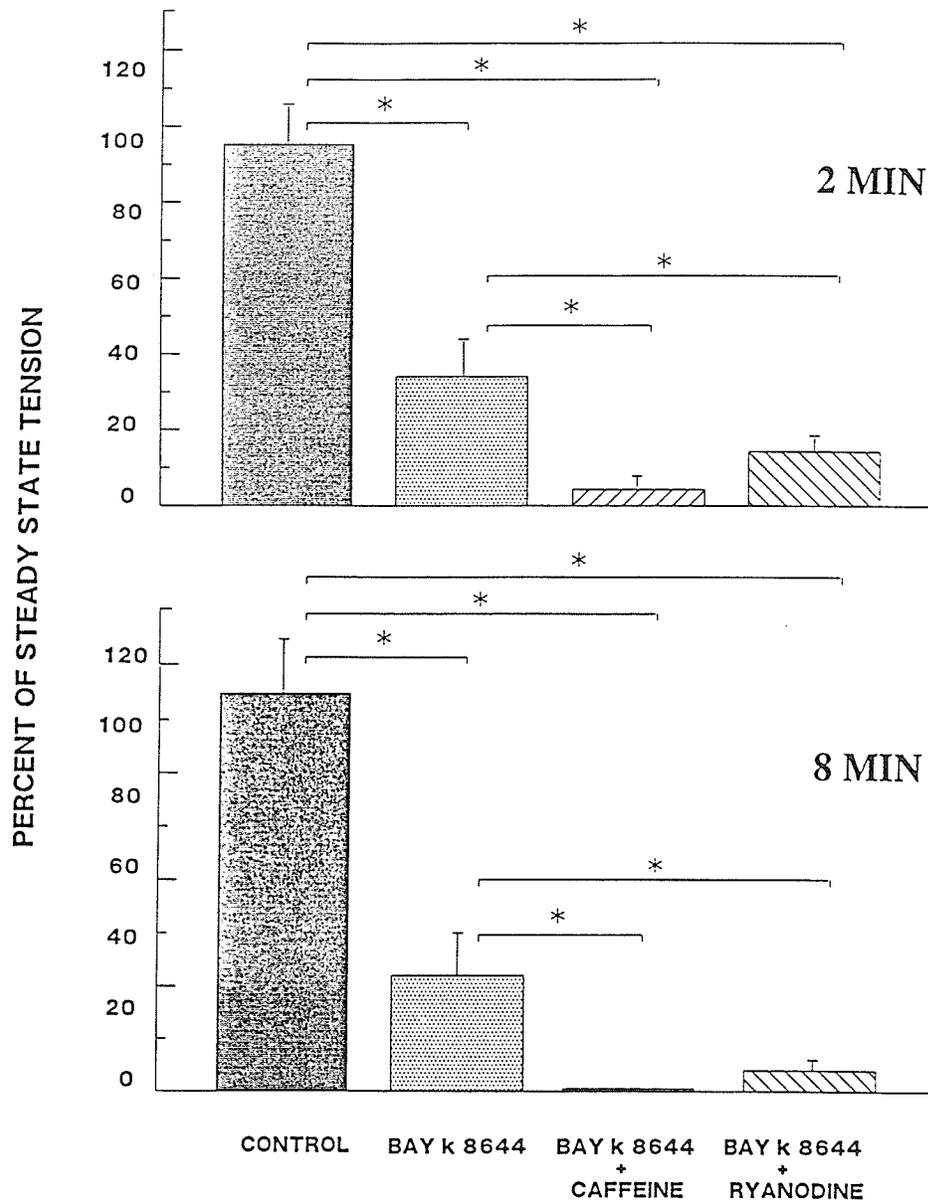


Figure 8. Pooled data from 6 rapid cooling contracture experiments showing the effect of combining BAY K 8644 (1 μ M) with caffeine (5 mM) or ryanodine (10 nM) on rapid cooling contractures obtained after 2 and 8 minutes of rest. Data is expressed as a percentage of contraction prior to initiating the rest interval. Significant differences between treated and untreated groups were determined by analysis of variance followed by Duncan's new multiple range test for multiple comparisons. * denotes $P < 0.5$

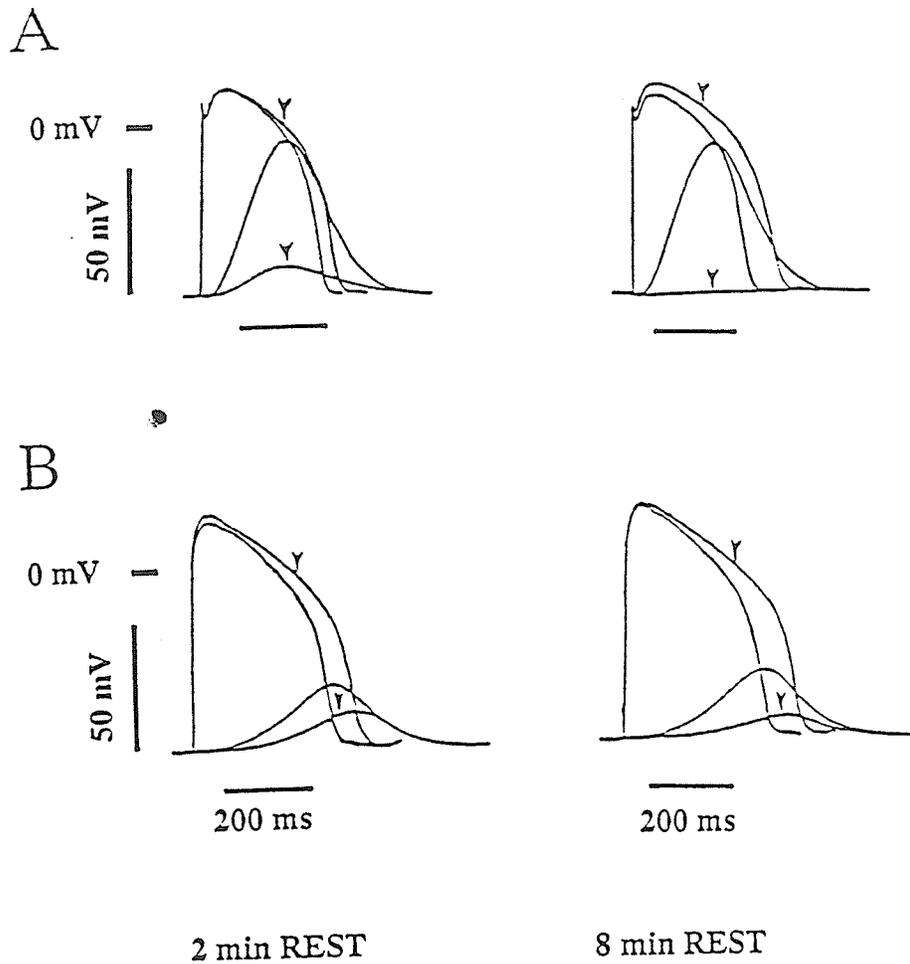


Figure 9. Simultaneous recording of action potentials and accompanying contractions from isolated trabeculae after treatment with 1 μ M BAY K 8644 (top panel) or BAY K 8644 and 3 mM caffeine (bottom panel) after 2 (left) and 8 (right) minutes rest. In each case, the postrest action potential and accompanying contraction (indicated by arrow) have been superimposed on the steady-state beat preceding the period of rest. The stimulation frequency during all electrophysiological studies was 0.5 Hz.

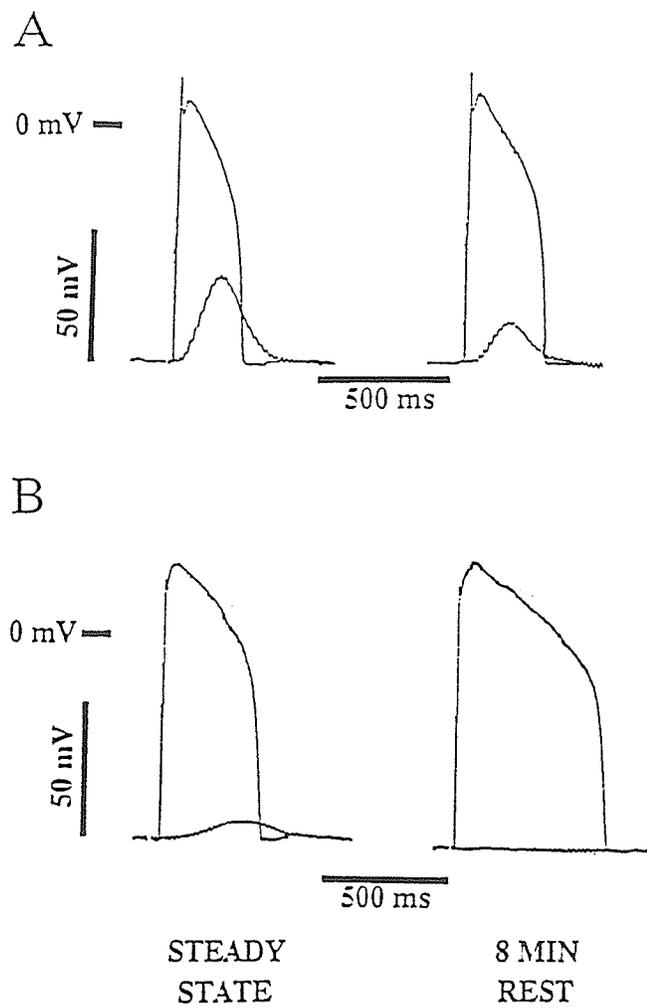


Figure 10. Simultaneous recording of action potentials and accompanying contractions from isolated trabeculae after treatment with 1 μ M BAY K 8644 (top panel) or BAY K 8644 and 10 nM ryanodine (bottom panel) in response to rhythmic stimulation (left) or after 8 minutes rest (right).

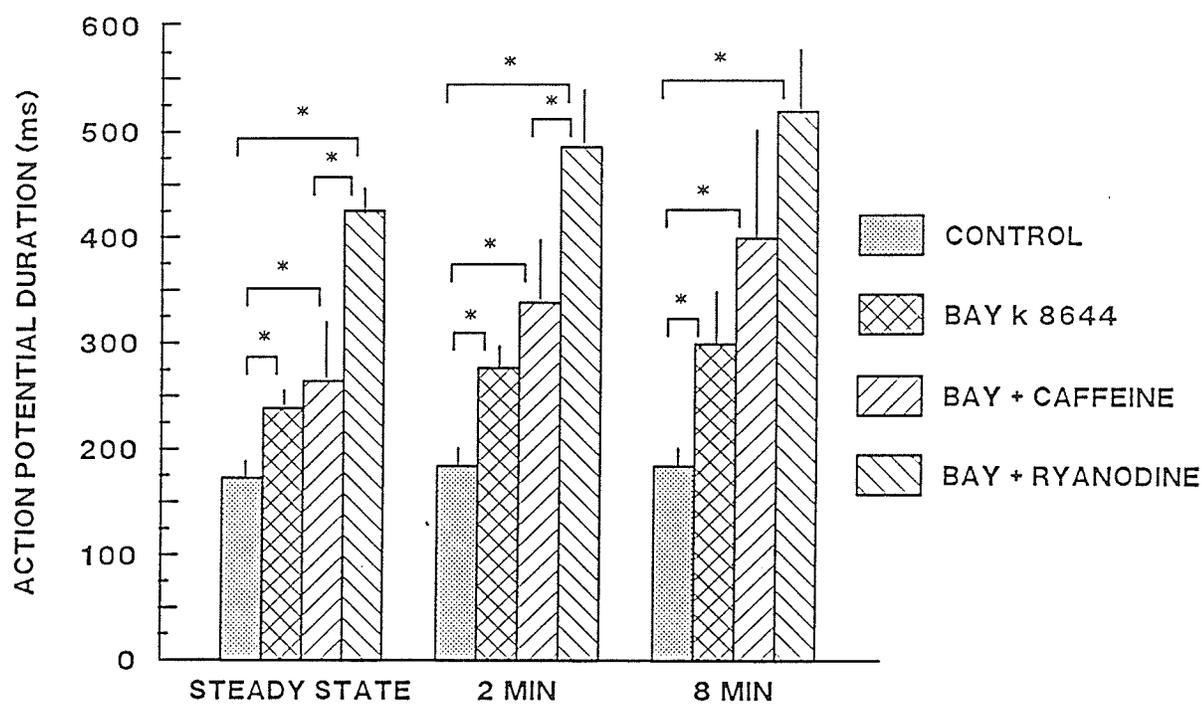


Figure 11. Pooled data from 7 experiments showing control action potential duration for 50% repolarization (densely shaded bars), the effect of Bay K 8644 (1 μ M; cross hatched bars) and a combination of BAY K 8644 with caffeine (3 mM; left diagonal striped) or ryanodine (10 nM; right diagonal striped). Measurements were made during steady-state contractions and after 2 and 8 minutes rest. Significance of differences between treated and untreated groups were determined by an analysis of variance followed by Duncan's new multiple range test for multiple comparisons. * denotes $P < 0.05$

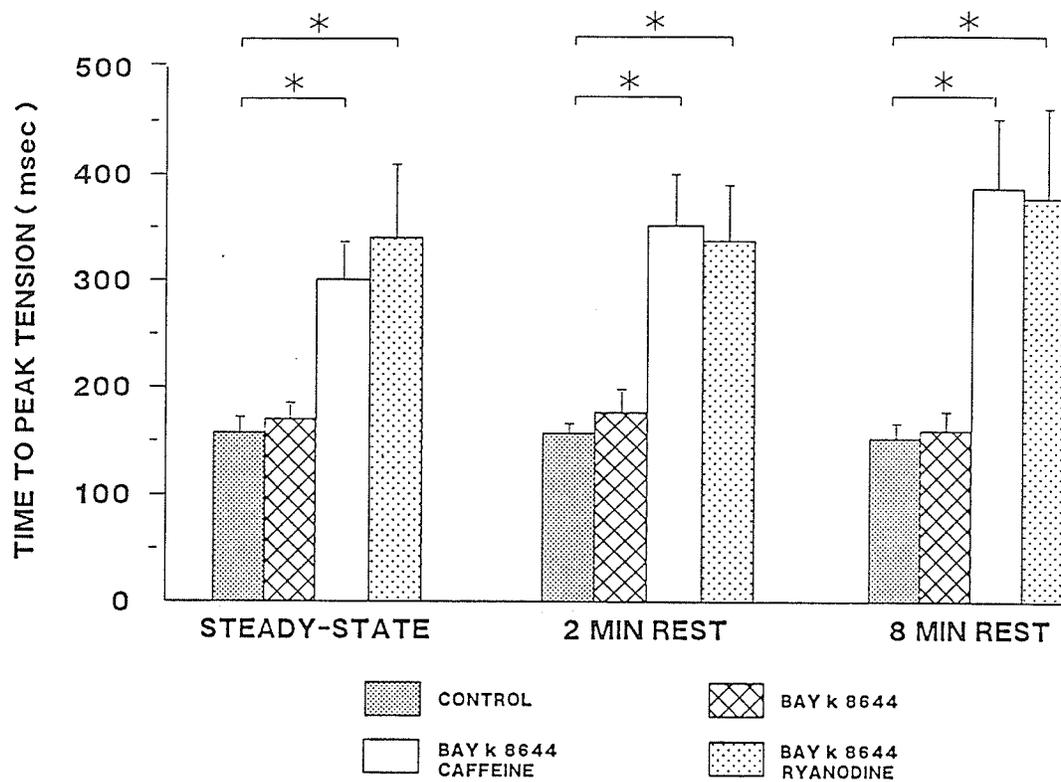


Figure 12. Pooled data from 7 experiments illustrating control time to peak tension (densely shaded bars), the effect of BAY K 8644 (1 μ M; cross hatched bars) and the combination of BAY K 8644 with caffeine (5 mM; white bars) or ryanodine (10 nM; lightly stippled bars). Measurements were made during steady-state contractions and after 2 and 8 minutes rest. Statistical analysis of this data was similar to the tests performed in earlier Figures. * denotes $P < 0.05$.

Table 4. Effect of BAY K 8644, caffeine and ryanodine on the peak and time course of isometric contraction in canine ventricular muscle.

Treatment	n	Percent Change (mean SEM)			
		TPT _{ss}	RT _{ss}	TPT _r	RT _r
BAY K 8644 (1 μ M)	5	107.0 \pm 6.7	116.6 \pm 8.5 *	112.9 \pm 10.4	129.8 \pm 11.5 *
Caffeine (5 mM)	6	124.5 \pm 1 *	135.3 \pm 7.1 *	113.0 \pm 3.5 *	134.0 \pm 10.6 *
Ryanodine (10 nM)	6	162.5 \pm 7.9 *	277.5 \pm 11.3 *	125.0 \pm 7.5 *	290.5 \pm 11 *

TPT = Time to peak tension

RT = Relaxation time

'ss' & 'r' denote steady-state and post-rest contractions respectively

* denotes $p < 0.05$ compared to control

**ANALYSIS OF THE INTERVAL-FORCE RELATIONSHIP IN
RAT AND CANINE VENTRICULAR MYOCARDIUM**

SECTION 2

SUMMARY

The mechanism of the negative force staircase in thin rat ventricular trabeculae was investigated and compared with the positive force staircase in dog ventricular muscles of comparable diameter. Increasing stimulus frequency from 0.2 to 0.5 and 1 Hz resulted in a progressive reduction in the amplitude of steady-state contraction that was demonstrated in both 1.25 and 2.5 mM $[Ca^{2+}]_o$. The negative staircase was associated with no change in the amplitude of postrest contraction or rapid cooling contracture at either $[Ca^{2+}]_o$ investigated. The results of these experiments suggest that reduced loading of the sarcoplasmic reticulum with Ca^{2+} is not a likely explanation for the negative staircase in this species and are consistent with a frequency-dependent increase in the refractoriness of the sarcoplasmic reticulum Ca^{2+} release process or a decrease in the trigger for Ca^{2+} release, assuming that the amount of Ca^{2+} present within the release site is constant from one frequency to the next. In contrast to the rat, canine ventricular muscle exhibited a positive force staircase, the slope of which depended on $[Ca^{2+}]_o$, as well as a frequency-dependent increase in the amplitude of postrest contraction and peak rapid cooling contracture. Data obtained from this latter series of experiments suggests that increased filling of the release pool within the sarcoplasmic reticulum with Ca^{2+} underlies the inotropic effect of high frequency stimulation in canine ventricular muscle.

INTRODUCTION

In the myocardium of most mammalian species, including cat, sheep, guinea pig, dog, and rabbit, twitch amplitude is typically augmented in response to a gradual increase in the rate of stimulation (e.g., positive staircase). In contrast, preparations of rat myocardium ranging from isolated perfused hearts to ventricular myocytes have been consistently reported to produce a negative force staircase at similar rates of stimulation (Kelly and Hofmann, 1960; Forester and Mainwood, 1974; Mitchell et al, 1985; Capogrossi et al, 1988). Two theories of excitation-contraction coupling specific for cardiac muscle have been proposed which assign different degrees of importance to transsarcolemmal Ca^{2+} entry for direct myofilament activation. The model originally proposed by Langer (1973, 1974; 1982) suggests that enough Ca^{2+} may enter through the plasma membrane during the action potential to directly activate the myofilaments. However, Fabiato and Fabiato (1975; 1978; 1983; 1985a-c) have put forward a different model in which both the magnitude and rate of transsarcolemmal Ca^{2+} entry serve primarily to control the amount of Ca^{2+} released from the sarcoplasmic reticulum (SR), and thereby the degree of myofilament activation. In either case, however, an increase in stimulation frequency should result in an increase in time-averaged Ca^{2+} entry across the sarcolemma into the myoplasm. Indeed, this explanation was the favoured hypothesis used to explain the positive staircase phenomenon prior to the demonstration of Ca^{2+} -induced release of Ca^{2+} in cardiac muscle (Koch-Weser and Blinks, 1963; Wood et al, 1969; Allen et al, 1976).

Experiments performed with the whole-cell voltage clamp technique on rat ventricular myocytes (Mitchell et al, 1985; 1987) have shown that an increase in frequency of rhythmic stimulation is associated with a reduction of action potential duration (APD) and amplitude of the slow inward Ca^{2+} current (I_{Ca}). It is of interest therefore, that similar directional changes in APD and peak I_{Ca} have been demonstrated in numerous species throughout the animal kingdom, where they have been associated with the development of a positive, rather than a negative staircase (for review, see Boyett and Jewel, 1980). Thus, the reduction of contractility in rat ventricle would appear to

take place under conditions usually associated with enhanced cellular Ca^{2+} influx, which should in theory lead to increased loading of the SR with activator Ca^{2+} (Wood et al, 1969; Gibbons and Fozzard, 1971; Fabiato, 1981; 1985c; Bridge, 1986; Clusin and Lee, 1987). This raises the question of whether the negative staircase in rat ventricle is a manifestation of altered loading of the SR with Ca^{2+} during steady-state stimulation or is perhaps due to some other mechanism which may influence the release process specifically.

Postrest tension development (Allen et al, 1976; Ragnarsdottir et al, 1982; Schouten and terKeurs, 1986; Bers, 1985; Lewartowski and Pytkowski, 1988, Bose et al, 1988a) and rapid cooling contractures (Kurihara and Sakai, 1985; Bridge, 1986; Bers et al, 1987; 1989) have been used extensively in recent years to estimate indirectly SR Ca^{2+} availability and release in ventricular muscle under varying physiological and pharmacological conditions. These techniques have been applied in the present experiments to independently assess SR Ca^{2+} content and release as a function of conditioning interval in both rat and canine ventricular myocardium. It is proposed that if transsarcolemmal Ca^{2+} entry is reduced with increasing rate of stimulation, postrest force production and cooling contracture amplitude would be expected to decline due to decreased loading of the SR. Conversely, if these estimates of SR Ca^{2+} availability are enhanced or remain unaltered despite changes in the amplitude of steady-state contraction, then reduced Ca^{2+} entry may not be responsible for the negative staircase. A preliminary report of part of this work has been presented to the American Physiological Society (Bouchard and Bose, 1988).

MATERIALS AND METHODS

Experimental Preparation

Trabeculae were isolated from the hearts of male Sprague Dawley rats (350-400 gm) and mongrel dogs of either sex (8-12 kg). Rats were decapitated under light ether anesthesia while dogs of either sex were anaesthetized with sodium pentobarbital (30 mg/kg i.v.). Hearts were rapidly excised and the coronary vasculature was flushed through the aorta with ice-cold Krebs-Henseleit (KH) solution. The KH solution had the following composition in mM: NaCl, 118; KCl, 4.7; CaCl₂, 2.5; MgSO₄, 1.2; KH₂PO₄, 1.4; NaHCO₃, 25; and glucose, 11. Thin, free-running right ventricular trabeculae (0.1-0.4 mm diameter) were tied with 8-0 silk thread (Matuda) and dissected free from the wall of the ventricle.

Tension Measurements

Following removal from the heart, muscles were transferred to a 2 ml horizontal recirculating tissue bath containing continuously oxygenated HEPES buffer (in mM: NaCl, 140; KCl, 4.7; MgCl₂, 1.0; HEPES ([4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid]]), 5; glucose, 10) maintained at 37°C and pH of 7.4. The muscle chamber was in series with a circuit of insulated teflon tubing forming one-half of the rapid cooling apparatus (see below). Muscles were stimulated with 2 ms pulses delivered at a continual (steady-state) frequency of 0.2 Hz by a stimulator (Grass model SD5) at an amplitude between 75-100% above threshold. After a 45-60 minute equilibration period the muscles were adjusted manually with a micrometer to their optimal length (L_{max}), where developed tension was maximal. Rest intervals ranging from 30 to 240 seconds were imposed on the otherwise constant stimulation pattern. When switching from one frequency to the next, at least 10 minutes were allowed for re-equilibration prior to initiating the following train of test intervals.

It has been suggested (Schouten and terKeurs, 1986; Gulch and Ebrecht, 1986) that the negative tension staircase often demonstrated in isolated rat heart may be the result of compromised metabolism in the core cells of the muscle at higher frequencies of stimulation. In

the study of Schouten and terKeurs (1986), isolated trabeculae from the right ventricle of rats having a "critical diameter" of 0.2 mm or less did not exhibit the negative staircase response, a finding the authors attributed to a lack of ischemia usually present in thicker preparations. In an attempt to address this problem, muscles used in the present investigation were also divided on the basis of their diameter. In addition, muscles isolated from rat hearts consistently occupied the lower diameter range (0.1-0.3 mm) when compared to canine trabeculae (0.2-0.4 mm).

Rapid Cooling Contractures

For these experiments, the trabeculae were mounted in a 2 ml bath in series with a two-reservoir recirculation apparatus. Continuously oxygenated HEPES buffer flowed to the bath by gravity from one of two reservoirs suspended above the muscle chamber. Flow rate into the muscle chamber was ~35 ml/min for the warmed circuit and ~40 ml/min for the cooled circuit, and was controlled by adjusting the tubing diameter and reservoir height. Rapid cooling contractures were elicited in the place of a regular beat (e.g., steady-state cooling contractures) or after variable rest intervals by switching the stream of solution entering the muscle chamber through a parallel by-pass system. Cooling of the solution surrounding the outer cells of the muscle (from 37°C to 0-2°C) occurred typically in <1 sec. Force production was measured with a force transducer (Grass FT-O3C) and contractions were recorded on a 4 channel polygraph (Grass model 7) for later analysis. Periods of rest identical to those used in the twitch tension experiments were re-examined with cooling contractures substituting for twitch contractions. After the completion of a given set of test intervals, 10 minutes were allowed at the next experimental frequency prior to commencing the next train of test intervals.

Statistics

Data were analysed with a repeated measures analysis of variance using the least-squares method of general linear modelling. The level of significance chosen for all blocked experiments was $P < 0.05$. When significance was found within the full model for the dependent variable, multiple range tests for the least-square means were performed to determine the level of significance.

RESULTS

RAT VENTRICLE

Frequency-Force Relationship: As discussed in the METHODS section, a number of recent studies have re-examined whether the negative force staircase represents the intrinsic response of this tissue, or is a better reflection of hypoxia in the cells at the core of the muscle preparation (Schouten and terKeurs, 1986; Gulch and Ebrecht, 1986). Prior to investigating the mechanisms tentatively underlying the staircase, an attempt was made to address this concern. Our first approach was to determine the effect of muscle thickness on tension developed in response to increasing the frequency of rhythmic stimulation. Secondly, the experimental protocol was repeated under identical conditions using isolated canine right ventricular trabeculae.

Figure 13 shows the effect of varying external Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$) on isometric force developed by rat right ventricular trabeculae. Increasing the stimulation frequency from 0.2 to 0.5 and 1.0 Hz produced a descending contractile staircase, where tension development was 100 , 77 ± 1.96 and $56 \pm 2.65\%$ of maximal steady-state force production respectively. The staircase remained unaltered when $[\text{Ca}^{2+}]_o$ was elevated to 2.5 mM. In this respect, the results are similar to the previous demonstration of a negative staircase in the range of $[\text{Ca}^{2+}]_o$ of 1.1-2.5 mM (Forester and Mainwood, 1974; Orchard and Lakatta, 1985; Gulch and Ebrecht, 1986). Previous studies have demonstrated that the slope of the staircase is particularly sensitive to $[\text{Ca}^{2+}]_o$ between stimulation frequencies of 0.1 and 3.0 Hz; as $[\text{Ca}^{2+}]_o$ is progressively lowered from 2.5 mM to 0.25 mM, the staircase response is converted from a continuous monophasic drop in force in the presence of 2.5 mM $[\text{Ca}^{2+}]_o$, to a biphasic response where the negative staircase observed at 0.1 to 0.5 Hz stimulation is followed by positive staircase as the rate of stimulation is increased from 0.5 to 3.0 Hz (Forester and Mainwood, 1974). The mean diameter of the muscles used in the present experiments was 0.2 mm, which was similar to the "critical diameter" suggested by earlier studies (Schouten and terKeurs, 1986) to avoid core hypoxia.

To determine the effect of muscle thickness on the frequency-force relation, muscles were grouped arbitrarily according to diameter (0.1-0.29 and 0.3-0.4 mm). This was done to see if the thinner muscles produced a qualitatively different frequency-force relation compared to the thicker preparations. Figure 14 shows that in the presence of 1.25 mM $[Ca^{2+}]_o$, an increase in the rate of stimulation produced a negative staircase in both groups. Although the thicker muscles did develop less tension at the fastest drive rate, this difference was not statistically significant. The correlation between frequency and developed tension remained the same when $[Ca^{2+}]_o$ was elevated to 2.5 mM. The data are in agreement with recent documentation of a negative frequency-force relation in both ultra-thin rat papillary muscles (<0.1 mm) under conditions of relatively low energy demand (0.125-2.0 Hz in Gulch and Ebrecht, 1986) and isolated rat ventricular myocytes (Mitchell et al, 1985; 1987; Capogrossi et al, 1988), which have little diffusional barrier for O_2 delivery.

Figure 15 illustrates the relationship between stimulation frequency and isometric tension developed by isolated trabeculae from canine myocardium. The mean diameter of the muscles in these experiments was greater than that obtained from the rat hearts (0.28 vs 0.2 mm respectively). However, unlike the rat trabeculae, a similar increase in the rate of stimulation was found to produce a positive force staircase in the canine trabeculae. Also different from the rat response, was the dependence of the slope of this relationship on $[Ca^{2+}]_o$. For example, increments of stimulation frequency in the upper portion of the frequency range (1.5-3.0 Hz) consistently augmented steady-state contractions in both Ca^{2+} -containing solutions, but only in 2.5 mM $[Ca^{2+}]_o$ was the same effect observed at low frequencies (0.5-1.5 Hz.).

Postrest Response: To test the possibility that the negative staircase in rat ventricular muscle might occur as a result of altered SR Ca^{2+} accumulation, postrest contractions were elicited after variable periods of rest as an index of SR Ca^{2+} loading. Potentiation of steady-state contraction amplitude after brief periods of rest has been previously reported in rat (Ragnarsdottir et al, 1982; Schouten and terKeurs, 1986), canine (Endoh et al, 1981; King and Bose, 1984; Hryshko et al,

1989a) and kitten (Koch-Weser and Blinks, 1963) ventricle, where it has been attributed to time-dependent redistribution of Ca^{2+} from an "uptake" site to a "release" site within the SR during the course of rest.

Figure 16 illustrates the typical contractile response of an isolated rat trabeculae to rest for 30-120 seconds. Increasing the stimulation frequency from 0.2 to 0.5 and 1.0 Hz slightly augmented the amplitude of the first postrest beat in the presence of 1.25 mM $[\text{Ca}^{2+}]_o$, despite the decline in steady-state contraction amplitude at these drive rates. This dissociation in the amplitude of steady-state and postrest contractions was reflected by the gradual elevation in the degree of rest-potential when contractions were expressed as a percentage of steady-state contraction. Pooled data illustrating this response have been furnished in Fig. 17.

Cardiac muscle from several mammalian species show the phenomenon of "rest-decay", whereby the decline in the amplitude of the first postrest beat with time has been associated with the loss of releasable Ca^{2+} from the SR (Sutko and Willerson, 1980; Bers, 1985; Bridge, 1986; Bose et al, 1988a) to the extracellular space (Hilgemann, 1986a,b; Bers and McLeod, 1986; Bers et al, 1987). Figs. 16 and 17 show the absence of this type of tension decay in isolated rat trabeculae, as the amplitude of the first postrest beat was not altered when the rest duration was gradually extended over the range of 30-240 seconds (240 seconds rest not shown). Increasing $[\text{Ca}^{2+}]_o$ to 2.5 mM had no effect on the restitution of postrest contraction observed in response to changes in the frequency of rhythmic stimulation or duration of rest. Thus, as shown in Fig. 17 (Panel B - lightly stippled bar), the amplitude of the first postrest contraction found throughout the range of rest intervals could be represented by a single value.

Rapid Cooling Contractures: The results obtained so far suggest that the content of Ca^{2+} located within the SR and the fraction released by postrest stimulation appear to remain unaffected with increasing frequency. This is contrary to what would be expected if the inverse relationship between the rate of stimulation and steady-state contraction was a reflection of reduced SR Ca^{2+}

loading. Rapid cooling contractures were elicited using a similar stimulation protocol to further assess SR Ca^{2+} availability.

Figure 18 depicts a representative response of isolated rat trabeculae to rapid cooling in the presence of 1.25 mM $[\text{Ca}^{2+}]_o$. The typical decrease in isometric contraction associated with a graded increase in the rate of stimulation is evident in each of the panels prior to cooling. A progressive increase in the rate of steady-state stimulation had no effect on the amplitude of RCCs elicited in the place of a regularly driven beat, despite the reduced amplitude of steady-state contractions preceding cooling. This was reflected by the stepwise increase in the amplitude of these contractures when they were expressed as a percentage of steady-state twitch amplitude associated with the individual drive rates. An identical response was obtained when $[\text{Ca}^{2+}]_o$ was elevated to 2.5 mM. If the steady-state RCCs are indeed indicative of the amount of Ca^{2+} present in the SR at the time when an evoked stimulus would have occurred, it may be concluded from these experiments that the negative tension staircase is not a reflection of reduced SR Ca^{2+} loading.

Further experiments were conducted to determine the magnitude of RCCs elicited after progressively longer periods of rest. Increasing the duration of rest in the presence of 1.25 mM $[\text{Ca}^{2+}]_o$ had no effect on the amplitude of RCCs obtained after 30, 120 and 240 seconds rest. Pooled data for cooling contractures elicited after 120 seconds rest have been provided in Panel B of Fig. 18. Similar to the results observed with postrest contraction, the inability of stimulus frequency to alter the amplitude of either steady-state or postrest RCC was not related to $[\text{Ca}^{2+}]_o$. The only difference we observed was that in the presence of 1.25 mM $[\text{Ca}^{2+}]_o$, an increase in the duration of rest produced a small but reliable increase in the amplitude of postrest RCCs. Conversely, when the rest response was re-examined in 2.5 mM $[\text{Ca}^{2+}]_o$, a similar increase in rest duration led to a small decrease of postrest RCC amplitude. Both of these trends, however, did not attain statistical significance. The absence of an "apparent" diastolic Ca^{2+} loss in rat cardiac muscle is different from that observed in other species (Kurihara and Sakai, 1985; Bridge, 1986; Bers et al, 1987), as mentioned earlier. Thus, these data imply that, not only does the SR of rat

cardiac cells not lose Ca^{2+} with time, but under certain experimental conditions, may instead gain it.

Figure 19 summarizes the relationship between developed tension and the entire range of test intervals studied, including both rhythmic stimulation (smallest test interval in each block of data) and the various rest intervals (30-240 seconds) at $[\text{Ca}^{2+}]_o$ of 1.25 mM. The lower panel represents the data in units of force, while the upper panel is a replotting of the data as a percentage of steady-state twitch amplitudes. In addition to the reduction of steady-state contraction, no change was observed in the pattern of normalized tension change for postrest contraction or rapid cooling contractures within a given block of data following an increase of stimulation frequency. Thus, increasing the rate of stimulation always resulted in decreased steady-state contraction, increased rest-potential, increased RCC amplitude, and a lack of time-dependent decay in the amplitude of postrest contraction or rapid cooling contracture with increasing rest duration. The dissociation of steady-state twitch amplitude from normalized postrest contraction and cooling contraction amplitude can be explained by the calculations in the lower panel. Here, a progressive increase in the rate of stimulation had no significant effect on either measure of contractility, thus explaining the augmented amplitude of these contractions when normalized for decreasing steady-state contraction.

With minor variation, the situation was similar when $[\text{Ca}^{2+}]_o$ was increased to 2.5 mM. The exception was the clear lack of rest-potential at the lowest frequency (0.2 Hz). For this reason it is tempting to speculate that the sensitivity of the mechanisms responsible for force production are inversely related to $[\text{Ca}^{2+}]_o$. This would be in accordance with the suggestion that the SR of rat cardiac muscle is saturated or nearly saturated with Ca^{2+} when the perfusate $[\text{Ca}^{2+}]$ is elevated above ~ 2.0 mM (Forester and Mainwood, 1974; Fabiato, 1981; Schouten et al, 1987; Capogrossi et al, 1988), which would in turn render the coupling of excitation and contraction more sensitive to beat frequency at $[\text{Ca}^{2+}]_o$ of 1.25 mM.

CANINE VENTRICLE

Frequency-force Relationship: Canine ventricular muscle yielded a positive tension staircase in response to increments of stimulation frequency from 0.5 to 3.0 Hz. As shown previously in Fig. 15, the degree of potentiation of steady-state contraction after switching to a higher drive rate was found to be dependent on $[Ca^{2+}]_o$. In the presence of 1.25 mM $[Ca^{2+}]_o$, little change in force production was observed in response altered beat frequency in the lower frequency range. However, when the muscles were bathed in 2.5 mM $[Ca^{2+}]_o$, a greater increase in steady-state tension development was observed when the rate of stimulation was increased. Therefore, unlike the contractile response of the rat trabeculae to increasing frequency, isolated muscle from canine heart produced a frequency-force relation which was more dependent on $[Ca^{2+}]_o$.

Postrest Response: A representative effect of stimulation frequency on postrest contraction in isolated canine trabeculae is shown in Panels A-C of Fig. 20. Responses in the presence of 1.25 mM and 2.5 mM $[Ca^{2+}]_o$ are shown in the top and bottom recordings of each pair, respectively. As was the case with steady-state contraction, the relationship between the amplitude of the first postrest beat and the preceding rate of stimulation was found to depend on $[Ca^{2+}]_o$. In the presence of 1.25 mM $[Ca^{2+}]_o$, a gradual increase of stimulation frequency resulted in a parallel increase in the amplitude of the first postrest contraction. Also illustrated in this Figure, is the bell-shaped relationship between postrest contraction amplitude and rest duration, which is characteristic of isolated canine myocardium (Endoh et al, 1981; King and Bose, 1984; Hryshko et al, 1989a,b).

When $[Ca^{2+}]_o$ was increased to 2.5 mM, peak rest-potentiation was shifted to an earlier test interval as the rate of stimulation was increased from 0.5 to 1.0 Hz. This is different from the response in the presence of 1.25 mM $[Ca^{2+}]_o$, where the test interval associated with peak rest-potentiation remained unaltered with the rate change. A further increase in drive rate to 1.5 Hz resulted in a subsequent elevation in the amplitude of the first postrest beat. The time-dependent

decay of postrest contraction which followed progressive lengthening of rest was found to be slightly enhanced with the higher drive rates.

The diagram on the bottom of Fig. 20 illustrates the effect of increasing the frequency of stimulation on postrest contraction after 30 seconds rest. Contractions have been expressed in this plot as a percentage of steady-state contractions at the individual frequencies, as opposed to being represented in units of force. Interestingly, an increase of frequency was found to reduce, rather than augment, the rest response in 2.5 mM $[Ca^{2+}]_o$, with opposing results in 1.25 mM $[Ca^{2+}]_o$. Inspection of these data expressed in units of force (Fig. 23) and the dependence of the frequency-force relation on $[Ca^{2+}]_o$ (Fig. 15) indicate that the rate-dependent decrease of postrest contraction in 2.5 mM $[Ca^{2+}]_o$ is associated with the greater inotropic effect of stimulation frequency on steady-state contraction.

Rapid Cooling Contractures: A representative response of isolated canine ventricular muscle bathed in 1.25 mM $[Ca^{2+}]_o$ to rapid cooling is illustrated in Fig. 21. The rate of rise of the cooling contractures in canine ventricle was similar to that observed in rat ventricle with the exception of being slightly more gradual in onset and with a less pronounced peak. The main difference in the time course of these contractures was the very low rate of decay of individual contractures obtained in the dog compared with those in the rat. Often, the cooling contracture persisted for 2-3 minutes whereas contractures obtained in rat under similar conditions were completed within 45-60 seconds. In this respect the data are similar to those reported for both rabbit and guinea-pig ventricle by Bridge (Bridge, 1986) and Bers (Bers, 1985; 1989; Bers et al, 1987). Increasing the rate of stimulation significantly enhanced peak cooling contracture amplitude, while producing little effect on the amplitude of contractures after normalization for steady-state contraction. The trend observed was similar to that observed for normalized postrest contractions, which were affected by the degree of significance associated with changes in steady-state contractions.

The effect of altering the frequency of stimulation on the amplitude of the postrest cooling contractures is shown in Fig. 22. Unlike the response of rat ventricle, lengthening the duration of

rest in canine ventricle produced a progressive decline in the amplitude of postrest cooling contractures, which began from the moment stimulation was terminated. Furthermore, the rate of decay of cooling contracture amplitude was not altered appreciably by either increasing the stimulus frequency or altering $[Ca^{2+}]_o$.

Figure 23 summarizes the complex interaction observed between twitch amplitude and stimulus interval and $[Ca^{2+}]_o$ in isolated canine trabeculae. Contrary to the results provided in Fig. 19 for rat ventricle, different interval-force relations were obtained in different $[Ca^{2+}]_o$ in canine ventricle. As shown in Panel A, increasing the rate of stimulation in the presence of 1.25 mM $[Ca^{2+}]_o$ augmented the amplitude of postrest contractions, despite producing minimal effects on steady-state contraction in the lower frequency range. This pattern was similar for changes observed in RCC amplitude from one frequency block to another. Increasing the duration of rest within a given frequency in canine trabeculae resulted in a significant decrease in the degree of rest-potential and cooling contracture amplitude. Panel B summarizes the interval-force relation in 2.5 mM $[Ca^{2+}]_o$. Unlike the response observed in 1.25 mM $[Ca^{2+}]_o$, the pattern of tension change was also dependent on the preceding rate of stimulation. Increasing stimulation frequency from 0.5 to 1.0 Hz was found to gradually shift peak rest-potential to a shorter test interval. A second increase of stimulus frequency to 1.5 Hz, did not shorten the test interval associated with peak rest-potential, as shorter rest durations were not examined. However, it did increase the twitch amplitude associated with the smallest test interval. The significant potentiation of steady-state twitch amplitudes found in response to increasing stimulation frequency in 2.5 mM $[Ca^{2+}]_o$, compared to that seen in 1.25 mM $[Ca^{2+}]_o$, was found to alter the pattern of normalized contractions. Instead of producing an increase in peak rest-potential and cooling contracture amplitude, increasing the rate of stimulation in 2.5 mM $[Ca^{2+}]_o$ caused a progressive decrease in the normalized amplitude of these contractions.

DISCUSSION

The purpose of this study was to relate changes in twitch amplitude of ventricular trabeculae from rat and dog at different rates of rhythmic stimulation with the degree of SR Ca^{2+} loading present under these conditions. The data obtained from these experiments support the conclusion that the negative staircase demonstrated in the rat ventricle is not a result of reduced loading of the SR with Ca^{2+} . The increasing discrepancy in the amount of Ca^{2+} present within pooled SR stores and that delivered to the myofilaments as the stimulus rate is enhanced suggests that there may be a rate-related change in the coupling of excitation to contraction, or alternatively, in the amount of Ca^{2+} translocated to the release site prior to the arrival of the next stimulus. Adequate resolution of Ca^{2+} recirculation through the SR requires estimates of both pooled SR stores and that Ca^{2+} present within the release site itself at various times after the termination of rhythmic stimulation. As such changes are likely to be the most profound at the time nearest to the interval between rhythmically stimulated beats (e.g., 2-10 sec), these results do not permit an estimate of Ca^{2+} movements within the SR at the time most critical to steady-state contraction, assuming that recycling of Ca^{2+} from an "uptake" site to a "release" site is faster than that demonstrated in canine ventricle. The covariance of steady-state contraction with cooling contracture and postrest contraction in response to different rates of stimulation in canine ventricle implies that the strength of isometric contraction is a good indicator of both global SR Ca^{2+} content and fractional release in this species.

Interval-force Relation in Rat Ventricle

The importance of the time course of the ventricular action potential and associated membrane currents in determining twitch force has been well established (Morad and Trautwein, 1968; Wood et al, 1969; Morad and Goldman, 1973; Wohlfart and Noble, 1982). In the majority of species, both the duration and plateau amplitude of the action potential are diminished upon an increase in the rate of stimulation (Boyett and Jewell, 1980). Despite the possible reduction of peak I_{Ca} associated

with individual action potentials, the inotropic effect of increasing frequency has been correlated with a parallel rate-dependent increase in the size of the intracellular Ca^{2+} transient (Allen and Blinks, 1978; Wier and Yue, 1986; Clusin and Lee, 1987), extracellular Ca^{2+} depletions (Bers, 1983; Hilgemann, 1986b), and SR-mediated rapid cooling (Kurihara and Sakai, 1985) or high potassium-low sodium contractures (Gibbons and Fozzard, 1971). Data obtained from skinned muscle fibres (Fabiato, 1985c) has suggested that both fast and slow components of the Ca^{2+} current are necessary for the positive staircase. The description of this phenomenon in the present experiments on canine trabeculae is consistent with the recent observation that the "L-type" Ca^{2+} current in canine ventricular myocytes shares similar activation and inactivation properties with those present in guinea-pig (Tseng, 1988), a species which also has a positive frequency-force relation.

The inactivation of I_{Ca} in rat ventricular cells has been shown to be both faster (Josephson et al, 1984; Mitchell et al, 1985; 1987) and more complete (Josephson et al, 1984) than currents of similar magnitude in other species. The brief time course of this current is compatible with the relatively short action potential duration recorded from multicellular preparations (Josephson et al, 1984; Mitchell et al, 1985; Schouten and terKeurs, 1986). It has been previously suggested that the absence of a slow component of Ca^{2+} influx (e.g., the large degree of steady-state inactivation of I_{Ca}) may be responsible for the negative staircase in rat ventricle (Fabiato, 1985c). Support for this statement was derived from the relatively fast inactivation of I_{Ca} in the rat (~80% in 15 ms) compared to the guinea-pig (~55% in 70 ms) and the demonstration of a negative force staircase in skinned canine purkinje fibers deprived of the slow loading component of a simulated Ca^{2+} current (cf. Fig.9 of Fabiato, 1985c). The increased rate of stimulation was proposed to lead to a reduction of SR Ca^{2+} content due to a decrease in refilling of the release pool between twitches. A number of experimental observations suggest this may not be the case in intact ventricular preparations. First, is the independence of peak cooling contracture amplitude or postrest contraction from stimulus frequency observed in the present experiments. Secondly, a much greater effect on peak I_{Ca} (~50%) is observed in isolated rat ventricular cells following an increase

in stimulus frequency from 0.3 to 3.0 Hz (Mitchell et al, 1985) compared to the almost negligible effect on I_{Ca} decay, particularly in the later stages of inactivation. Furthermore, application of ryanodine produces little effect on peak I_{Ca} or its decay (Mitchell et al, 1985), in spite of converting the negative to a positive staircase in intact muscle preparations (Temma and Akera, 1986; Kort and Lakatta, 1988b) as well as isolated myocytes (Mitchell et al, 1985). Thus, although the Ca^{2+} current of rat lacks the loading component found in other species, it seems that due to the relative stability of current inactivation from low to high frequency stimulation, that changes of I_{Ca} peak (e.g., the trigger for release) may be more likely to explain the negative staircase.

The observations made in this study also suggest that unlike ventricular muscle from most species, the amplitude of steady-state contraction is not a reliable indicator of global SR Ca^{2+} content in the rat. This is typified by the parallel and rate-dependent increase in twitch and cooling contracture amplitude in canine, but not rat ventricular muscle. Because of this difference, the results imply that the extent of Ca^{2+} release from the SR may not be mediated simply by the degree of absolute loading. For example, in the presence of ryanodine, preparations of rat cardiac muscle ranging from intact muscle to dispersed isolated cells uniformly exhibit a positive rather than a negative staircase (Mitchell et al, 1985; Temma and Akera, 1986; Kort and Lakatta, 1988b). Moreover, in previous work we have demonstrated that canine ventricular trabeculae also show a positive force staircase under the influence of similar concentrations of ryanodine (Bose et al, 1988a). Hence, a site that may be involved in the production of the negative staircase may in fact be the coupling of the plasma membrane with the SR at the junctional membrane- Ca^{2+} -release channel complex.

A second possible explanation for the descending tension staircase is raised by data obtained from experiments measuring the change of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) under voltage clamp conditions. In guinea pig ventricular myocytes there is a parallel bell-shaped voltage-dependence for minimal and peak activation of ryanodine-sensitive $[Ca^{2+}]_i$ transients and verapamil-sensitive membrane currents (Beuckelmann and Wier, 1989). Data obtained from these experiments suggest that $[Ca^{2+}]_i$ transients occurring as a result of Ca^{2+} release from the SR and

the amplitude and duration of I_{Ca} may be activated and co-regulated over the same range of membrane potentials in isolated guinea pig ventricular cells. The results of this type of experiment are different in rat cardiac cells (Cannell et al, 1987). Here, I_{Ca} peak shares a similar bell-shaped dependence on membrane potential to that of the guinea pig, activating at about -40 mV and producing a peak at -10 mV. However, increase in $[Ca^{2+}]_i$ in the rat occurs at a more polarized membrane voltage than that needed for activation of I_{Ca} in the rat (Cannell et al, 1987) or for the voltage-dependence of $[Ca^{2+}]_i$ in isolated cells from the guinea pig (Beuckelmann and Wier, 1989). Another finding was that repolarization of the membrane after progressively increasing durations of voltage clamp had the ability to terminate the rise of $[Ca^{2+}]_i$. This has direct implications with respect to the interval-force relation in rat cardiac muscle, as it suggests that changes in the duration of transmembrane action potential may directly influence the time course of the intracellular Ca^{2+} transient, independent of its effect on I_{Ca} . Thus, one possible explanation for the negative inotropy associated with increments of stimulus frequency in the rat may be the decrease in the duration of the action potential associated with this intervention (Watanabe et al, 1983; Mitchell et al, 1985; Schouten and terKeurs, 1986). If, as suggested by Cannell et al (1987), the release of Ca^{2+} is partially modulated by intramembrane charge movement, the reduced duration of membrane depolarization at higher rates of stimulation could theoretically shorten the course of Ca^{2+} release from the SR. Although a number of reports have indicated that changes in the duration of voltage-clamp pulses are associated with accompanying changes in the duration of the $[Ca^{2+}]_i$ transient (Barcenaz-Ruiz et al, 1987; Cannell et al, 1987; Bueckelmann and Wier, 1989; Callawaert et al, 1990), the possibility that charge movement within the membrane of cardiac cells directly controls the time course of Ca^{2+} release has yet to be determined (Fabiato, 1989).

Excessive intracellular Ca^{2+} loading has been previously demonstrated to inhibit force production by causing non-homogeneous Ca^{2+} release from the SR (Allen et al, 1985; Fabiato and Fabiato, 1975; 1978; Fabiato, 1981; Stern et al, 1984; Kort and Lakatta, 1988b). In this scheme, force development is reduced by a combination of two factors: (a) a decrease in the amount of Ca^{2+} present in the release site of the SR, and (b) an increase in series compliance due to regional

asynchronous release of Ca^{2+} from the SR. Cross-species experiments using scattered light intensity fluctuations (SLIF) to estimate asynchronous Ca^{2+} -mediated diastolic myofilament oscillations have found these to be most pronounced in rat myocardium compared to other species investigated and are facilitated by increasing $[\text{Ca}^{2+}]_o$ (Stern et al, 1985; Kort and Lakatta, 1988b). Thus, it is possible that time-averaged transsarcolemmal Ca^{2+} entry could be transiently facilitated by increasing stimulation rate in rat myocardium, as seems to be the case in canine ventricle. As discussed by Mitchell et al (1985), the frequency of regional spontaneous asynchronous Ca^{2+} release from the SR would also be augmented in this setting due to continual intracellular accumulation of Ca^{2+} . This model would predict that the SR membrane would become increasingly "refractory" for subsequent stimulus-dependent Ca^{2+} release. However, recent experiments using this technique have shown that SLIF are not present in rat papillary muscle unless the interval between beats exceeds 5 seconds (Kort and Lakatta, 1988a). This result suggests that spontaneous Ca^{2+} release from SR stores does not occur unless the stimulus frequency is 0.2 Hz, or less. It is of interest that the time course of restitution of SLIF after terminating stimulation and their relation to preceding patterns of electrical stimulation were accelerated in 2.5 mM compared to 1.25 mM $[\text{Ca}^{2+}]_o$. The demonstration that SLIF are not present during diastolic intervals within the 0.2 -1.0 Hz range and independence of the actual staircase from $[\text{Ca}^{2+}]_o$ in the 1.25 mM-2.5 mM range suggest that frequency-dependent focal SR Ca^{2+} release may not be the primary mechanism underlying the negative staircase.

The observation that postrest contraction is more sensitive (in terms of percentage potentiation) to stimulus frequency when perfusate Ca^{2+} is lowered to 1.25 mM is consistent with the proposal that the SR of rat ventricle may be saturated with Ca^{2+} at concentrations equal to or greater than ~2.0 mM (Forester and Mainwood, 1974; Fabiato, 1981; Schouten, 1985; Capogrossi et al, 1988). Other evidence in support of this proposal includes the relatively small difference observed in the amplitude of peak steady-state cooling contractures following an increase of $[\text{Ca}^{2+}]_o$ from 1.25 to 2.5 mM, despite large changes in the strength of steady-state contraction, and the $[\text{Ca}^{2+}]_o$ -sensitivity of shifts in the degree of rest-potentiation with increasing duration of rest. For example,

gradually extending the duration of rest prior to resuming stimulation was associated with a very slight but measurable decrease in the amplitude of the first postrest contraction, in the presence of 2.5 mM $[Ca^{2+}]_o$. However, after lowering $[Ca^{2+}]_o$ to 1.25 mM, similar test intervals were associated with a progressive increase in the amplitude of the first postrest contraction. These observations suggest that the capacity of the SR to accumulate and store Ca^{2+} during a period of rest may increase as the concentration of Ca^{2+} in the bathing medium decreases, or that the cell is actually gaining $[Ca^{2+}]_o$ during this time. Rest behaviour of this type has been recently reported in rat papillary muscles (Shattock and Bers, 1989), where augmented postrest contractions were correlated with net extracellular Ca^{2+} depletion during the period of rest. The enhanced cellular Ca^{2+} repletion inferred to occur during quiescence was not observed in similar preparations of isolated rabbit ventricular muscle and was explained by assuming species differences in the activity and direction of Na^+-Ca^{2+} exchange during both rest and muscle activation. This was supported by the demonstration that intracellular sodium activity (a_{Na}^i) was considerably higher in rat ventricle than that reported to exist in other species. Our observation that the amplitude of both postrest contraction and cooling contractures were well maintained following extended rest agree with that conclusion and extend the interpretation further to mechanical events associated with different rates of stimulation and $[Ca^{2+}]_o$. However, the independence of steady-state cooling contracture amplitude from changes in stimulus frequency and rest interval does not support the prediction (Shattock and Bers, 1989; see also Lukas and Bose, 1986) that net cellular Ca^{2+} loss associated with stimulation eventually leads to reduced SR Ca^{2+} content at higher rates of stimulation.

Interval-force Relation in Canine Ventricle

In preparations of canine ventricle, an increase in rate of stimulation resulted in a stepwise increase in both steady-state and postrest contraction. This behaviour might be expected if increasing stimulation were to result in a parallel increase in transsarcolemmal Ca^{2+} influx, as discussed above. In this respect the results reported here are compatible with those of Endoh et al

(1981) who described a positive staircase for both steady-state and postrest contractions in the 0.1-0.5 Hz range. It is important to stress that the pattern of tension change which occurred in response to altered stimulus interval in canine trabeculae reported here was different when the data were expressed relative to contractions developed at individual frequencies of stimulation. The lack of significant effect of stimulus frequency on scaled contractions in the presence of 1.25 mM $[Ca^{2+}]_o$ and the decline of such values in 2.5 mM $[Ca^{2+}]_o$ suggests that, in parallel with increasing strength of contraction, the processes coupling the various fluxes of Ca^{2+} across the sarcolemma and the subsequent release of Ca^{2+} from intracellular storage sites become more predisposed to maintaining high levels of myoplasmic Ca^{2+} as rate of stimulation is increased. This explanation is consistent with the demonstration that it is not only the magnitude but also the rate of simulated transsarcolemmal Ca^{2+} influx which has the capacity to exert pronounced effects on the extent of Ca^{2+} release from the SR (Fabiato, 1983; 1985a-c). Thus, in addition to enhanced intracellular Ca^{2+} release it is quite possible that with augmented loading of the SR or a progressive decrease in the duration of diastole, a proportionately larger percentage of Ca^{2+} present at or near the inner surface of the membrane accompanying an increase of stimulation frequency may itself be directed to the myofilaments for tension development.

As shown in Fig. 21, the magnitude of SR Ca^{2+} release stimulated by rapid cooling was enhanced by increments of stimulus frequency. Similar to results obtained from the rest experiments, the pattern of tension change was altered when the data were expressed in terms of force production occurring at the individual drive rates. Regardless of the test interval studied or $[Ca^{2+}]_o$, the cooling contracture amplitude was always greatest when elicited in the place of a regularly driven beat, as opposed to those elicited following a period of rest. A marked decline in the magnitude of cooling contractures with increasing quiescence in this manner has been reported in a number of other species (Bridge, 1986; Bers et al, 1987; Bers, 1989; Bers et al, 1989; Hryshko et al, 1989c; Bouchard et al, 1989), where it was presumed to occur due to a continual loss of Ca^{2+} from the SR. Leakage of Ca^{2+} as such from SR stores of saponin-skinned guinea pig ventricular fibers (Kitazawa, 1984) has been reported to occur with a rate constant comparable

with that observed in canine (present study) and rabbit ventricular myocardium (Bridge, 1986). The lack of effect of stimulus frequency on the slope of cooling contracture decay suggests that the loss of Ca^{2+} from stores located within the SR is a fundamental event which is regulated by the content of Ca^{2+} present in this organelle. Interestingly, the pattern of postrest tension decay was more pronounced when measured by electrical stimulation, as opposed to that seen with cooling. This can be partially explained by assuming that the rate of Ca^{2+} loss from the SR is enhanced primarily by increasing the total content of Ca^{2+} present within pooled compartments. It has been frequently proposed that loss of Ca^{2+} in this manner from the SR may in large part be mediated through the activity of the forward mode of sarcolemmal Na^+ - Ca^{2+} exchange (Sutko et al, 1986a; Bridge, 1986; Hryshko et al, 1989a,c; Bers and Bridge, 1989).

Based on available evidence it is reasonable to suggest that the amount of Ca^{2+} present within the SR at a given moment in the cardiac cycle is the product of several influx and efflux processes. Thus, a prime determinant of SR Ca^{2+} loading during rhythmic stimulation is the time-averaged influx of Ca^{2+} across the sarcolemma, the integral of which per given unit time is likely to be affected by the rate of stimulation. It is unknown if the trigger for SR Ca^{2+} release, be it the rate and amplitude of cumulative transsarcolemmal Ca entry (Fabiato, 1983; 1985a-c; Callewaert et al, 1989; Nabauer et al, 1990; Valdeomillos et al, 1990; Leblanc and Hume, 1990) or membrane voltage change and accompanying gating currents (Fabiato, 1982; Cannell et al, 1987; Field et al, 1988; Bean and Rios, 1989), is altered by different conditioning intervals in different species. As a result, it is likely that Ca^{2+} released in response to an action potential may not simply reflect the degree of SR Ca^{2+} loading *per se*, but also modulation by the trigger mechanism. A reduction of steady-state contraction could result from a decreased trigger for release (e.g., a relatively large decrease in peak I_{Ca} , or early plateau currents such as I_{NaCa} or I_{to} , compared to that seen in species with an ascending staircase) despite the lack of change in global SR Ca^{2+} content, such as that estimated by postrest contraction amplitude or rapid cooling. In this respect, experiments designed to quantitate the contribution of "T-type" and "L-type" Ca^{2+} currents or Ca^{2+} entry through the reverse mode of the sarcolemmal Na^+ - Ca^{2+} exchange process to rate-dependent

changes in whole-cell Ca^{2+} conductance, as well as possible alterations of steady-state activation and inactivation properties of these current components in species with opposing staircase responses would be of great benefit in dissecting out the contribution of functional changes in the trigger for SR Ca^{2+} release in intact cells. A second possibility may be a frequency-dependent increase in refractoriness of the SR Ca^{2+} release process. As discussed above, this could result from an increase in the frequency of spontaneous Ca^{2+} release from the SR (Allen et al, 1985; Ishide et al, 1984; Stern et al, 1985; Bose et al, 1988b) or an increase in the degree of steady-state inactivation of the Ca^{2+} -induced release of Ca^{2+} process, as demonstrated elegantly by Fabiato (1983; 1985b). A third possibility is that the SR Ca^{2+} release process in rat ventricle is more sensitive to changes of membrane potential than is cardiac muscle from other species. If so, then rate-dependent shortening of an already brief action potential could act in a previously unrecognized manner to curtail the time course of Ca^{2+} release from the SR. Whether such a voltage-dependence might reside solely within the release process of the SR or the junction of the SR with the sarcolemma at the t-tubule is not clear at the present time, although the demonstration that tight electrical coupling of the SR to the surface membrane at the diadic junction is greater in rat than in guinea-pig or frog single ventricular cells (Moore et al, 1986) is consistent with the latter.

An important caveat underlying the three explanations offered above for the dissociation of steady-state contraction and steady-state cooling contracture is the necessity of assuming that the amount of Ca^{2+} present within the release pool of the SR is constant from one frequency to the next. Although it has been recently demonstrated that intracellular Ca^{2+} content in rat ventricle is augmented during the course of diastole (Kort and Lakatta, 1988b; Shattock and Bers, 1989), other studies have shown that time-dependent recirculation of Ca^{2+} within the SR during similar test intervals is also responsible for the mechanical restitution curve in this species (Ragnarsdottir et al, 1982; Schouten et al, 1987; Stern et al, 1988). Thus, it is possible that the stimulus for contraction at higher frequencies may simply arrive too fast for adequate repriming of the release pool, and as a result the amplitude of steady-state contraction declines at higher drive rates.

In conclusion, we have shown that the amplitude of postrest contraction and rapid cooling contractures are dissociated from steady-state twitch amplitude with increasing rate of stimulation in rat ventricular muscle. The data support the hypothesis that the negative staircase is due neither to a reduction of nor excessive loading of the SR and is consistent with a rate-dependent perturbation of the SR Ca^{2+} release process or a decline in the amount of Ca^{2+} located within the release site of the SR. The relationship between stimulus frequency and force production in canine ventricular muscle was found to be clearly different from that observed in the rat. The variability in the pattern of tension change when the data are expressed in units of absolute force production compared to that observed for the same values calculated as a percentage of steady-state contraction suggests that (a) the processes linking the movement of Ca^{2+} across the sarcolemma with the release of Ca^{2+} from the SR may become more tightly coupled as the rate of stimulation is enhanced and/or (b) a greater percentage of Ca^{2+} entering the muscle cell upon depolarization of the membrane may directly contribute to tension development.

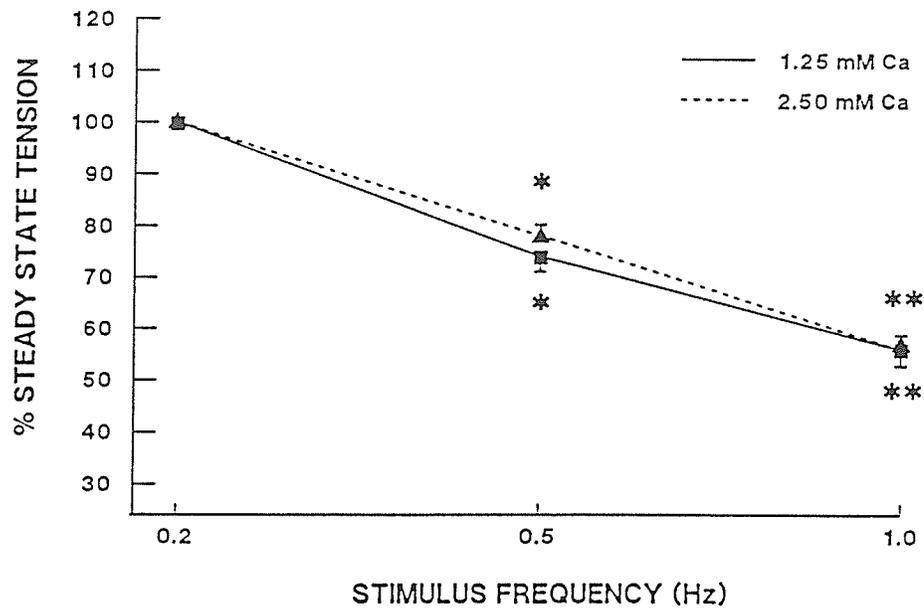


Figure 13. Pooled data illustrating the effect of $[Ca^{2+}]_o$ on the frequency-force relation in isolated rat ventricular muscle. Force production has been expressed as percentage of contraction amplitude found at 0.2 Hz. *denotes $P < 0.05$.

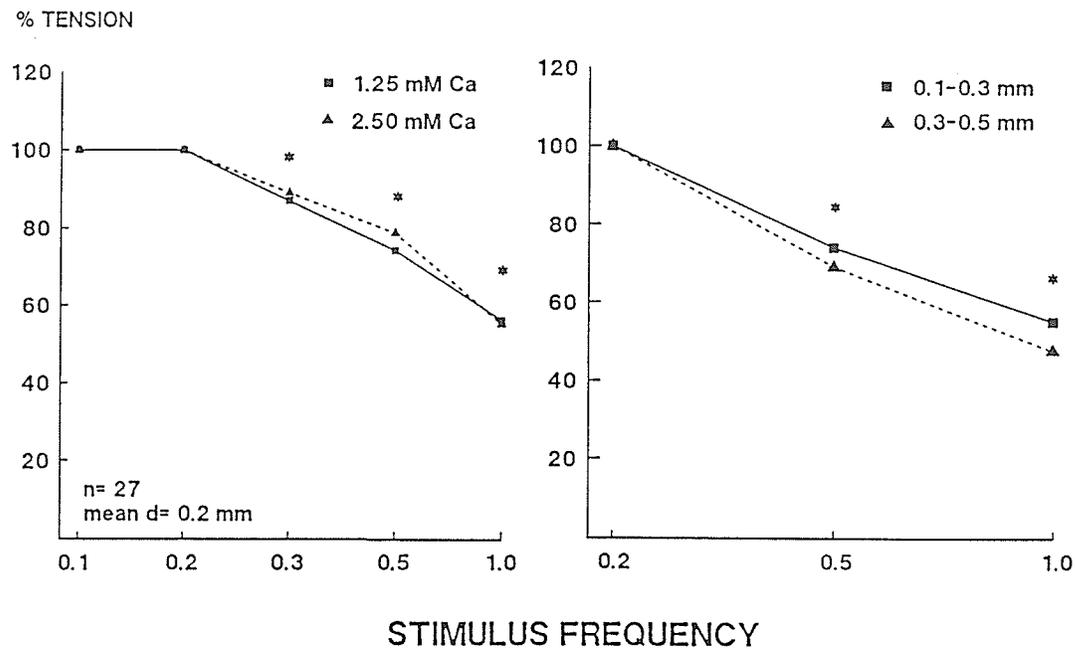


Figure 14. Effect of muscle diameter on the frequency-force relation. The panel on the left shows the pooled responses of 27 right ventricular trabeculae having a mean diameter of 0.2 mm to increased rate of stimulation in both 1.25 and 2.5 mM $[Ca^{2+}]_o$. The panel on the right illustrates the effect of dividing the muscles on the basis of their diameter, with the cut-off point set arbitrarily at 0.3 mm. The response was identical in 1.25 and 2.5 mM $[Ca^{2+}]_o$.

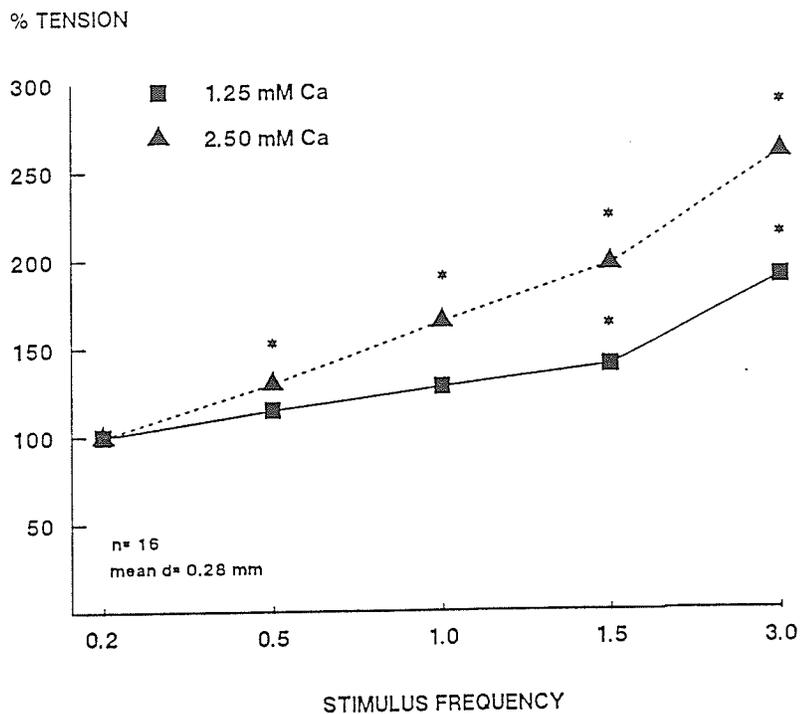


Figure 15. Pooled data showing the effect of $[Ca^{2+}]_o$ on the frequency-force relation obtained in 16 isolated canine right ventricular trabeculae. Note that the mean diameter of the muscles in these experiments was greater than that shown in the previous figure for experiments performed on rat ventricle. No significant difference was found in the variability of the data around the means of the two respective groups.

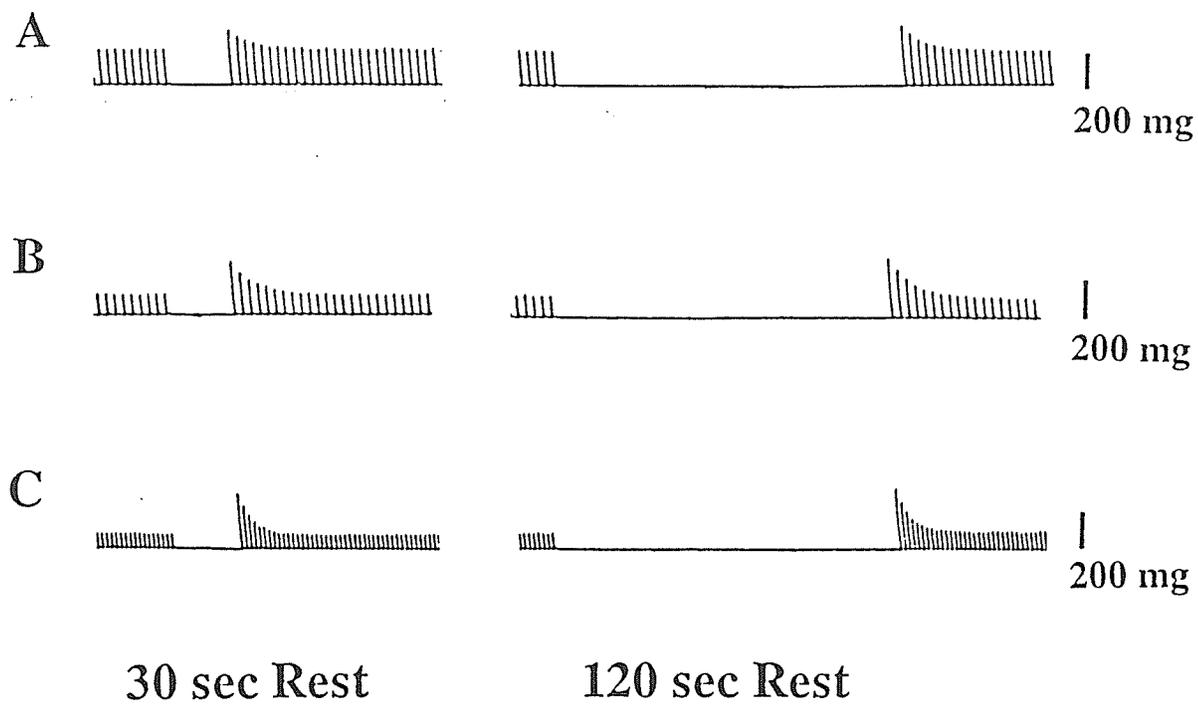


Figure 16. Effect of stimulus frequency on postrest contraction in isolated rat ventricle after 30-120 sec rest in the presence of 1.25 mM $[Ca^{2+}]_o$. Rate of rhythmic stimulation in these experiments was 0.2 (Panel A), 0.5 (Panel B) and 1.0 (Panel C) Hz. Vertical calibration bar on the right of each trace represents force development of 200 mg.

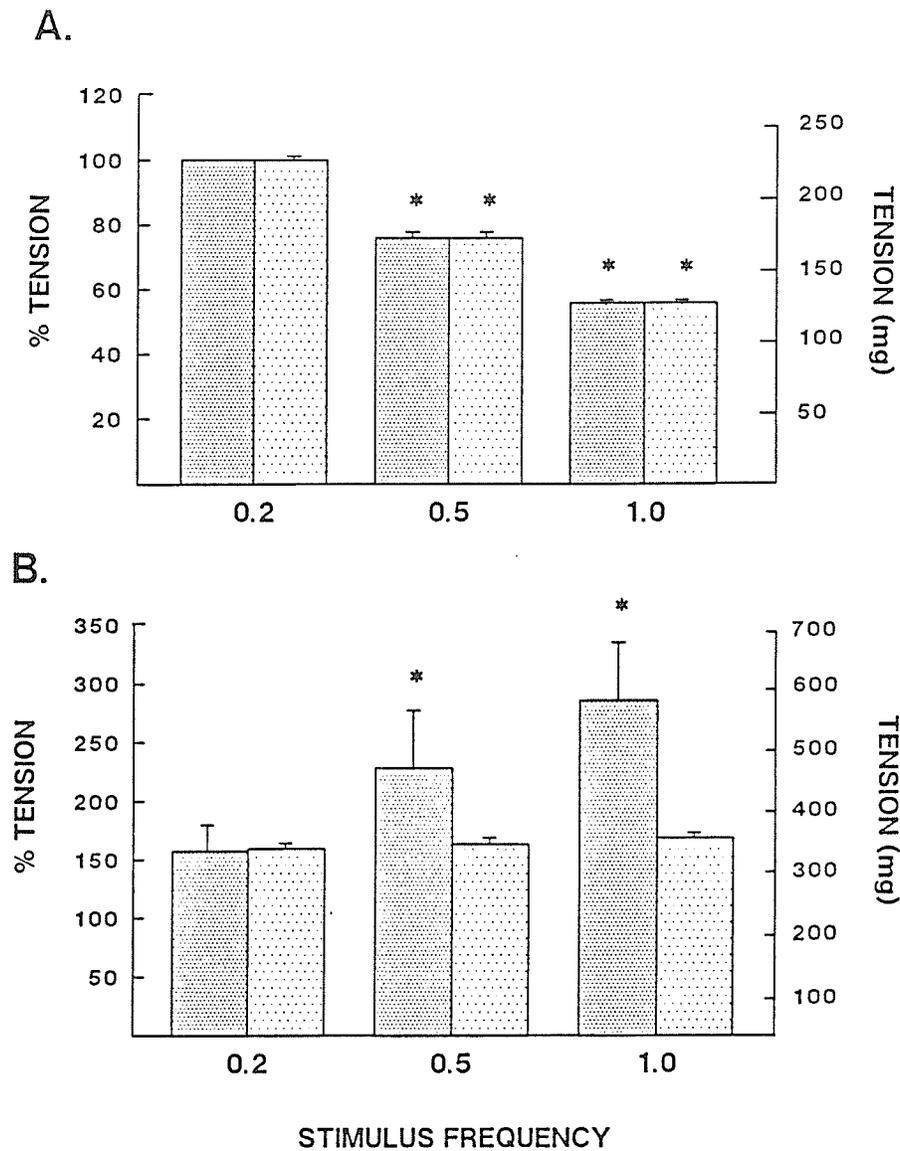
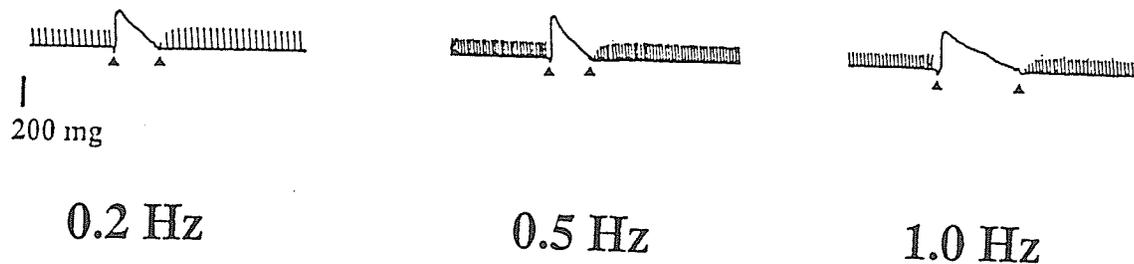


Figure 17. Pooled data from rat ventricle illustrating the frequency-dependent dissociation of steady-state (Panel A) and postrest (Panel B) contraction amplitude. The bars with dense shading represent contractions expressed as a percentage of steady-state contraction amplitude observed at each individual frequency, while the remaining bars represent contractions expressed in units of absolute force production. $[Ca^{2+}]_o$ was 1.25 mM.

A



B

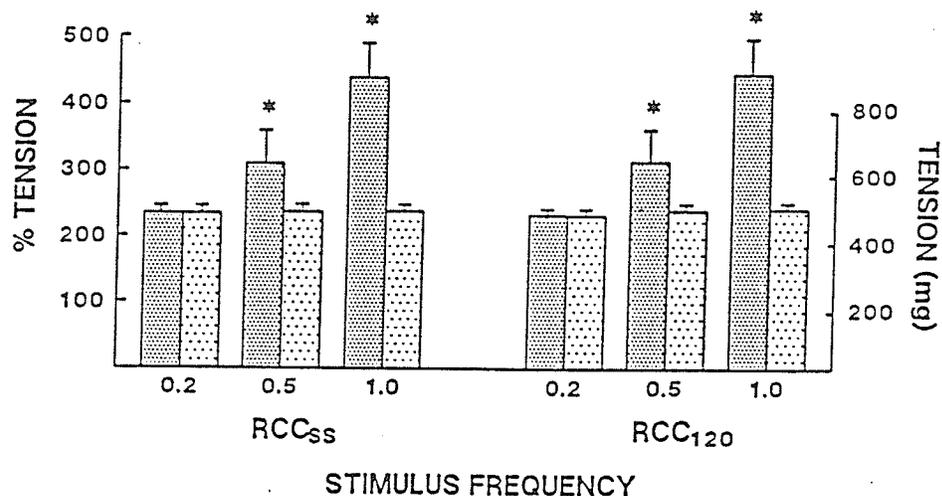


Figure 18. Effect of stimulation frequency on the amplitude of peak RCC in isolated rat ventricular muscle perfused with 1.25 mM $[Ca^{2+}]_o$. Increasing the frequency of stimulation had no effect on the amplitude of steady-state RCC (Panel A), despite accompanying changes of steady-state contraction. Pooled data for cooling contractures elicited in response to rhythmic stimulation and after 120 sec rest is shown in Panel B. Data have been expressed as a percentage of the steady-state contraction (dense bars) or in units of force (lightly stippled bars).

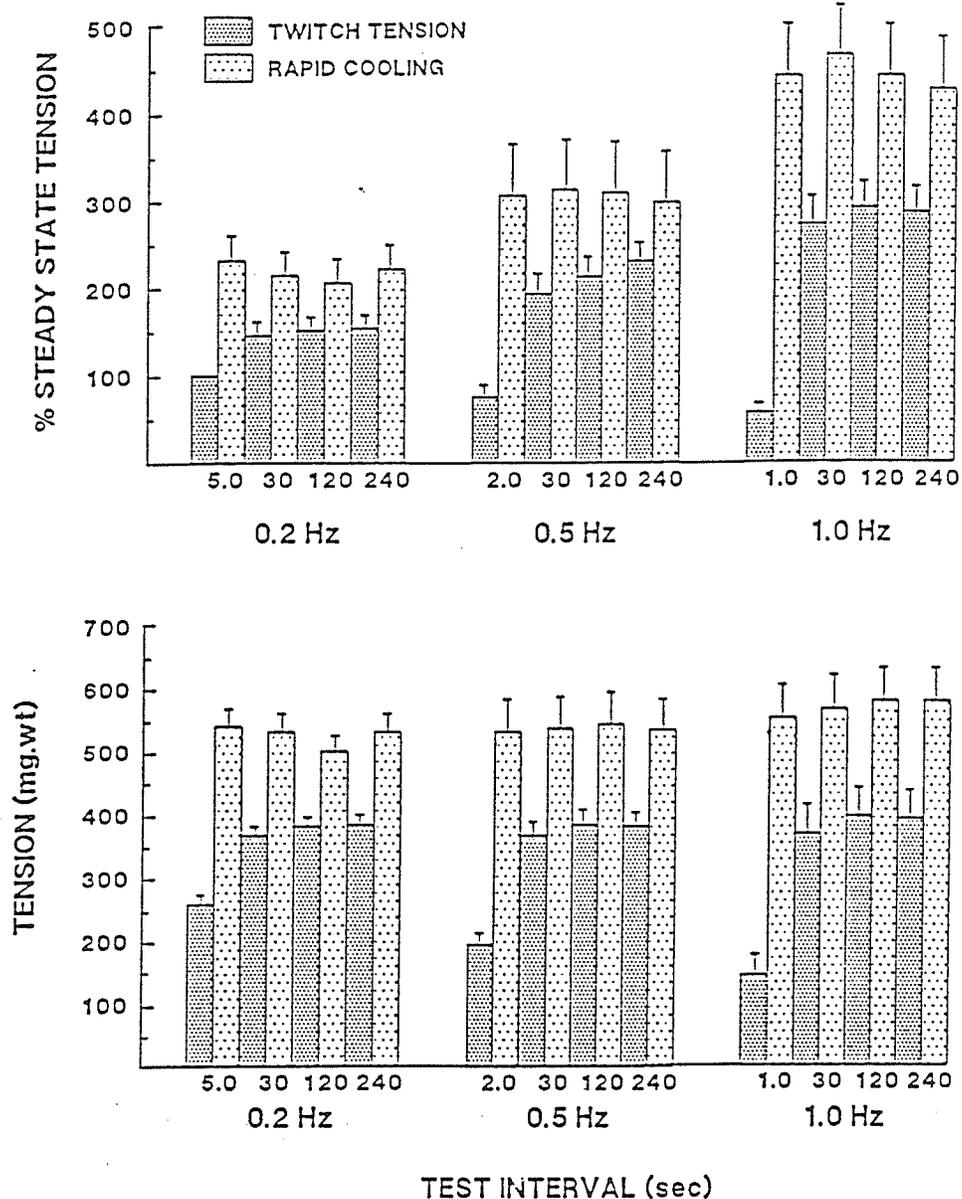


Figure 19. Summary of the effect of stimulus frequency on force developed by isolated rat trabeculae in response to electrical stimulation or rapid cooling. $[Ca^{2+}]_o$ in these experiments was 1.25 mM. Each block of data represents the steady-state (smallest rest interval) and postrest (30, 120 and 240 seconds) response to both forms of stimulation. Increased frequency significantly augmented the normalized twitch and RCC amplitude from group to group, but the pattern of tension change within a given frequency block remained unaltered. No such interaction was observed for absolute tensions shown in the lower panel, where the only statistically significant difference was that found for the decrease in steady-state contraction.

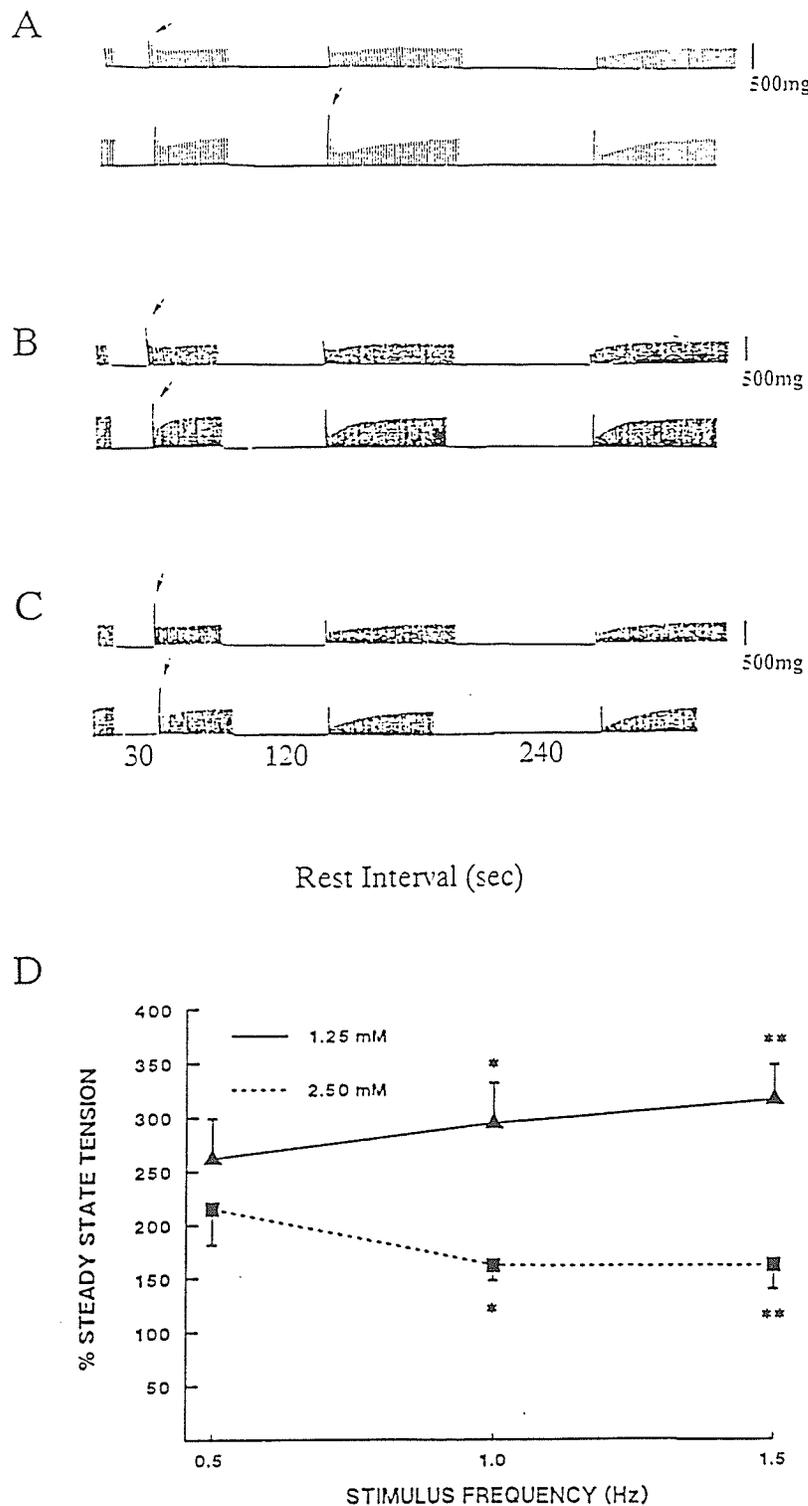


Figure 20. Effect of stimulus frequency on the degree of rest-potential in isolated canine ventricular muscle. Panels A-C illustrate the change in twitch amplitude following resumption of stimulation at 0.5, 1.0 and 1.5 Hz. The top and bottom traces of each pair show the response in 1.25 mM and 2.5 mM $[Ca^{2+}]_o$ following 30, 120 and 240 sec rest. Arrows indicate the peak response in each series of test intervals. The pooled data shows the dependence of normalized twitch amplitudes following 30 sec rest on $[Ca^{2+}]_o$.

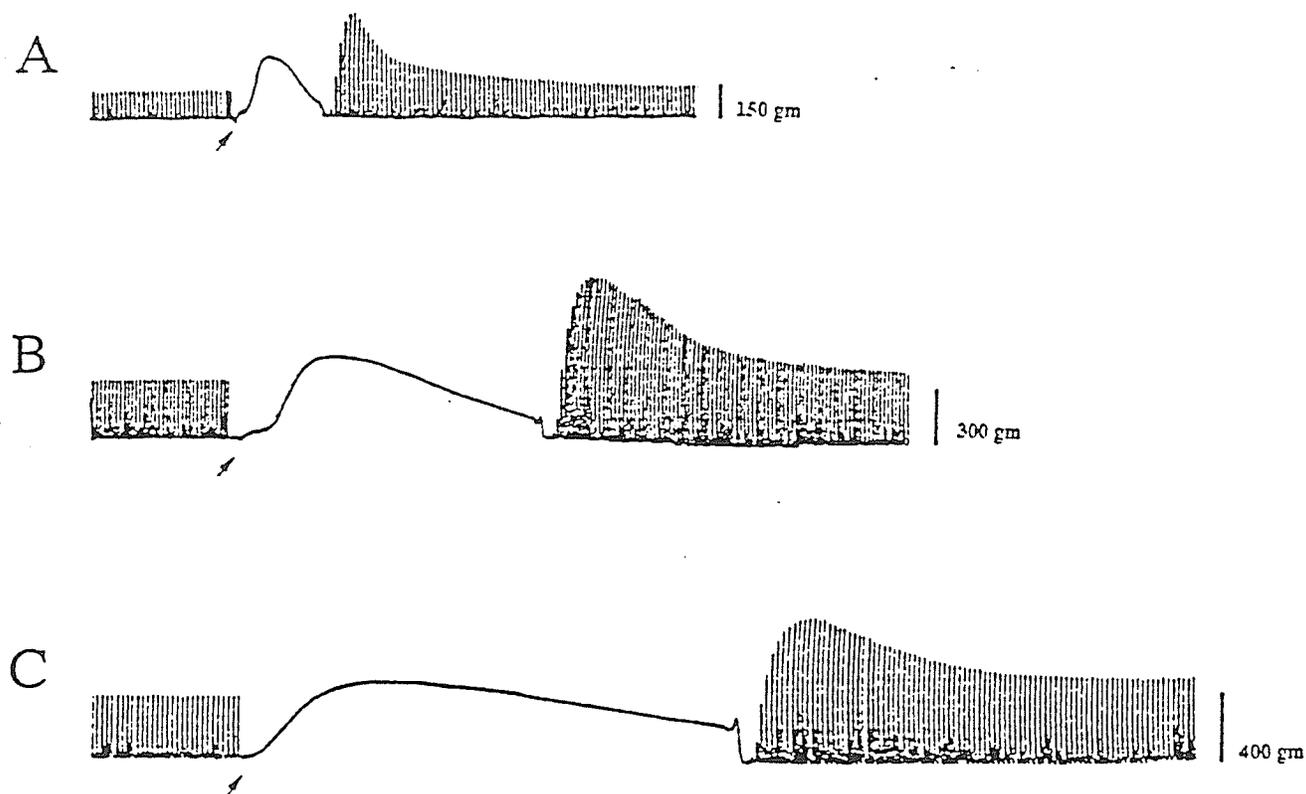


Figure 21. Effect of stimulus frequency on the time course of rapid cooling contracture in isolated canine ventricle. Arrows and re-warming spikes indicate the onset and termination of the switch in bathing solutions. Panels A-C represent the response to cooling following stimulation at 0.5, 1.0 and 1.5 Hz. $[Ca^{2+}]_o$ was 2.5 mM.

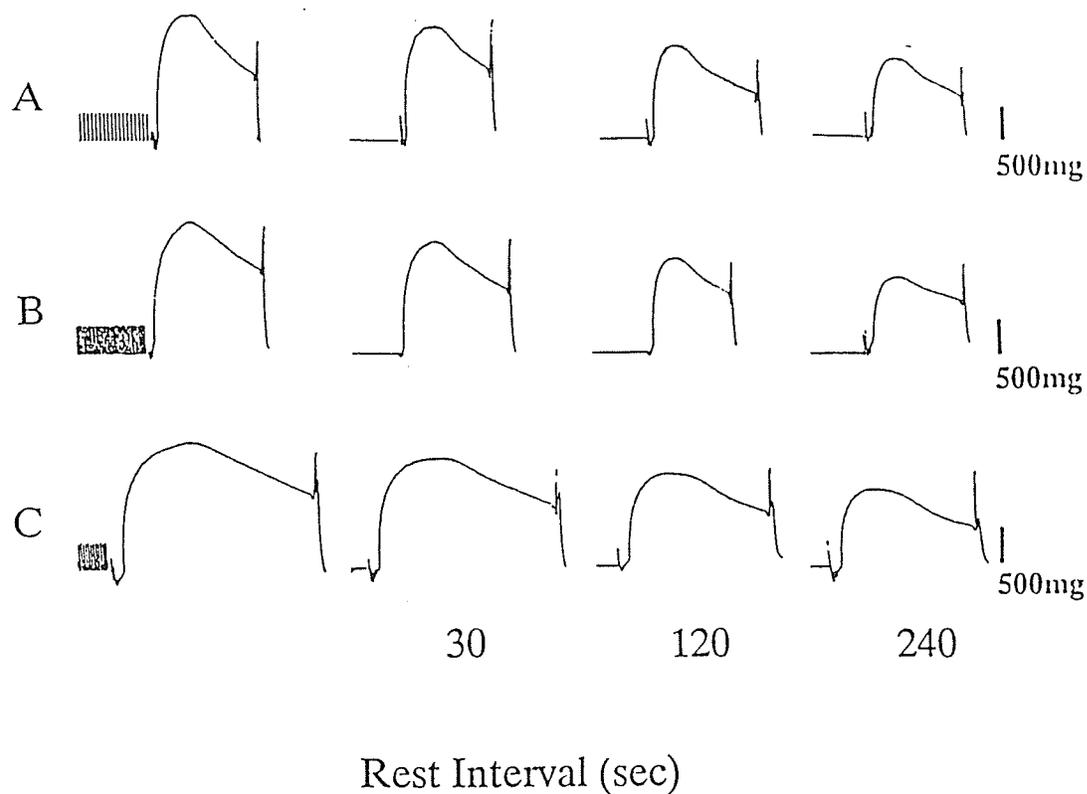


Figure 22. Time-dependent decay of RCC amplitude in canine ventricular muscle with increasing duration of rest. $[Ca^{2+}]_o$ in this experiment was 2.5 mM. RCC amplitude decreased monotonically as the duration of rest preceding cooling was gradually extended over the course of 240 sec. Decay of RCC amplitude began from the moment rhythmic stimulation was terminated and remained unaffected by altering the rate of rhythmic stimulation preceding cooling.

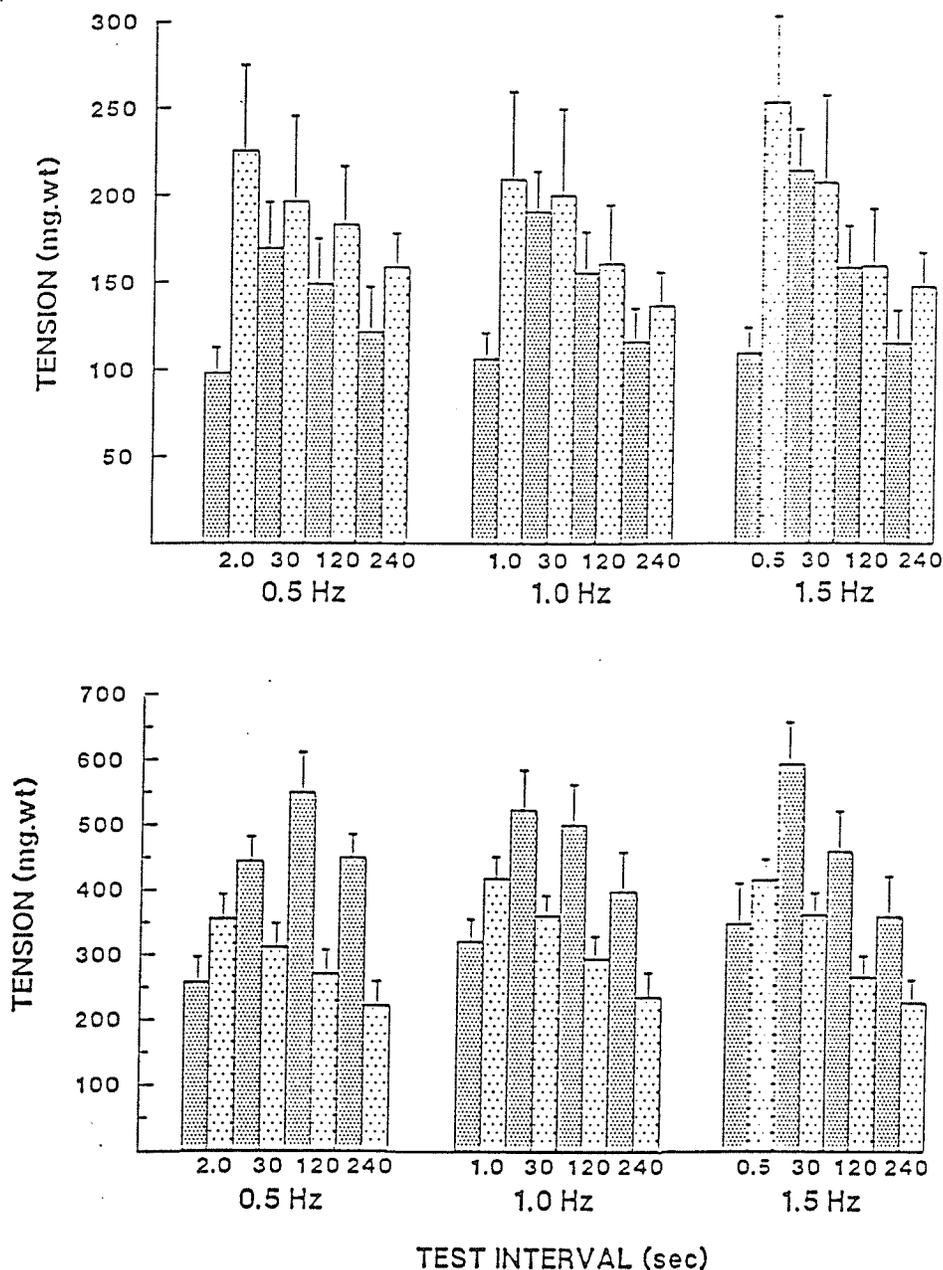


Figure 23. Summary of the effect of stimulation frequency on force developed by isolated canine trabeculae in response to electrical stimulation or rapid cooling. In the presence of 1.25 mM $[Ca^{2+}]_o$ (top Panel), increased frequency of stimulation significantly augmented postrest contraction at a given test interval with little effect on steady-state contraction. As in the rat, the pattern of tension change within a given frequency block remained unchanged with increasing frequency of stimulation. However, in contrast to the rat, the amplitude of both postrest contraction and cooling contractions declined with increasing rest duration, the slope of which remained unaffected by the rate of stimulation. The bottom panel shows the response in 2.5 mM $[Ca^{2+}]_o$ where an increase in frequency exerted a significant inotropic effect on the steady-state contraction. One notable difference from the response in 1.25 mM $[Ca^{2+}]_o$, was the effect of drive rate to shorten the time required to produce peak rest-potential.

**CONTRIBUTION OF SARCOLEMMAL Na^+ - Ca^{2+} EXCHANGE
TOWARD DEVELOPED TENSION IN CANINE VENTRICULAR MUSCLE**

SECTION 3

SUMMARY

Experiments were conducted to test the hypothesis that rate-dependent changes in the strength of contraction in isolated canine ventricular muscle are related to accompanying alterations of both intracellular Ca^{2+} release and loading, and shifts in the dominant mode of operation of the sarcolemmal Na^+ - Ca^{2+} exchange process. Our approach has been to describe the quantitative relationship between Ca^{2+} availability within the sarcoplasmic reticulum (SR) and the strength of contraction in solutions containing varying amounts of $[\text{Ca}^{2+}]_o$ and $[\text{Na}^+]_o$ during alterations in the rate and rhythm of stimulation. Fifty percent substitution of bathing Na^+ with Li^+ produced a positive inotropic response on contraction which was paralleled by a similar increase of Ca^{2+} uptake into and release from the SR, which was estimated indirectly by the magnitude of rapid cooling contractures (RCC) and postrest contractions. Lowering $[\text{Na}^+]_o$ also had the effect of prolonging the duration of contraction, inhibiting the postrest-decay of RCC amplitude and enhancing the amplitude of the second in a set of paired cooling contractures. The positive staircase of steady-state contraction, steady-state RCC and postrest contraction in control solutions was altered after lowering $[\text{Na}^+]_o$ in such a fashion that a graded increase of stimulation frequency lead to a marked negative staircase of contraction, while steady-state RCC amplitude and postrest contraction remained unaltered or increased. Experiments designed to probe the underlying mechanism of this apparent uncoupling of SR Ca^{2+} availability and release suggested that it was not a consequence of a slowed rate constant for Ca^{2+} transport between intracellular compartments, or the absolute size of the intracellular Ca^{2+} storage pool, but appeared to be related, at least in part, to Ca^{2+} -induced inactivation of the release process. Application of ryanodine (10 nM) and caffeine (3 mM) in the presence of low- $[\text{Na}^+]_o$ solutions depressed contraction and restored the positive staircase, the slope of which was dramatically enhanced in the presence of ryanodine. Inhibition of sarcolemmal Ca^{2+} entry in the presence of 70 mM $[\text{Na}^+]_o$ (2 μM Nifedipine) depressed contractile strength and greatly potentiated the negative staircase. Conversely, 1 μM BAY K 8644 significantly increased contractile force, and restored the positive

staircase response. These results indicate that rate-dependent changes of both contraction and intracellular Ca^{2+} loading require the presence of a functional Na^+ - Ca^{2+} exchange process. The possibility is discussed that net cellular Ca^{2+} gain related to the activity of the exchanger may affect the strength of contraction during high-frequency stimulation by either altering the trigger for intracellular Ca^{2+} release, or making Ca^{2+} available directly to the myofilaments.

INTRODUCTION

The relationship between sarcolemmal Na^+ - Ca^{2+} exchange and the contractile state of cardiac muscle has received much attention since the demonstration that a significant fraction of cellular Ca^{2+} efflux from isolated preparations of sheep and calf ventricles was dependent on the ratio of $[\text{Ca}^{2+}]_o/[\text{Na}^+]_o^2$ in the bathing medium (Reuter and Seitz, 1968). Despite the absence of specific pharmacological inhibitors, a number of important experimental links have been established recently between cell contraction and $[\text{Na}^+]_o$ (Reuter, 1974; Watanabe et al, 1986; Bers, 1987), intracellular Na^+ activity, a_{Na}^i (Cohen et al, 1982; Eisner et al, 1984; Brill et al, 1987; Boyett et al, 1987), as well as various time, voltage, and stimulation-dependent ionic currents attributed to an electrogenic Na^+ - Ca^{2+} exchange process (Kimura et al, 1986; Mechman and Pott, 1986; Hume and Uehara, 1986a,b; Lipp and Pott, 1988; Terrar and White, 1989; Egan et al, 1989; Shimoni and Giles, 1989). In addition, a number of theoretical models based on an exchange stoichiometry of $3\text{Na}^+ : 1\text{Ca}^{2+}$ have been developed which focus on thermodynamic changes in the equilibrium potential for the exchanger (E_{NaCa}) during the cardiac cycle (Mullins, 1979, 1981; Noble, 1985; 1986). This approach has provided valuable insight into how transmodal exchange of cellular Na^+ for Ca^{2+} might influence myoplasmic Ca^{2+} both during and following contraction of cardiac tissues (Difrancesco and Noble, 1985; Hilgemann and Noble, 1987; Bers, 1987; Shattock and Bers, 1989).

Evidence is now available to support the proposal made in previous years by Langer (1968, 1974) that rate-dependent changes of myoplasmic Ca^{2+} and force of contraction may be related, in part, to the effect of repetitive stimulation on a_{Na}^i (Boyett et al, 1987; Wang et al, 1988). Contractile alterations of this nature have been subsequently correlated with the activity of a Na^+ - Ca^{2+} exchange mechanism which can presumably increase the strength of contraction by either facilitating $[\text{Na}^+]_i$ -dependent Ca^{2+} entry or inhibiting $[\text{Na}^+]_o$ -dependent Ca^{2+} efflux (Brill et al, 1987; Boyett et al, 1987; Terrar and White, 1989; Miura and Kimura, 1989; Leblanc and Hume, 1990). It has been demonstrated under a variety of experimental conditions that cell contraction

during steady-state stimulation is not always proportional to trans-sarcolemmal Ca^{2+} entry into the cell during the peak of the Ca^{2+} current, I_{Ca} (Reuter, 1968; New and Trautwein, 1972; Fabiato, 1983; London and Krueger, 1986; duBell and Houser, 1989) or the amount of Ca^{2+} present within pooled sarcoplasmic reticulum (SR) compartments during stimulation protocols designed to "clamp" SR Ca^{2+} at a particular level (Lipsius et al, 1982; Meyers et al, 1988; duBell and Houser, 1989). Thus, a source of Ca^{2+} separate from that entering the cell during the flow of I_{Ca} or that released from the SR may be responsible for maintaining high levels of myoplasmic Ca^{2+} during the course of contraction under such conditions.

Although it is now generally accepted that the concentration of intracellular free Ca^{2+} can be influenced directly by sarcolemmal Na^+ - Ca^{2+} exchange at certain points in the cardiac cycle, there is also a large body of evidence to support the role of Ca^{2+} released from intracellular stores in governing contraction on a beat to beat basis (Koch-Weser and Blinks, 1963; Wood et al, 1969, Allen et al, 1976; Fabiato, 1978; 1983; 1985a-c; London and Krueger, 1986; duBell and Houser, 1989; Nabaeur et al, 1989). Thus, it seems plausible that peak myoplasmic Ca^{2+} levels reached during contraction may be mediated interatively by both Ca^{2+} release from the SR and Ca^{2+} located at or near the inner surface of the membrane due to a gradient effected by changes in modal activity of the exchanger, and that the ratio of Ca^{2+} contribution from either source depends on the previous stimulation history. The purpose of the present study was to test the hypothesis that frequency-dependent changes in the force of steady-state contraction occurs concomittant with, but not entirely a result of, changes in intracellular Ca^{2+} loading *per se*. Our principal approach has been to describe the quantitative relationship between SR Ca^{2+} availability during different patterns of electrical stimulation and force of contraction. The possibility that frequency-dependent alterations of contractile strength are related to sarcolemmal Na^+ - Ca^{2+} exchange has been tested by repeating various stimulation protocols after reducing the trans-membrane driving force for the exchange process. Preliminary results from part of this work have been presented to the Biophysical Society (Bouchard and Bose, 1990).

MATERIALS AND METHODS

The methods employed in this study have been fully described elsewhere (Bouchard et al, 1989; Bouchard and Bose, 1989; 1991). To summarize, thin free-running right ventricular trabeculae were dissected from the hearts of mongrel dogs weighing 6-12 kg. The length of muscles used in this study ranged from 3-5 mm and the diameter ranged from 0.2-0.5 mm. The normal physiological solution used to perfuse the preparations contained the following (mM): NaCl, 140; KCl, 4.7; MgCl, 1; CaCl, 2.5; HEPES ([4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid]), 5; and glucose 10. Solutions were titrated to pH 7.4 at 37°C with NaOH. The muscles were suspended in a 2-ml horizontal chamber which formed part of a 2-circuit parallel by-pass recirculation system in which two sets of physiological solutions were maintained at 37°C and 0-2°C. One end of the preparation was tied to a fixed steel post and the other end was attached to a force transducer (Grass FT O3C).

Active force was measured in response to variation of the interval between beats or perfusate temperature. Alterations were made in the rate of rhythmic stimulation as well as following the resumption of stimulation after different periods of rest (postrest contraction). Frequency-force relations were obtained simply by comparing the amplitude of steady-state contraction at the different drive rates, while restitution curves for postrest contraction were determined by comparing the amplitude of contraction following rest with those preceding it. In this way it was possible to construct a family of rest response curves at the different rates of stimulation. Mechanical restitution curves were constructed also for contractions elicited both in response to premature stimuli delivered at coupling intervals ranging between 100 to 1000-ms, and for the following postextrasystolic contractions. This stimulus protocol was repeated with different basic cycle lengths (BCL) preceding the delivery of individual premature excitations, following which the interval separating the premature contraction from the following postextrasystolic contraction was held constant at a duration equal to that found between preceding steady-state contractions. As described previously for intact canine (Yue et al, 1985) and ferret ventricular preparations (Wier

and Yue, 1986), the observed recovery of extrasystolic contraction occurred with a monoexponential time course, which was fitted best by a modified Levenberg-Maquand algorithm of the form $A_0 + A_1 * \exp^{-T/\tau}$. Time constants for full recovery of extrasystolic contractions were determined by the fitted monoexponential function, after which they were analyzed with a repeated measures analysis of variance (ANOVA) to determine the extent of quantitative differences associated with a given experimental sequence (e.g., BCL dependence within a given perfusion solution) or between treatments (e.g., alteration of $[Na^+]_o$ or $[Ca^{2+}]_o$). As a final qualitative measure of intracellular Ca^{2+} handling, the contractile response of the preparation to rapid lowering of perfusate temperature was employed as an assay to probe for the amount of Ca^{2+} located within pooled SR compartments. We have used a protocol similar to that described originally for cardiac muscle by Kurihara and Sakai (1986) and Bridge (1986), whereby rapid cooling contractures are elicited by manually diverting a stream of warmed solution (35-37°C) normally used to perfuse the muscle into a parallel circuit and allowing a stream of cooled solution (0-2°C) to flow through the muscle chamber in the absence of electrical stimulation. Manual diversion of solutions from one parallel circuit to the next at flow rate of ~35 ml/min permitted very rapid (< 1 sec) switching of bathing media, without appreciable mechanical artifact or perturbation of resting muscle length. Cooling contractures were obtained both in the place of a regularly driven beat (e.g., steady-state cooling contracture) and after identical rest intervals to those described above for postrest contraction.

Reduction of $[Na^+]_o$ to 70 mM was accomplished in the majority of experiments with equimolar replacement of NaCl in the bathing solution with LiCl, although in some experiments choline Cl or sucrose were used. Lithium was chosen as the main substituent for Na^+ due to its ability to reduce the magnitude of membrane currents attributed to $Na^+ - Ca^{2+}$ exchange in intact ventricular muscle (Schouten and terKeurs, 1986), isolated Purkinje fibres (Bril and Man, 1989), and freshly dispersed ventricular myocytes (Mitchell et al, 1984; Giles and Shimoni, 1989; Bridge et al, 1990). A second reason for choosing Li^+ was that it can also act successfully as a substrate to stimulate efflux of H^+ from a variety of tissues, including Purkinje fibres (Ellis and MacLeod, 1985), renal tubules (Ives et

al, 1983), and red blood cells (Parker, 1986). Although the values reported for the apparent K_m and V_{max} for Na^+ - Li^+ exchange are smaller than those demonstrated for Na^+ - H^+ exchange (Montrose and Murer, 1988), this property of Li^+ was beneficial for our purposes because the increased intracellular free Ca^{2+} concentration and force of contraction accompanying such a large reduction of $[\text{Na}^+]_o$ would be expected to result in significant acidification of the cell interior due either to the effect of reduced extracellular Na^+ on Na^+ - H^+ exchange or displacement of protons from intracellular binding sites (Deitmer and Ellis, 1980; Bountra et al, 1988). Fifty percent reduction of $[\text{Na}^+]_o$ was chosen over more complete substitution to leave the exchange process functioning with enough residual activity present to permit shifts in modal activity to occur during the change in E_{NaCa} accompanying the action potential, such as that described by Mullins (1979, 1981) and Noble (Noble, 1985; Difrancesco and Noble, 1985; Hilgemann and Noble, 1987). Furthermore, the possibility exists that excessive removal of $[\text{Na}^+]_o$ may result in a significant reduction in the capacity of the exchange process to shift from a thermodynamic mode which favours cellular Ca^{2+} efflux during diastole to one which favours Ca^{2+} influx (or decreased efflux) during the action potential, due to the differences in values reported for the $K_m[\text{Na}^+]_o$ and $K_m[\text{Na}^+]_i$ of the exchange process (Miura and Kimura, 1989) or competition for the binding site at the external face of the membrane by external Ca^{2+} ions (Phillipson et al, 1982, 1985). A final consideration in choosing the degree of $[\text{Na}^+]_o$ substitution was to circumvent complications demonstrated previously to arise from intracellular Ca^{2+} overload (Fabiato, 1983; Allen et al, 1985; Stern et al, 1988; Bose et al, 1988a,c) which would itself affect both the degree of activity and direction of Na^+ - Ca^{2+} exchange (Kurihara and Sakai, 1985; Hilgemann, 1986a,b; Bers, 1987; Bers and Bridge, 1988). Whenever possible, self-controlled experiments were designed such that paired t-tests could be conducted to determine the effects of a given intervention on muscle performance (Steel and Torrie, 1960). On occasions when this was not possible, data analysis was performed using a repeated measures analysis of variance (ANOVA). The level of significance chosen for all blocked experiments was $P < 0.05$. Data are presented throughout as the mean \pm S.E.M.

RESULTS

Interval-force relationship in 140 mM $[\text{Na}^+]_o$

Figure 24 illustrates the typical response of canine right ventricular muscle to an increase in the rate of rhythmic stimulation. The positive force staircase of steady-state contraction is shown in the top panel, which also depicts the effect of stimulus frequency on the time course of isometric contraction. Peak contraction was enhanced in this preparation by 25% and 40% following an increase in the rate of stimulation from 0.5 to 1.0 and 1.5 Hz. This increase in the strength of contraction was accompanied by a similar rate-dependent increase of pooled SR Ca^{2+} stores estimated by rapid cooling and postrest stimulation. In the case of the muscle shown in the middle panel, peak steady-state cooling contracture was increased by 20% and 25% at 1.0 and 1.5 Hz respectively, while steady-state contraction was augmented by 26% and 37%. An almost identical effect of altering the rate of stimulation was observed on the amplitude of the first postrest contraction following a 30 sec rest interval. For the muscle shown here, steady-state contraction was increased by 50% and 67% while the corresponding postrest contractions were increased by 26% and 53%. In addition to the effects demonstrated on peak contraction, alterations in the frequency of stimulation modestly reduced the time to peak force by $5.5 \pm 0.2\%$ and $10.0 \pm 0.36\%$, while time required for complete relaxation of the twitch was shortened by $5.0 \pm 0.25\%$ and $9.0 \pm 0.5\%$ at 1.0 and 1.5 Hz, respectively.

Averaged data for ten such experiments have been provided in Fig. 25. The amplitude of steady-state and postrest contractions along with peak steady-state rapid cooling contractures have been plotted against the rate of stimulation. All values in this analysis have been calculated as a percentage of the corresponding responses observed at 0.5 Hz, where for the most part, they were found to be the smallest in all muscles studied (cf. Fig. 30). As illustrated for the 3 individual muscles in the recordings shown in Fig. 24, increasing the stimulation frequency from 0.5 to 1.0 and 1.5 Hz resulted in a significant increase in the mean strength of contraction of $18 \pm 4\%$ and $44 \pm 8\%$. Peak steady-state cooling contractures were increased $10 \pm 3\%$ and $32 \pm 5\%$ above the

values obtained at 0.5 Hz. Postrest contractions were augmented $15 \pm 8\%$ and $30 \pm 5\%$ at similar rates of stimulation. Analysis of variance indicated that the increase of steady-state contraction was significantly greater ($P < 0.05$) than the accompanying increase of either peak steady-state cooling contracture or postrest contraction, with no further difference detected between the frequency-dependence of steady-state cooling contracture and postrest contraction. These results are similar in part to those reported previously with the use of rapid cooling (Kurihara and Sakai, 1985) or high extracellular K^+ contractures (Gibbons and Fozzard, 1971), and suggest that as the frequency of stimulation is enhanced, intracellular free Ca^{2+} levels during the course of contraction may be either enhanced or maintained by Ca^{2+} originating from sources other than the SR. Hilgemann and Noble (1987) have proposed that time-averaged Ca^{2+} entry through a combination of voltage-dependent channels and coupled transport mechanisms could be balanced by the combination of sequestration into the SR and extrusion out of the cell during relaxation and diastole. Experimental data supports this proposal (Hilgemann, 1986a,b; Boyett et al, 1987; Wang et al, 1988; Terrar and White, 1989; Egan et al, 1989; Bridge et al, 1990) and suggests it is also possible that the balance would be shifted towards net cellular Ca^{2+} gain as the rate of stimulation is enhanced due to the reduced ability of the exchanger to extrude Ca^{2+} during the abbreviated diastolic interval. Depending on the extent of frequency-dependent shortening of action potential duration in canine ventricle (Geenway and Miller, 1979; Robinson et al, 1987), an increase in the time spent by the muscle in a *relatively* depolarized state should alone result in a progressive increase in myoplasmic Ca^{2+} due to a gradual shift in the dominant mode of the exchanger to one favouring net cellular Ca^{2+} gain, due to the $3Na^+ : 1Ca^{2+}$ stoichiometry of the exchange process (Mullins, 1981; Reeves and Hale, 1984; Noble, 1986; Bridge et al, 1990). To determine whether a portion of the rate-dependent increase of steady-state contraction or pooled SR Ca^{2+} stores was related to the activity of the $Na^+ - Ca^{2+}$ exchange mechanism, the effect of reducing the trans-membrane Na^+ gradient on the steady-state contractile properties and their frequency-dependence was assessed.

Effect of $[\text{Na}^+]_o$ reduction on steady-state contractility

The effects of $[\text{Na}^+]_o$ depletion on the general contractile properties of canine ventricle are illustrated in Fig. 26. Muscles were washed twice and re-equilibrated in solutions in which 50% of the bathing Na^+ was replaced with Li^+ . The points at which the recirculating solution was exchanged with fresh Li^+ -substituted solution are indicated by the arrows in the top recording and are typical for most of the preparations studied. Following the second wash, the muscle was stimulated rhythmically at 0.5 Hz until peak contraction reached a new steady-state level. This usually took place within 30 minutes, during which time no change in resting tension was observed in any of the experiments. In a total of 17 preparations studied with various protocols, reduction of $[\text{Na}^+]_o$ from 140 to 70 mM increased the mean strength of steady-state contraction by $91.5 \pm 4\%$.

Previous work conducted on ventricular tissues lacking appreciable development of the sarcotubular network (e.g., rabbit ventricle) by Bers (1987) has shown that a graded reduction of trans-sarcolemmal Na^+ gradient produces an inotropic effect on contraction which persists in the presence of either caffeine (10 mM) or ryanodine (0.1-0.5 mM), indicating that enhanced release of Ca^{2+} from SR stores is not the primary means by which the strength of contraction is enhanced under similar experimental conditions to those employed here. In addition to the changes in steady-state contraction, Fig. 26 illustrates further changes in pooled SR Ca^{2+} content induced by $[\text{Na}^+]_o$ withdrawal in canine ventricle. For the muscle shown in the middle panel, the amplitude of peak steady-state cooling contracture was increased by 100% following reduction of $[\text{Na}^+]_o$ to 70 mM, which is similar to the group mean of $90 \pm 7\%$. As illustrated in the lower panel, similar observations were made with respect to the $[\text{Na}^+]_o$ -sensitivity of postrest contraction. Here, the amplitude of the first postrest beat was nearly doubled after the reduction of $[\text{Na}^+]_o$, which was consistent with the inotropic effect on steady-state contraction. An analysis of variance indicated that the $[\text{Na}^+]_o$ -dependence of the increase of steady-state contraction ($91.5 \pm 4\%$), steady-state cooling contracture ($90 \pm 7\%$) and postrest contraction ($110 \pm 8\%$) were not statistically different from one another, although the trend was such that the inotropic effect of lowering $[\text{Na}^+]_o$ on steady-state contraction was less than that associated with changes of SR Ca^{2+} availability. When

the amplitude of steady-state cooling contractures were calculated as a percentage of preceding steady-state contractions, lowering $[\text{Na}^+]_o$ had no effect on the ratio of peak contraction to peak cooling contracture: the values were $85 \pm 4\%$ and $79.3 \pm 5.6\%$ for 140 and 70 mM $[\text{Na}^+]_o$, respectively. Hence, in contrast to species showing poor postrest contraction such as rabbit (Sutko et al, 1986; Bers, 1987) or guinea-pig (Lewartowski and Pytkowski, 1988), reduction of $[\text{Na}^+]_o$ in canine ventricle results in an inotropic effect on steady-state contraction that does not occur independent of changes in the degree of intracellular Ca^{2+} loading, and as will be shown later, both ryanodine and caffeine markedly depress steady-state contraction when applied after lowering $[\text{Na}^+]_o$. The greater tendency of $[\text{Na}^+]_o$ withdrawal to augment different functional pools of Ca^{2+} located within the SR of canine ventricle, compared with that seen in the rabbit, may be related to the greater development of the sarcotubular network in canine ventricle (Sommer and Johnson, 1979, 1982), as suggested by the greater degree of rest-potential and larger ratio of steady-state cooling contracture to steady-state contraction in the dog compared with rabbit or guinea-pig at similar rates of stimulation and $[\text{Ca}^{2+}]_o$ (Kruta and Braveny, 1961; Endoh et al, 1982; Bers, 1985, 1989; Kurihara and Sakai, 1985; Bridge, 1986; Bose et al, 1988a; Bouchard and Bose, 1989).

Effect of $[\text{Na}^+]_o$ reduction on cellular Ca^{2+} efflux

This series of experiments was performed to determine whether select indices of cell function normally attributed to sarcolemmal $\text{Na}^+-\text{Ca}^{2+}$ exchange were affected in the manner expected following reduction of the transmembrane Na^+ gradient. Previous studies have indicated that the amplitude of postrest cooling contractures in rabbit (Bridge, 1986; Bers 1989), guinea-pig (Kurihara and Sakai, 1985; Bers et al, 1989) and canine ventricular muscle (Hryshko et al, 1989b; Bouchard and Bose, 1989) are reduced in a time-dependent fashion following the termination of rhythmic stimulation. The rate at which this Ca^{2+} is lost to the extracellular space appears to be coupled to the activity of the $\text{Na}^+-\text{Ca}^{2+}$ exchange process, as estimates of both intracellular Ca^{2+} content (Bridge, 1986; Lewartowski and Pytkowski, 1987; Bers and Bridge, 1988; Pytkowski, 1988; Bers et

al, 1989) and extracellular Ca^{2+} concentration (Hilgemann, 1986a,b; Bers et al, 1987) following extended rest more closely approximate those demonstrated immediately after termination of rhythmic stimulation following the reduction of $[\text{Na}^+]_o$ or inhibition of the Na^+ pump by acetylthiocholine. We observed a similar response in canine ventricle, as shown in the top panel of Fig. 27. In these experiments, rhythmic stimulation was terminated and rest intervals of either 30 or 60 sec interpolated before the onset of cooling. The dashed lines above the individual recordings are for reference and represent the peak amplitude of steady-state cooling contractures. On termination of stimulation at 0.5 Hz, a gradual increase in the period of time preceding cooling led to a significant decay of postrest cooling contractures from 100% steady-state to $80 \pm 6\%$ and $69 \pm 6\%$ following 30 and 60 sec rest, respectively. As shown in the panel below, reduction of $[\text{Na}^+]_o$ to 70 mM was found to prevent the time-dependent decay of postrest cooling contractures, as the amplitude of cooling contractures following 30 and 60 sec rest were $97 \pm 4\%$ and $90.3 \pm 4\%$ of peak steady-state contracture. Similar results were obtained at 1.0 and 1.5 Hz stimulation in nine other paired experiments.

Eliciting a second cooling contracture immediately after terminating the first contracture has been proposed to reflect the amount of Ca^{2+} taken up into the SR during the rewarming period of the preceding cooling contracture (Hryshko et al, 1989c). Although it is conceivable that a significant fraction of the Ca^{2+} released in response to the second cooling stimulus may indeed arise from residual Ca^{2+} stores within the SR not released during the first cooling period, data from other studies comparing fractional relaxation ($t_{1/2}$) components of the rewarming phase associated with membrane Ca^{2+} ATPase activity and Na^+ - Ca^{2+} exchange activity (Bers and Bridge, 1989) combined with differences in the peak and rate of change of $[\text{Ca}^{2+}]_i$ during cooling contractures and twitch contractions (Bers et al, 1989) support this proposal. Thus, conditions which inhibit transmembrane Na^+ - Ca^{2+} exchange should elevate myoplasmic Ca^{2+} during rewarming and allow the SR to accumulate a greater amount of Ca^{2+} , provided the kinetics of this process are not rate-limiting. For canine ventricular muscle bathed in 140 mM $[\text{Na}^+]_o$, the peak of the second cooling contracture (P2) was $50 \pm 5\%$ that of the first contracture (P1) following

termination of stimulation at 0.5 Hz. As shown in the lower right panel of Fig. 4, reduction of $[\text{Na}^+]_o$ to 70 mM significantly elevated the amplitude of P2 to $63 \pm 5\%$ that of P1. This antagonistic effect of low- $[\text{Na}^+]_o$ perfusion was slightly enhanced following an increase in the rate of stimulation preceding the paired cooling protocol. Following the termination of stimulation at 1.5 Hz, P2 amplitude was $52.5 \pm 4\%$ in the presence of 140 mM $[\text{Na}^+]_o$ and $70 \pm 6\%$ that of P1 after reducing $[\text{Na}^+]_o$ to 70 mM.

In addition to its effects on peak contraction, reduction of $[\text{Na}^+]_o$ significantly prolonged the duration of contraction by $16 \pm 0.9\%$ ($P < 0.05$), $21 \pm 1.2\%$ ($P < 0.05$) and $21.5 \pm 1.3\%$ ($P < 0.05$) at 0.5, 1.0 and 1.5 Hz, respectively. In combination with the significant lengthening of contraction, the effects of $[\text{Na}^+]_o$ -withdrawal on paired cooling contractures or those elicited after variable rest intervals suggest that efflux of myoplasmic Ca^{2+} out of the cells of the preparation due to the activity of the Na^+ - Ca^{2+} exchange was significantly inhibited. As discussed earlier, this suggests that under the present experimental conditions the exchange process is still functioning in a relatively physiological manner (e.g., rest-decay of postrest contraction and rapid cooling contracture does occur to some extent and contraction is not overly prolonged), and is not contributing to the development of intracellular Ca^{2+} overload (e.g., no increase of resting tension, aftercontractions or mechanical alternans were observed, even at the fastest frequencies of stimulation).

Interval-force relationship in 70 mM $[\text{Na}^+]_o$

The typical response of canine ventricular muscle to an increase in the rate of rhythmic stimulation in the presence of 70 mM $[\text{Na}^+]_o$ is illustrated in Fig. 28. In striking contrast to the response observed prior to reducing $[\text{Na}^+]_o$, increasing the stimulation frequency from 0.5 to 1.0 and 1.5 Hz led to a negative staircase of steady-state contraction. In addition to its effects on peak contraction, increasing the rate of stimulation had little effect on the amplitude of steady-state cooling contractures. An example of this uncoupling of peak developed tension and intracellular Ca^{2+} content is shown in the middle panel of Fig. 28., which illustrates that while an increase in the

rate of stimulation from 0.5 to 1.0 and 1.5 Hz depressed steady-state contraction to 85% and 65%, peak steady-state cooling contracture amplitude was reduced by only 3% and 5%. Similar observations were made with respect to the frequency-dependence of postrest contraction. In response to a 30 sec test interval, the amplitude of the first postrest beat was progressively enhanced as the stimulation frequency was increased. This occurred despite the fact that pooled SR Ca^{2+} stores were slightly diminished. In the recording shown in the bottom panel, the amplitude of postrest contraction was enhanced by 15% and 25% as the rate of stimulation was increased from 0.5 through 1.5 Hz, while steady-state cooling contracture in this muscle remained unaltered. In this respect, reduction of $[\text{Na}^+]_o$ to 70 mM in canine ventricle results in a similar frequency-dependence of steady-state contraction, steady-state rapid cooling contracture and postrest contraction as similar preparations of rat ventricular muscle perfused with solutions containing physiological concentrations of extracellular Na^+ (Kelly and Hoffmann, 1960; Forrester and Mainwood, 1974; Orchard and Lakatta, 1985; Bouchard and Bose, 1989). Interestingly, the reduction of $[\text{Na}^+]_o$ had no effect on the rate-dependent shortening of contraction. Although we did not pursue this finding further, this result appears to suggest that frequency-dependent alterations in the time course of contraction may not be significantly related to either the contractile state of the muscle preparation, or coupled transmembrane fluxes of Na^+ and Ca^{2+} .

Averaged data from ten experiments have been provided in Fig. 29. Increasing the rate of stimulation resulted in a significant decrease of peak isometric contraction to $84 \pm 8\%$ ($P < 0.05$) at 1.0 Hz, and $64 \pm 4\%$ ($P < 0.05$) at 1.5 Hz. The negative staircase of steady-state contraction was accompanied by a small, but significant, increase in postrest contraction of $10 \pm 6\%$ ($P < 0.05$) at 1.0 Hz and $18 \pm 2\%$ ($P < 0.05$) at 1.5 Hz, and a slight reduction of peak steady-state cooling contracture amplitude of $2 \pm 5\%$ and $7 \pm 10\%$ at 1.0 and 1.5 Hz, respectively. The results of these experiments provide strong evidence for the conclusion that a $[\text{Na}^+]_o$ -dependent process is indeed responsible for a significant proportion of elevated myoplasmic Ca^{2+} levels which occur during high-frequency stimulation in canine ventricular muscle, and also raise the possibility that this Ca^{2+} can be either made available to the myofilaments for contraction independent of Ca^{2+} release from SR stores, or

in turn modulates the release of Ca^{2+} itself from the SR during the course of a given action potential in a manner similar to that described in guinea-pig ventricular cells by Leblanc and Hume (1990).

Tissue Heterogeneity: The data presented above suggest that the depression of contraction by high-frequency stimulation following reduction of $[\text{Na}^+]_o$ to 70 mM may occur despite variability in Ca^{2+} released from intracellular stores. This proposal is consistent with some interesting differences in the intrinsic contractile properties of canine ventricular muscle. In six preparations studied with various protocols in the cooling apparatus, a negative staircase of steady-state contraction was demonstrated in the control perfusate containing 140 mM $[\text{Na}^+]_o$ and 2.5 mM $[\text{Ca}^{2+}]_o$. Of these six muscles, three of the preparations produced postrest contractions which were nearly identical to those shown in Fig. 25 for muscles which displayed a positive staircase (Type IIa), while the remaining three showed poor or no postrest-potential (Type IIb). This is not a novel observation, as a negative frequency-force relation in the presence of strong postrest contraction has been reported previously in canine Purkinje fibres perfused with solutions containing 130 mM $[\text{Na}^+]_o$ and 2.0 mM $[\text{Ca}^{2+}]_o$ (Boyett et al, 1987). Figure 30 illustrates one example each of the typical response of Type II muscles showing both strong and weak postrest contraction to a progressive increase in the rate of rhythmic stimulation. The recordings furnished in the upper portion of each pair show the frequency-dependence of steady-state cooling contracture amplitude while those below indicate the contractile response to resuming electrical stimulation after 30 sec rest.

For three Type IIa muscles, an increase in frequency of stimulation from 0.5 to 1.0 Hz had no effect on the strength of contraction. A subsequent increase to 1.5 Hz depressed steady-state contraction to $75 \pm 0.7\%$ of the corresponding values at 0.5 Hz. Following a 30 sec test interval, the amplitude of the first postrest contraction was reduced by $4 \pm 0.35\%$ and $5 \pm 0.45\%$ at stimulation frequencies of 1.0 and 1.5 Hz. An almost identical effect was noted for peak steady-state cooling contractures, which were reduced by $5 \pm 0.48\%$ at both of the faster drive rates. As indicated by the

traces in the top panel, Type IIa muscles produced significantly greater postrest-potential than the Type IIb muscle shown in the lower panel, despite similarities in the frequency-dependence of contraction. The values for mean potentiation following stimulation at 0.5 Hz were $156 \pm 8\%$ ($n=3$) and $-2.3 \pm 1.0\%$ ($n=3$) for type IIa and IIb muscles, respectively. The ratio of peak steady-state cooling contracture to steady-state contraction in type IIa preparations was $0.78 \pm 0.06\%$. Reduction of $[\text{Na}^+]_o$ to 70 mM increased the strength of contraction in response to stimulation at 0.5 Hz by $199 \pm 15\%$. Following an increase of stimulation frequency to 1.0 and 1.5 Hz, the amplitude of steady-state contraction was decreased by $10 \pm 0.9\%$ and $24 \pm 2\%$. Similar to muscles in which a positive staircase could be demonstrated in 140 mM $[\text{Na}^+]_o$, the rate-dependent decrease of steady-state contraction was accompanied by a steady increase in the amplitude of postrest contraction, while steady-state cooling contractures remained unaltered.

Increasing the frequency of rhythmic stimulation from a basal rate of 0.5 Hz to 1.0 and 1.5 Hz in Type IIb muscles resulted in a moderate decrease of contraction to $97 \pm 3\%$ and $82 \pm 5\%$. Changes in the amplitude of steady-state contraction were accompanied by a similar decrease in the size of pooled Ca^{2+} stores estimated by rapid cooling. For the three muscles with poor postrest contraction this amounted to a $6 \pm 0.6\%$ and $18 \pm 10\%$ depression at 1.0 and 1.5 Hz. Surprisingly, the amplitude of postrest contractions remained unaffected in response to the increase of stimulation frequency. This result is somewhat unexpected, as estimates of pooled SR Ca^{2+} content declined in parallel with the amplitude of steady-state contraction. The ratio of steady-state cooling contracture to contraction was $0.75 \pm 0.62\%$ following stimulation at 0.5 Hz, which was very close to the $0.78 \pm 0.06\%$ demonstrated in Type IIa muscles. Lowering $[\text{Na}^+]_o$ to 70 mM resulted in a $68 \pm 5\%$ increase in the strength of contraction elicited by a train of stimulation at 0.5 Hz. The ratio of peak steady-state cooling contracture to steady-state twitch amplitude was significantly reduced to $0.58 \pm 0.04\%$, suggesting that type IIb muscles share a similar dependence on SR Ca^{2+} stores as demonstrated previously for rabbit ventricle (Bers, 1987). The negative staircase of steady-state contraction was consistently produced in these preparations, with a slope which was not different than observed for Type IIa muscles. Changes in the amplitude of steady-state contraction occurred

concomitant with a $5 \pm 0.4\%$ and $10 \pm 1.0\%$ reduction of peak steady-state cooling contracture, while postrest contraction remained unaltered by the rate change.

The results of such experiments would imply that although contraction in type IIa muscles appears to depend to a greater extent on intracellular Ca^{2+} stores than Type IIb muscles, variability in the degree of SR development can not be used as a method to predict the direction of the frequency-force relation for both Type I and Type II muscles. This conclusion is based on the lack of correlation between the slope of the staircase response and (a) the ratio of steady-state cooling contracture to steady-state contraction, (b) degree of rest-potential, (c) the rate-dependence of postrest contraction or steady-state rapid cooling contracture, or (d) the degree of inotropy following reduction of $[\text{Na}^+]_o$. The data have led us to conclude that differences observed in the direction and slope of the staircase do not reflect differences in the dependence of contraction on quantitative Ca^{2+} release from the SR, and are more likely to be related to differences in action potential configuration, such as those previously described for ventricular preparations from rat (Watanabe et al, 1983; Mitchell et al, 1984), rabbit (Shattock et al, 1987), guinea-pig (Watanabe et al, 1985) and newborn dogs (Spach et al, 1989). This is supported by the fact that all of the preparations were dissected from similar regions of the right ventricle, had comparable lengths (3.5-5.0 mm) and diameters (0.2-0.4 mm), and produced similar degrees of maximal force (20-30 mN/mm²). An important observation in this respect is that, although significant differences were observed in the rate-dependence of steady-state contraction between Type I, Type IIa and Type IIb muscles, lowering $[\text{Na}^+]_o$ converted each of the three muscle types into a state where the amplitude of steady-state contraction appeared to become progressively more independent of Ca^{2+} released from SR stores. Previous work has shown that contraction can be quantitatively uncoupled from intracellular Ca^{2+} stores in a similar fashion by either overloading of the SR with Ca^{2+} (Allen et al, 1985; Fabiato, 1985) or exceeding the rate constant for Ca^{2+} transport between various functional compartments within the SR (Orchard and Lakatta, 1985; Boyett et al, 1987; Schouten et al, 1987; Capogrossi et al, 1988).

Effect of altered ionic composition on the apparent recovery of the SR Ca^{2+} -release process

Data obtained from experiments conducted with the laser light scattering technique have indicated that complete (Lakatta and Lappe, 1981; Stern et al, 1983; Kort and Lakatta, 1984; 1988a,b) or partial removal of external sodium (Ishide et al, 1984; Bose et al, 1988c) from the perfusate results in an increase in microscopic diastolic myofilament oscillations in a wide range of isolated ventricular tissues due to enhanced spontaneous release of Ca^{2+} from the SR. The demonstration by Fabiato that an increase in bulk Ca^{2+} concentration surrounding the SR inhibits Ca^{2+} -induced release of Ca^{2+} in skinned canine Purkinje fibres maintained at 22°C (Fabiato, 1982; Fabiato, 1985b) raises the possibility that the inhibition of cellular Ca^{2+} efflux in the presence of lowered $[\text{Na}^+]_o$ might be feeding back in a similar fashion to inhibit SR Ca^{2+} release during high frequency stimulation. This explanation would be consistent with the gradual increase in rest-potential as the stimulation rate is enhanced, as sufficient time would have elapsed during the 30 sec rest period for the SR Ca^{2+} -release channels to pass through their ~ 3.5 sec refractory phase (cf. Fabiato, 1985b). We attempted to test this possibility by interpolating premature stimuli at different coupling intervals and observing the rate and extent of recovery of the extrasystolic (ES) and post-extrasystolic (PES) contractions in the presence of different concentrations of extracellular Na^+ and Ca^{2+} . This protocol was repeated in a total of 48 preparations obtained from 34 animals at different basic cycle lengths which corresponded to steady-state stimulation at 0.5, 1.0 and 1.5 Hz.

Reduction of $[\text{Na}^+]_o$: Figure 31 illustrates the typical recovery of both ES and PES contractions following premature excitations delivered at coupling intervals ranging from 150 to 900 ms. The control solution contained 140 mM $[\text{Na}^+]_o$ and 2.5 mM $[\text{Ca}^{2+}]_o$. External sodium was lowered to 70 mM in the initial experiments with equimolar sucrose substitution, as we have found in past work that 50% substitution of Na^+ with sucrose uniformly increases the amplitude of scattered light intensity fluctuations in discrete frequency bands between 0.3-10 Hz in quiescent canine ventricular muscle (Bose et al, 1988c). In this protocol, each ES/PES pair was preceded by 100 steady-state contractions at the different basic cycle lengths. For this particular muscle, this

was 2000 ms (0.5 Hz) and the ES contraction recovered back to the level of the preceding steady-state contraction by 700 ms, with a time constant for exponential recovery ($\tau = 66.6\%$) of 550 ms. Peak PES potentiation occurred at 200 ms, which corresponded to the first coupling interval at which an ES contraction could be elicited. The averaged results from 12 muscles at basic cycle lengths of 2000 ms (0.5 Hz), 1000 ms (1.0 Hz) and 668 ms (1.5 Hz) have been furnished in the top panel of Fig. 32. Each point represents the amplitude of the ES contraction divided by that of the preceding steady-state contraction. Curves were fitted to the data points with a fifth-order polynomial function generated by a commercially available graphics package (Slidewrite +; Advanced Graphics Software Inc., Sunnyvale, CA). At a basic cycle length of 2000 ms, the mean amplitude for ES contractions elicited at 100, 150, 200, 300, 400, 500, 600, 700, 800, and 900 ms were 0 , $.005 \pm .0036$, $.21 \pm .02$, $.348 \pm .033$, $.564 \pm .04$, $.694 \pm .037$, $.76 \pm .04$, $.828 \pm .04$, $.880 \pm .037$ and $.91 \pm .027$, respectively. The time constant for recovery of ES contractions was 551 ± 42 ms. Following reduction of the basic cycle length to 1000 ms, the mean amplitude of ES contractions at identical coupling intervals were 0 , $.003 \pm .021$, $.241 \pm .022$, $.403 \pm .032$, $.615 \pm .03$, $.749 \pm .025$, $.882 \pm .026$, $.940 \pm .017$, $.960 \pm .014$ and $.986 \pm .068$, respectively. The time constant was significantly ($P < 0.05$) reduced to 448 ± 18 ms. A further reduction of the basic cycle length to 668 ms led to a situation where pulses applied at test intervals > 700 ms were no longer stimuli for extrasystolic contractions *per se*. This was evident in the restitution of both ES and PES contractions, which were more or less completed within 600 ms. The mean amplitude for ES contractions elicited at coupling intervals of 100, 150, 200, 300, 400, 500, 600, 700, 800, and 900 ms were 0 , $.034 \pm .018$, $.246 \pm .028$, $.432 \pm .035$, $.634 \pm .033$, $.819 \pm .024$, $.958 \pm .024$, $.994 \pm .006$, $.994 \pm .006$ and 1 , respectively. The recovery constant was slightly reduced to 442 ± 20 ms.

The lower panel in Fig. 9 illustrates the effect of varying the coupling interval for premature excitation on the recovery of the following post-extrasystolic contraction. Each point in this plot is the product of dividing the amplitude of the PES contraction by the amplitude of the final steady-state contraction preceding the ES/PES pair. Unlike the recovery of the ES contraction, a significant rate-dependent alteration of the PES profile was found. Following stimulation with a

basic cycle length of 2000 ms, the mean amplitude of PES contractions elicited at coupling intervals of 100, 150, 200, 300, 400, 500, 600, 700, 800 and 900 ms were $1.02 \pm .024$, $1.41 \pm .159$, $2.4 \pm .19$, $1.84 \pm .195$, $1.5 \pm .091$, $1.26 \pm .05$, $1.16 \pm .045$, $1.1 \pm .024$, $1.06 \pm .026$ and $1.04 \pm .017$, respectively. As was the case for steady-state contraction, enhancing the rate of stimulation prior to interpolating the extrasystole resulted in a progressive increase in the degree of post-extrasystolic potentiation. Mean contraction amplitude at a basic cycle length of 1000 ms was $1.03 \pm .017$, $1.5 \pm .22$, $2.74 \pm .25$, $1.95 \pm .1$, $1.53 \pm .07$, $1.3 \pm .05$, $1.18 \pm .035$, $1.04 \pm .014$, $1.02 \pm .013$ and $1.01 \pm .011$ at coupling intervals of 100, 150, 200, 300, 400, 500, 600, 700, 800 and 900 ms. Further reduction of the basic cycle length to 668 ms significantly increased the mean potentiation to $1.05 \pm .017$, $1.84 \pm .28$, $2.9 \pm .21$, $1.92 \pm .12$, $1.37 \pm .055$, $1.18 \pm .025$, $1.08 \pm .026$, 1 , $1.005 \pm .004$, and $1.02 \pm .01$ at identical test intervals.

At all three of the basic cycle lengths studied, peak post-extrasystolic potentiation was observed at 200 ms, which was also the first coupling interval following which a clearly observable extrasystolic contraction could be elicited. For coupling intervals >200 ms, an inverse relationship between the amplitude of the ES and PES contractions ensued, similar to that described previously for isolated preparations of canine (Yue et al, 1985) or ferret ventricle (Wier and Yue, 1986). Thus peak potentiation of the PES contraction always took place when the amplitude of the preceding contraction was smallest and as the size of the ES contraction recovered back to the steady-state level, the amplitude of the following contraction declined back toward that same steady-state level. It should be remembered that, in addition to the effects on the recovery of ES and PES contractions, reducing the basic cycle length also enhanced the size of the myoplasmic Ca^{2+} transient associated with steady-state contractions (e.g., the rate-dependent increase of steady-state contraction, steady-state rapid cooling contracture, and postrest contraction) and therefore the faster restitution of the ES contraction appears to take place under conditions associated with an apparent elevation, rather than a reduction, of intracellular $[\text{free Ca}^{2+}]$.

Figure 33 illustrates the pattern of recovery of ES and PES contractions after lowering $[\text{Na}^+]_o$ to 70 mM. Despite the increase in the general contractile properties of the preparation (see Table 5),

reduction of $[Na^+]_o$ did not exert an inhibitory effect on the time-dependence of recovery of the ES contraction at different basic cycle lengths. At a basic cycle length of 2000 ms and coupling intervals of 100, 150, 200, 300, 400, 500, 600, 700, 800, and 900 ms the mean amplitudes of the ES contraction were 0, $.005 \pm .003$, $.108 \pm .014$, $.422 \pm .032$, $.655 \pm .031$, $.788 \pm .027$, $.864 \pm .021$, $.904 \pm .02$, $.917 \pm .02$ and $.943 \pm .016$, respectively. Recovery was 66.6% complete by 423 ± 20 ms. At the basic cycle rate corresponding to 1.0 Hz (1000 ms), the mean amplitude of contraction at identical coupling intervals was 0, $0.17 \pm .016$, $.128 \pm .015$, $.445 \pm .031$, $.707 \pm .024$, $.841 \pm .02$, $.905 \pm .016$, $1 \pm .08$, 1 ± 0 and 1 ± 0 , respectively. The time constant for recovery was modestly reduced to 390 ± 13 ms. As was observed before $[Na^+]_o$ withdrawal, a final reduction of the basic cycle length to 668 ms significantly enhanced the rate of recovery of the ES contraction. At coupling intervals of 100, 150, 200, 300, 400, 500, 600, 700, 800 and 900 ms, mean ES contraction was 0, $.012 \pm .006$, $.142 \pm .011$, $.438 \pm .03$, $.727 \pm .02$, $.877 \pm .015$, $.96 \pm .01$, $.998 \pm .002$, $.995 \pm .003$ and 1 ± 0 , respectively. The time constant of recovery was 386 ± 12 ms.

The lower panel of Fig. 33 illustrates the frequency-dependence of recovery of the PES contraction in 70 mM $[Na^+]_o$. In contrast to the pattern of recovery in 140 mM $[Na^+]_o$, strong post-extrasystolic potentiation was not observed subsequent to the reduction of $[Na^+]_o$ and the small amount of potentiation that could be demonstrated was poorly dependent on the preceding frequency of stimulation. At a basic cycle length of 2000 ms. the mean amplitudes of the PES contraction at coupling intervals of 100, 150, 200, 300, 400, 500, 600, 700, 800 and 900 ms were $1 \pm .007$, $1.17 \pm .067$, $1.24 \pm .06$, $1.13 \pm .03$, $1.06 \pm .02$, $1.02 \pm .02$, $1 \pm .003$, $1 \pm .005$, $1 \pm .003$ and $1 \pm .008$, respectively. Following a decrease of the basic cycle length to 1000 ms, mean PES contraction amplitudes at identical coupling intervals were $1 \pm .005$, $1.2 \pm .06$, $1.31 \pm .056$, $1.16 \pm .03$, $1.1 \pm .03$, $1.03 \pm .008$, $1 \pm .005$, $1.01 \pm .006$, $1 \pm .004$ and $1 \pm .004$. Potentiation of the PES contraction was significantly enhanced by the final reduction of basic cycle length to 668 ms. For coupling intervals of 100, 150, 200, 300, 400, 500, 600, 700, 800 and 900 ms contraction amplitude was $1 \pm .005$, $1.33 \pm .061$, $1.49 \pm .09$, $1.22 \pm .04$, $1.11 \pm .025$, $1.04 \pm .019$, $1.01 \pm .005$, $1 \pm .003$, $1 \pm .004$ and $1 \pm .004$, respectively. Analysis of variance indicated that the pattern of potentiation and de-potentiation of

the PES contraction within the 500-900 ms range was not related to the basic cycle length preceding the ES/PES pair. For intervals between 100-400 ms, a significant interaction was noted only for the 300 ms interval, which represented a residual effect of the greater degree of potentiation at the coupling interval of 200 ms at a basic cycle length of 668 ms.

Figure 34 illustrates the sensitivity of the frequency-dependent recovery of ES and PES contractions to alterations of $[Na^+]_o$. Each point on the respective curves represents the difference contraction obtained by subtracting the response in 70 mM $[Na^+]_o$ from the equivalent response obtained in 140 mM $[Na^+]_o$. For the ES contraction shown in the top panel, paired t-tests performed at each coupling interval and basic cycle length indicated that the pattern of recovery of contraction was not greatly affected by $[Na^+]_o$ withdrawal. The only point significantly different from unity was the first coupling interval at which a significant ES contraction could be elicited. At all three test frequencies the amplitude of contraction at 200 ms in the presence of 140 mM $[Na^+]_o$ was significantly greater ($P < 0.05$) than the equivalent response in 70 mM $[Na^+]_o$. It is not readily apparent whether this difference represents a significant inhibition of the SR Ca^{2+} release machinery in the presence of low $[Na^+]_o$ or a lowering of the membrane threshold for excitation, as all points before and after the 200 ms coupling interval were nearly identical to unity. Sheu and Fozzard (1982) have shown that a 40-80% reduction of $[Na^+]_o$ produces a hyperpolarization of ~ 5 mV in sheep ventricular muscle, which could conceivably increase the electrical threshold for membrane excitation enough to cause the small shift noted in our experiments. However, this does not explain the maintained difference at coupling intervals > 200 ms or the fact that peak post-extrasystolic potentiation was reduced to such an extent. The results were much different when difference contractions for the PES response were calculated. As expected on the basis of the above results, the largest difference in low $[Na^+]_o$ solution was the large reduction in the degree of post-extrasystolic potentiation. At the basic cycle length corresponding to 0.5 Hz (2000 ms), the amplitude of contraction was significantly reduced at coupling intervals between 200 and 900 ms. A reduction of the basic cycle length to 1000 ms shifted this difference to earlier intervals between

200 and 800 ms and this shift was enhanced further yet by the final decrease of the basic cycle rate to 668 ms, where significance was demonstrated at coupling intervals between 100 and 600 ms.

The differential sensitivities of the recovery of ES and PES contractions and their respective frequency-dependence suggest that lowering $[Na^+]_o$ has a more profound effect on the cellular mechanisms responsible for post-extrasystolic potentiation (e.g., Ca^{2+} entry during the ES beat and ensuing changes in the degree of SR Ca^{2+} loading and rate constant for Ca^{2+} transport) than those responsible for the time-dependent recovery of the extrasystolic contraction (e.g., recovery of the action potential and time constant for the recovery of SR Ca^{2+} -release channel). The apparent lack of Ca^{2+} -induced inactivation of SR release is surprising given the demonstration of this process in skinned canine Purkinje fibres (Fabiato, 1985b). The absence of an intact sarcolemmal membrane in skinned muscle preparations, combined with the observation of Boyett et al (1987) that the slope of the staircase response in intact canine Purkinje fibres is in a negative, rather than a positive, direction implies that the cellular mechanisms responsible for fine-tuning Ca^{2+} release may be slightly different in areas of the heart concerned primarily with impulse conduction or after removal of the sarcolemma. To further test this possibility, the level of free myoplasmic Ca^{2+} during steady-state stimulation was lowered by decreasing the concentration of Ca^{2+} in the perfusate ($[Ca^{2+}]_o$) while keeping $[Na^+]_o$ constant at 140 mM. It was postulated that if the above speculation was correct, then similar observations should be made with respect to the sensitivity of recovery of the ES and PES contractions to different basic cycle lengths as were made during experiments where $[Na^+]_o$ was altered.

Reduction of $[Ca^{2+}]_o$ The pattern of recovery for ES and PES contractions in the presence of 1.25 mM $[Ca^{2+}]_o/140$ mM $[Na^+]_o$ is shown in Fig. 35. Data have been averaged from 17 experiments carried out under identical conditions as those shown in Figs. 31-34. Despite the reduction of steady-state contraction by more than half the corresponding values obtained in 2.5 mM $[Ca^{2+}]_o$, the frequency-dependence of recovery of ES contraction was similar to that demonstrated in Figs. 32 and 33 for muscles perfused with 2.5 mM $[Ca^{2+}]_o$ (see Table 5).

Following stimulation at a basic cycle length of 2000 ms, the mean amplitude of ES contractions elicited at coupling intervals of 100, 150, 200, 300, 400, 500, 600, 700, 800 and 900 ms were 0.009 ± 0.007 , $.115 \pm .02$, $.329 \pm .023$, $.571 \pm .025$, $.732 \pm .022$, $.842 \pm .024$, $.878 \pm .016$, $.91 \pm .016$ and $.936 \pm .015$, respectively. The time constant for recovery of ES contraction was 480 ± 14.6 ms. Reduction of the basic cycle length to 1000 ms resulted in ES contractions with a mean amplitude of 0, $.001 \pm .02$, $.125 \pm .02$, $.356 \pm .029$, $.6 \pm .03$, $.764 \pm .03$, $.862 \pm .019$, $.939 \pm .014$, $.973 \pm .015$ and $.99 \pm .003$ at identical coupling intervals. Contraction recovered to 66.6% by 460 ± 18 ms. Similar to the response obtained in $2.5 \text{ mM } [\text{Ca}^{2+}]_o$, the final reduction of the basic cycle length to 668 ms resulted in a significantly faster recovery of the ES contraction. The mean amplitude of contraction at coupling intervals of 100, 150, 200, 300, 400, 500, 600, 700, 800 and 900 ms were 0, $.006 \pm .006$, $.123 \pm .023$, $.382 \pm .039$, $.657 \pm .024$, $.844 \pm .02$, $.943 \pm .017$ 1 ± 0 , 1 ± 0 and 1 ± 0 respectively. The time constant of recovery was 427 ± 16 ms.

The lower panel of Fig. 35 illustrates the frequency-dependence of recovery for the accompanying PES contractions. The degree to which these contractions were potentiated at a given coupling interval was significantly enhanced following the reduction of $[\text{Ca}^{2+}]_o$. Also in contrast to the equivalent response in $2.5 \text{ mM } [\text{Ca}^{2+}]_o$ was the insensitivity of both the peak of the potentiation curve as well as the rate of recovery back to the steady-state level to the basic cycle length preceding the ES/PES pair. This appears to be related to the amount of Ca^{2+} available for release from the SR, as increasing the rate of rhythmic stimulation resulted in a positive staircase of steady-state contraction for muscles bathed in 2.5 mM but not $1.25 \text{ mM } [\text{Ca}^{2+}]_o$. In response to stimulation with a basic cycle length of 2000 ms, the mean amplitude of PES contractions following interpolation of an extrasystole at coupling intervals of 100, 150, 200, 300, 400, 500, 600, 700, 800 and 900 ms were $1.04 \pm .027$, $1.28 \pm .14$, $3.33 \pm .42$, $2.4 \pm .21$, $1.63 \pm .08$, $1.35 \pm .046$, $1.23 \pm .03$, $1.14 \pm .02$, $1.07 \pm .017$ and $1.07 \pm .018$, respectively. A decrease of the basic cycle length to 1000 ms yielded contractions with a mean amplitude of $1.04 \pm .03$, $1.2 \pm .11$, $3.11 \pm .42$, $2.36 \pm .17$, $1.6 \pm .08$, $1.34 \pm .04$, $1.18 \pm .02$, $1.11 \pm .016$, $1.04 \pm .01$ and $1 \pm .007$ at identical coupling intervals. A final reduction of the basic cycle length to 668 ms resulted in contractions of $1.04 \pm .027$, $1.37 \pm .15$,

3.18±.41, 2.23±.13, 1.5±.045, 1.2±.02, 1.07±.016, 1.02±.017, 1.02±.012 and 1.04±.014 following interpolation of an extrasystole at coupling intervals of 100, 150, 200, 300, 400, 500, 600, 700, 800 and 900 ms.

The sensitivity of recovery of the ES and PES contractions to variation of $[Ca^{2+}]_o$ and basic cycle length is shown in Fig. 36. Difference contractions were calculated as described for Fig. 34 by subtracting the response obtained in 2.5 mM $[Ca^{2+}]_o$ from that obtained after lowering $[Ca^{2+}]_o$ to 1.25 mM. Paired t-tests conducted at each coupling interval and basic cycle length indicated that none of the points was significantly different from unity during recovery of the ES contraction. This provides supportive evidence for the earlier suggestion that the single difference contraction observed at the 200 ms coupling interval following reduction of $[Na^+]_o$ may have been a result of a shift in the threshold for membrane excitation, as lowering $[Ca^{2+}]_o$ would not be expected to significantly alter the resting membrane potential. As was noted in experiments where $[Na^+]_o$ was altered, large difference contractions were calculated for the PES response following the reduction of $[Ca^{2+}]_o$. At a basic cycle length of 2000 ms lowering $[Ca^{2+}]_o$ significantly increased the amplitude of PES contractions elicited at coupling intervals between 200 and 700 ms. Similar to the effect of altering $[Na^+]_o$, decreasing the basic cycle length to either 1000 ms or 668 ms shifted this difference to earlier coupling intervals between 200 and 500 ms. While a portion of this shift can be explained on the basis of previous reports of faster repriming of the release pool concomitant with increased stimulation frequency in canine ventricle (Endoh and Iijima, 1981; Bose et al, 1988a), the inverse relationship demonstrated between peak post-extrasystolic potentiation and stimulation frequency in Fig. 35 can not. As was pointed out earlier, the independence of this response from the preceding basic cycle length may be related to the lack of effect of stimulation frequency on steady-state contraction or steady-state contracture.

A summary of the relationship between the rate of recovery of ES contractions in perfusing solutions with different ionic compositions and the level of intracellular Ca^{2+} loading and release during contraction is shown in Table 5. Alteration of $[Na^+]_o$ or $[Ca^{2+}]_o$ was shown with different stimulation protocols to have much more variable effects on the strength of steady-state

contraction and rapid cooling contracture than on the time-dependence of recovery for ES contractions, which appeared to be more invariantly dependent on the preceding rate of stimulation. Even within a given perfusion solution, the slope or direction of the frequency-force relationship was not related to the rate of recovery of the ES contraction. For example, an increase in the rate of stimulation from 0.5 to 1.0 and 1.5 Hz resulted in either a significant positive staircase (2.5mM $[Ca^{2+}]_o$ /140mM $[Na^+]_o$), no significant change in contraction amplitude (1.25mM $[Ca^{2+}]_o$ /140mM $[Na^+]_o$) or a negative staircase (2.5mM $[Ca^{2+}]_o$ /70mM $[Na^+]_o$) which was accompanied by either a significant increase (2.5mM $[Ca^{2+}]_o$ /140mM $[Na^+]_o$) or no change (1.25mM $[Ca^{2+}]_o$ /140mM $[Na^+]_o$, 2.5mM $[Ca^{2+}]_o$ /70mM $[Na^+]_o$) in the amount of Ca^{2+} present within pooled SR compartments estimated by rapid cooling. Despite the variable frequency-dependence of contraction, the amount of time required for ES contractions to recover to 66.6% of steady-state was related to peak myoplasmic Ca^{2+} levels in an inverse fashion: as first $[Ca^{2+}]_o$ was elevated and then $[Na^+]_o$ decreased, the amplitude of steady-state contraction and rapid cooling contractures were significantly enhanced, and the time constant of recovery decreased; regardless of the basic cycle length employed. The possibility may still exist that intracellular Ca^{2+} levels high enough to suppress contraction were not reached in our conditions, as no aftercontractions were observed and resting tension was not elevated during high frequency stimulation, as is often observed in normal $[Na^+]_o$. If the diastolic Ca^{2+} concentration was not sufficiently raised to inactivate a significant population of release channels in the manner predicted by the Ca^{2+} -inactivation hypothesis, then the results given in Table 5 would imply that the negative staircase demonstrated in the presence of 70 mM $[Na^+]_o$ was not due to inhibited SR release. In this respect the results are similar to the inability of photoreleased Ca^{2+} immediately after depolarization-induced Ca^{2+} release to suppress contraction in voltage clamped guinea-pig ventricular myocytes (Nabauer and Morad, 1990; see also Fabiato, 1989).

Effect of $[Na^+]_o$ withdrawal on mechanical restitution

In isolated rat ventricular muscle perfused with physiological concentrations of Na^+ , a progressive increase in the rate of stimulation leads to a negative staircase of steady-state contraction (Kelly and Hoffman, 1960; Forester and Mainwood, 1974; Capogrossi et al, 1988) and the peak of the systolic Ca^{2+} transient (Orchard and Lakatta, 1985), while the amount of Ca^{2+} present within the SR remains unaltered or slightly decreased (Bouchard and Bose, 1989; 1991). Although it is possible that a portion of the negative staircase in thicker multicellular preparations may be due to hypoxia in the core cells of the muscle (Schouten and terKeurs, 1986; Gulch and Ebrecht, 1986), numerous reports have appeared which suggest that the rate of recirculation between functional pools within the SR may be one of the limiting factors in determining the amount of Ca^{2+} available for release at higher rates of stimulation (Ragnarsdottir et al, 1982; Schouten et al, 1987; Kort and Lakatta, 1988b; Kim and Smith, 1988; Stern et al, 1988). To determine if this may be the reason for the negative staircase following reduction of $[Na^+]_o$ in canine ventricle, we extended the range of rest intervals to include those close to the interval between rhythmically stimulated contractions at different basic cycle lengths. By selecting test intervals between 2.5 and 120 sec, the effect of lowering $[Na^+]_o$ on different phases of the restitution curve could be determined, similar to those computed for guinea-pig, rabbit and rat heart (Kruta and Braveny, 1961; Koch-Weser and Blinks, 1963; Schouten et al, 1987; Capogrossi et al, 1988).

The protocol used to assess the restitution of postrest contraction and the accompanying alterations of pooled SR Ca^{2+} content is shown in Fig. 37. Rest intervals ranging from 2.5 to 120 sec were imposed on an otherwise constant train of stimulation at 0.5 Hz (top panel) or 1.0 Hz (bottom panel). The top recording in each of the four sets of data depicts the response of the muscle to postrest electrical stimulation. The dashed lines above each family of postrest contractions are for reference and represent peak restpotentiation at 0.5 Hz stimulation before $[Na^+]_o$ withdrawal. The bottom recording in each of the panels illustrates the response to rapid cooling immediately after terminating rhythmic stimulation and after 30 and 120 sec rest. The

arrows indicate the peak of the cooling contracture elicited after a 120 sec rest interval at 0.5 Hz stimulation within a given $[\text{Na}^+]_o$. The two panels on the left illustrate the response in 140 mM $[\text{Na}^+]_o$ and the two on the right following reduction of $[\text{Na}^+]_o$ to 70 mM.

Termination of stimulation at 0.5 Hz in the presence of 140 mM $[\text{Na}^+]_o$ resulted in a gradual increase in the amplitude of the first postrest contraction as the rest interval preceding resumption of stimulation was extended from 2.5 sec through 120 sec. In most of the preparations the strength of contraction remained relatively stable following a test interval of 2.5 sec, and as a result the portion of the restitution process between 2 and 5 sec was fairly flat (e.g., the basic cycle length was equal to 2000 ms at 0.5 Hz). Throughout the entire time when contraction amplitude was increasing to the maximum point on the restitution curve, the amount of Ca^{2+} present within pooled SR compartments estimated by rapid cooling was declining in a similar time-dependent fashion. This is illustrated in the lower recording, where the amplitude of cooling contractures begins to decline from the moment stimulation is terminated (see also Fig. 27. A doubling of stimulation frequency to 1.0 Hz led to a decrease in the period of time required to attain peak postrest contraction. A comparison of the restitution process in the upper and lower left panels shows that peak rest-potential of 20 mN/mm², which required 120 sec at 0.5 Hz stimulation, was surpassed after only 10 sec rest at the faster rate of stimulation. The leftward shift in the restitution process was associated with an increase in the rate of Ca^{2+} loss from the SR. This is illustrated by the corresponding cooling recordings, which show that despite a 20% increase in the amplitude of steady-state rapid cooling contracture, the amplitude of cooling contractures elicited after 120 sec rest were nearly identical at the two frequencies (e.g., compare the peak of postrest cooling contracture at 1.0 Hz with arrow).

Reduction of $[\text{Na}^+]_o$ to 70 mM produced qualitative changes in the restitution of contraction which would not have been expected if the rate constant of Ca^{2+} transport within the SR were limiting the strength of contraction at the higher frequencies of stimulation. In the presence of 70 mM $[\text{Na}^+]_o$, a gradual extension of the rest interval resulted in a time-dependent increase in the amplitude of contraction that was not monophasic as was observed in 140 mM $[\text{Na}^+]_o$. Short rest

intervals between 2.5 and 10 sec led to a slight, but rapid, potentiation of contraction which was followed by a more delayed and gradual component of restitution. Unlike the response in 140 mM $[\text{Na}^+]_o$ changes in the amplitude of contraction were not accompanied by a significant decay of postrest rapid cooling contracture. Increasing the rate of stimulation to 1.0 Hz led to an increase in the degree of rest-potentiation associated with either component of restitution, due partially to the reduction in the strength of steady-state contraction. However, despite the negative inotropy associated with faster stimulation the absolute magnitude of contraction at all test intervals was significantly greater than the corresponding contractions at 0.5 Hz, as illustrated by the dashed line in the lower right panel. The panel below illustrates the time-dependence of peak rapid cooling contracture following 1.0 Hz stimulation, and as was observed at 0.5 Hz, little decay of postrest cooling contracture was observed. As a result, the rate-dependent alteration of the restitution process following reduction of $[\text{Na}^+]_o$ occurs in the absence of concomittant changes in the content of pooled SR Ca^{2+} stores, in a fashion which is almost identical to that shown in Figs. 28 and 29 for steady-state contraction.

Figure 38 illustrates averaged restitution curves constructed from paired experiments performed on nine muscles before and after lowering $[\text{Na}^+]_o$ from 140 to 70 mM. The y-intercept for each plot represents mean steady-state contraction at 0.5 Hz. Following termination of stimulation at 0.5 Hz, the amplitude of postrest contraction in the presence of 140 mM $[\text{Na}^+]_o$ increased monotonically as the rest interval was gradually extended from 2.5 to 5, 10, 30, 60 and 120 sec. Increasing the stimulation frequency to 1.0 and 1.5 Hz led to a biphasic restitution curve. The observed shift in the restitution curve can be explained on the basis of either an increase of myoplasmic Ca^{2+} concentration during steady-state stimulation (Orchard and Lakatta, 1985) or the rate constant of Ca^{2+} transport within the SR at a given stimulation frequency (Schouten et al, 1987); and it seems that both mechanisms are likely contributing to the altered pattern of mechanical restitution under these experimental conditions. Evidence for this conclusion includes (a) the rate-dependent increase in pooled SR Ca^{2+} content estimated by rapid cooling (Figs. 24 and 25), (b) the observation that despite changes in the amplitude of steady-state cooling contracture, postrest

cooling contractures elicited after 120 sec rest were nearly identical at different stimulation frequencies (Fig. 37), and as will be shown later, (c) the magnitude of post-extrasystolic potentiation was increased in a frequency-dependent manner (Figs. 31-34). For rest intervals between 5 and 30 sec, the rate of Ca^{2+} efflux from the SR appears to be closely matched by the rate constant for Ca^{2+} recirculation, as little change in postrest contraction was observed during this time, despite the continual "leak" of Ca^{2+} from the SR. However, as the duration of rest was extended beyond 30 sec, the strength of postrest contraction gradually declined. This was particularly evident at 1.5 Hz stimulation, where the rate constant for the decay of postrest cooling contractures was greatest compared to those observed at 1.0 and 0.5 Hz.

The lower panel illustrates the effects of $[\text{Na}^+]_o$ withdrawal on the time and frequency-dependence of postrest contraction. In contrast to the response in 140 mM $[\text{Na}^+]_o$, restitution curves consisting of at least two distinct components were obtained at all three test frequencies. Following 0.5 Hz stimulation, the first rapid phase of potentiation was complete within ~ 10 sec. This was followed by a delayed component of restitution during which time the strength of contraction increased up until the longest test interval studied. An increase in the rate of stimulation to 1.0 and 1.5 Hz significantly shortened the time required to complete both phases of restitution. An important feature of the restitution process following reduction of $[\text{Na}^+]_o$, was that an increase in the rate of stimulation enhanced the peak of both the rapid and delayed components of restitution from their respective basal values at 0.5 Hz in a fashion similar to that observed prior to lowering $[\text{Na}^+]_o$. A late declining phase was only observed following prolonged rest at the fastest rate of stimulation. Therefore, the major difference in the frequency-dependence of restitution after lowering $[\text{Na}^+]_o$ was the presence of a strong delayed component (rather than alteration of the initial rapid phase), suggesting that despite the inotropic effect of $[\text{Na}^+]_o$ withdrawal, the SR is not saturated with activator Ca^{2+} during steady-state stimulation at 1.0 and 1.5 Hz. Although the frequency-dependence of rest-potentiation was not as strong in preparations perfused with 70 mM $[\text{Na}^+]_o$ compared with that observed in 140 mM $[\text{Na}^+]_o$, the absence of an inverse correlation between postrest contraction and stimulation frequency suggests that the rate

constant for unidirectional Ca^{2+} transport between different functional pools of the SR is not the limiting factor responsible for the negative staircase shown in Figs. 28 and 29.

Pharmacological investigation into the contribution of SR Ca^{2+} release in low- $[\text{Na}^+]_o$

To further investigate the ability of the SR to modulate the frequency-dependence of contraction in low- $[\text{Na}^+]_o$ solutions, various pharmacological agents were applied to either increase or decrease Ca^{2+} entry into the cell under conditions in which the intracellular buffering capacity for Ca^{2+} remained constant, or to alter the ability of the SR to sequester and/or retain its load of Ca^{2+} during the diastolic interval, and hence the magnitude of Ca^{2+} release in response to stimulation. Figure 39 illustrates the effects of caffeine (3 mM) and ryanodine (10 nM) on the depressed staircase response following reduction of $[\text{Na}^+]_o$ with equimolar sucrose substitution. In these experiments, the slope of the staircase was first dropped down by reducing $[\text{Na}^+]_o$ to 70 mM, which was then followed by the addition of either caffeine or ryanodine to the perfusate from stock solutions. We have found in previous experiments that both drugs induce a profound effect on the ability of the SR to store and release Ca^{2+} in canine ventricular muscle perfused with physiological concentrations of Na^+ (Bose et al, 1988a; Hryshko et al, 1989a; Bouchard et al, 1989). The differential ability of either agent to accelerate the diastolic loss of intracellular Ca^{2+} , or in the case of caffeine to also inhibit SR Ca^{2+} sequestration (and hence to completely abolish steady-state cooling contractures), seems to be consistent with the ability of either agent to alter the gating kinetics and conductance of the 90 pS SR Ca^{2+} -release channels from canine ventricle incorporated into planar lipid bilayers (Rousseau et al, 1987; Rousseau and Meissner, 1989).

Following the reduction of $[\text{Na}^+]_o$, application of either drug significantly depressed the amplitude of steady-state contractions elicited in response to 0.5 Hz stimulation. Caffeine depressed peak contraction to $40 \pm 15\%$ controls values, whereas ryanodine markedly reduced contraction to $25 \pm 15\%$ of the pretreatment values. Despite the negative inotropic effect on muscle contractility, caffeine restored the frequency-dependence of contraction to a point almost identical to the response observed prior to lowering $[\text{Na}^+]_o$. The inability of cardiac SR to efficiently buffer

intracellular [free Ca^{2+}] in the presence of caffeine (Weber and Herz, 1968; Fuchs, 1969; Bers and MacLeod, 1987; Hryshko et al, 1989a,c; Bouchard et al, 1989), suggests that the volume of Ca^{2+} normally sequestered into intracellular storage sites might be forced to remain in the cytosol; a situation which may be compounded by both the abbreviated diastolic interval during high-frequency stimulation and inhibition of the exchanger itself (cf. Fig. 27). This is supported in the present experiments by the small but reliable increase of resting tension at the faster rates of stimulation following treatment with caffeine, compared with control muscles at a similar stage in the experiment. Application of ryanodine had a similar chronotropic effect, but dramatically enhanced the slope of the positive staircase without producing any change in the level of resting tension.

Blockade of sarcolemmal Ca^{2+} channels by nifedipine (2 μM) in the presence of 70 mM $[\text{Na}^+]_o$ depressed contraction amplitude in response to stimulation at 0.5 Hz by $72.6 \pm 5\%$. A graded increase in the rate of stimulation from 0.5 to 1.0 and 1.5 Hz resulted in a marked depression of contractile force to $72.2 \pm 5\%$ and $44.7 \pm 6\%$ that observed at 0.5 Hz. Conversely, increasing the amount of Ca^{2+} entering the cell during the action potential by administration of 1 μM racemic BAY K 8644 increased contraction amplitude during 0.5 Hz stimulation in low- $[\text{Na}^+]_o$ solution by $108 \pm 20\%$. Like the response to caffeine, BAY K 8644 restored the positive staircase when administered to muscles previously exposed to 70 mM $[\text{Na}^+]_o$. Contraction amplitude at 1.0 and 1.5 Hz stimulation was $118 \pm 3\%$ and $129.4 \pm 4.7\%$ that observed at 0.5 Hz stimulation. An interesting observation was that the ability of BAY K 8644 to restore the frequency-dependence of contraction in the presence of 70 mM $[\text{Na}^+]_o$ was almost 2-fold greater in Type IIa or Type IIb muscles. Although the influence of each dihydropyridine compound on the staircase response is likely complicated to a certain degree by its respective use-dependence (Sanguinetti and Kass, 1984; Thomas et al, 1985c), this latter observation would imply that the opposite directional responses obtained with either drug may be related to accompanying alterations in the degree of intracellular Ca^{2+} loading and release.

Figure 40 shows the frequency-dependence of contraction following reduction of $[\text{Na}^+]_o$ to 70 mM with different substituents in the presence of 2.5 mM $[\text{Ca}^{2+}]_o$. As described above, increasing the rate of stimulation from 0.5 Hz to 1.0 and 1.5 Hz in control solutions (140 mM $[\text{Na}^+]_o$ /2.5 mM $[\text{Ca}^{2+}]_o$) resulted in an $18 \pm 4\%$ and $44 \pm 8\%$ increase of contractile strength. Following reduction of $[\text{Na}^+]_o$ to 70 mM by substitution with Li^+ , peak contraction at 1.0 and 1.5 Hz was depressed by $16 \pm 1.5\%$ and $36 \pm 3\%$. Depletion of $[\text{Na}^+]_o$ using either choline chloride or sucrose resulted in a very different situation, where the muscles were converted into a state where they were almost completely insensitive to alterations in the rate of stimulation. Substitution of external Na^+ with choline chloride (n=4) depressed peak contraction by $2 \pm 0.2\%$ and $2.8 \pm 2\%$ at 1.0 and 1.5 Hz, respectively. Using sucrose to substitute for external Na^+ ions (n=19) decreased peak contraction by $1 \pm 2\%$ and $0.5 \pm 3\%$.

DISCUSSION

The aim of this study was to examine the contribution of sarcolemmal Na^+ - Ca^{2+} exchange toward the interval-dependence of contraction in an intact preparation of mammalian ventricular muscle. Combined with the parallel effect of $[\text{Na}^+]_o$ reduction on the strength of contraction and either steady-state cooling contracture or postrest contraction, the sensitivity of contraction to inhibition of SR function subsequent to lowering $[\text{Na}^+]_o$ suggests that the inotropic effect of this intervention is due primarily to enhanced availability and release of Ca^{2+} from intracellular stores. Changes in the strength of contraction which accompany alterations in the rate of stimulation in the presence of low- $[\text{Na}^+]_o$ solutions and the relationship of these changes to the availability of Ca^{2+} for release from the SR have revealed the requirement of the positive staircase of steady-state contraction on a functional Na^+ - Ca^{2+} exchange mechanism. That the magnitude of steady-state cooling contractures remained insensitive to stimulation frequency following the reduction of $[\text{Na}^+]_o$ to 70 mM suggests that a significant fraction of the rate-dependent increase of SR Ca^{2+} availability observed in 140 mM $[\text{Na}^+]_o$ is related also to perturbations in the dominant mode of the exchange process. The results also raise the possibility that net cellular gain of Ca^{2+} related to the function of the exchanger may affect the strength of contraction during high-frequency stimulation by either altering the trigger for intracellular Ca^{2+} release, or by making Ca^{2+} available directly to the myofilaments.

Effect of $[\text{Na}^+]_o$ -reduction on steady-state contraction

The increase of steady-state contraction amplitude observed in this and other studies (Sheu and Fozzard, 1982; Sutko, Bers and Reeves, 1986; Bers, 1987; Pytkowski, 1988; Horackova, 1989) following partial replacement of $[\text{Na}^+]_o$ with either Li^+ or sucrose could be brought about by a number of cellular processes which affect specifically buffering of Ca^{2+} by the SR. These include alterations of trans-sarcolemmal Ca^{2+} extrusion during both relaxation of the twitch as well as during the diastolic interval, kinetics of Ca^{2+} transport within the SR, and the continual "leak" of

Ca^{2+} which occurs from the release compartment of the SR into the myoplasm. As the Na^+ - Ca^{2+} exchange process has been found to mediate both the extrusion of myoplasmic Ca^{2+} during relaxation of contraction (Bridge et al, 1988; 1989; Bers and Bridge, 1989) and the loss of cellular Ca^{2+} from the SR into the extracellular space during the diastolic interval (Hilgemann et al, 1984; Kitazawa, 1984; Bers and MacLeod, 1986; Sutko et al, 1986; Hilgemann, 1986a,b; Bridge, 1986; Lewartowski and Pytkowski, 1988; Bers et al, 1987; 1989; Hryshko et al, 1989c), a reduction in the driving force for the inward mode of the exchange process would be expected to lead to a net cellular gain of Ca^{2+} during steady-state stimulation. This would be particularly so if the leak predominated during the diastolic interval, the duration of which is increased significantly following the reduction of $[\text{Na}^+]_o$ (Mitchell et al, 1984; Schouten and terKeurs, 1985; Bril and Man, 1989; Horackova, 1989). A number of experimental observations from the present work agree with this proposal, including the prolonged relaxation phase of contraction, and the inhibition of myoplasmic Ca^{2+} extrusion both preceding the initiation of postrest cooling contractures (Fig. 27A) and following the termination of cooling in the paired cooling protocol (Fig. 27B). As mentioned above, in addition to Ca^{2+} entry across the sarcolemma, the amount of Ca^{2+} available for release in response to stimulation is also affected by rate at which the release compartment of the SR is replenished with Ca^{2+} during the diastolic interval (Lipsius et al, 1982; Fabiato, 1985b; London and Krueger, 1986; Meyers et al, 1988; Capogrossi et al, 1988; Kort and Lakatta, 1988b). On the basis of both experimental evidence and modelling studies, Schouten et al (1987) have suggested that the rate constant for unidirectional Ca^{2+} transport between functional compartments of the SR is strictly proportional to the volume of Ca^{2+} located within the uptake compartment. Our results agree with that proposal. As shown in Fig. 38, both the peak and rate of mechanical restitution were enhanced after lowering $[\text{Na}^+]_o$ to 70 mM in a manner similar to that induced by increasing the stimulation frequency in 140 mM $[\text{Na}^+]_o$. Alterations in the transient peak of the mechanical restitution curve can be explained by the 90% increase in steady-state cooling contracture amplitude alone, which according to the above hypothesis (Schouten et al, 1987) should enhance the rate of Ca^{2+} recirculation within a given period of time. An interesting

observation, however, was that lowering $[Na^+]_o$ also resulted in a second, more delayed, component of restitution. It is likely that this increase in the recirculating fraction of Ca^{2+} available for release in the presence of low- $[Na^+]_o$ solution simply reflects the added component of Ca^{2+} uptake resulting from the inhibition of sarcolemmal Ca^{2+} extrusion during the extended diastolic interval (e.g., the inward mode of the exchanger).

A second possible mechanism which could also contribute to the rise of contraction amplitude during perfusion with low- $[Na^+]_o$ solutions may be an accompanying shift of the reversal potential for the Na^+ - Ca^{2+} exchange process (E_{NaCa}) during the action potential (Mullins, 1979; 1981; Noble, 1984; 1986; Difrancesco and Noble, 1985; Hilgemann and Noble, 1987; Bers, 1987). According to the equation $E_{NaCa} = 3E_{Na} - 2E_{Ca}$ (Mullins, 1979; Noble, 1986; Kimura et al, 1987), we calculate that the reversal potential for the exchanger is approximately -12 mV, using values of 6 mM, 150 nM, 110 mM and 1.45 mM for a_{Na}^i , a_{Ca}^i , a_{Na}^o , a_{Ca}^o , respectively (Lee and Fozzard, 1975; Sheu and Fozzard, 1982; Bers and Ellis, 1982; Brill et al, 1986; Boyett et al, 1987; Wang et al, 1988). Reduction of $[Na^+]_o$ to 70 mM would be expected to result in a proportionately smaller decrease of a_{Na}^i of 15-17% to ~5 mM (Sheu and Fozzard, 1982), with an accompanying increase of a_{Ca}^i to ~200 nM (Marban et al, 1980; Dahl and Isenberg, 1980; Bers and Ellis, 1982; Sheu and Fozzard, 1982). Under these conditions, E_{NaCa} would be increased to -37.5 mV, with the greatest deviation from E_m during contraction taking place within the first 50 ms of the action potential (e.g., Egan et al, 1989). Even with the accompanying reduction of action potential duration (Schouten and terKeurs, 1985; Brill and Man, 1989; Horackova, 1989) and resting membrane potential (Sheu and Fozzard, 1982) in response to lowering $[Na^+]_o$, an increase of approximately 200% of the theoretical quantity $E_{NaCa} > E_m$ during the first 5-50 ms of the action potential may significantly alter the trigger for release of Ca^{2+} from intracellular stores (Leblanc and Hume, 1990). If so, this possibility would be tempered significantly by the decrease of peak I_{Na} (Carmeliet, 1987) and rate of rise of the action potential (Mitchell et al, 1984; Hume and Uehara, 1985) expected following partial removal of $[Na^+]_o$. A decrease of membrane Na^+ conductance of this nature would have the effect of reducing overshoot of the action potential (Hume and Uehara, 1985; Leblanc and

Hume, 1990), and hence the area defined by the term $E_{NaCa} > E_m$. Although we have no experimental evidence to support this proposal, Gruver, Katz and Messinneo (1990; see also Carafoli, 1983) have demonstrated that the kinetics of the exchanger are sufficiently fast to account for the necessary ionic fluxes required to alter the trigger for Ca^{2+} release from the SR, and the subsequent rise of intracellular [free Ca^{2+}] (Leblanc and Hume, 1990). Moreover, inward currents carried by the exchanger during the plateau and repolarization phases of the action potential (Egan et al, 1989; Giles and Shimoni, 1989; Terrar and White, 1989) would be facilitated by lowering external Na^+ due to the accompanying increase of intracellular free Ca^{2+} . There is a good chance that this effect would be significant in our experiments, as Giles and Shimoni (1989) have demonstrated that inward currents carried by the exchanger (I_{EX} ; see also Egan et al, 1989) predominate in tissues possessing relatively well developed SR. Other, more circumstantial evidence in support of this proposal comes from the large increase in strength of contraction that follows the reduction of $[Na^+]_o$ observed in those species which do not possess a well developed sarcotubular network, such as frog ventricle (Chapman and Neidergerke, 1970; Morad and Cleeman, 1987), rabbit ventricle (Sutko, Bers and Reeves, 1986; Bers, 1987) or guinea-pig ventricle (Pytkowski, 1988; Horackova, 1989). Additional evidence from the present work comes from the observation that, despite marked differences in the interval-dependence of contraction in Type I, Type IIa and Type IIb muscles in the presence of 140 mM $[Na^+]_o$, all three muscle sub-types exhibited a significant inotropic response to reduction of $[Na^+]_o$ to 70 mM, and shared a nearly identical frequency-dependence once this effect had been established.

Effect of $[Na^+]_o$ -reduction on the interval-dependence of contraction

Contribution of SR Ca^{2+} release: The present work suggests that the rate-dependent increase of SR Ca^{2+} loading seen in this and other studies on mammalian ventricle (Gibbons and Fozzard, 1971; Kurihara and Sakai, 1985; Bouchard and Bose, 1989) likely reflects accompanying changes of both the dominant mode of the Na^+ - Ca^{2+} exchange process, and the integral of the Ca^{2+} current per unit time, expressed as Q_{Ca}/min . An important observation in support of this

proposal is the insensitivity of rapid cooling contracture amplitude to changes in the preceding rate of stimulation in 70 mM, but not 140 mM, $[Na^+]_o$. The restoration of the positive force staircase after treatment with BAY K 8644 (1 μ M) and the rate-dependent decrease of contraction amplitude in the presence of similar concentrations of nifedipine suggests that, even in the presence of an altered Na^+ gradient, the strength of contraction is still proportional to quantitative release of Ca^{2+} from the SR (e.g., Figs. 24, 25 and 40). Further evidence in support of this proposal comes from the effects of caffeine and ryanodine on the slope of the staircase response. This was most evident in the presence of ryanodine, which unlike caffeine, does not inhibit uptake of Ca^{2+} by the SR (Bers et al, 1987; 1989; Bouchard et al, 1989). In low concentrations, ryanodine dramatically accelerates the loss of cellular Ca^{2+} (Hilgemann et al, 1984; Bers and MacLeod, 1986; Hilgemann, 1986; Bers et al, 1987) by binding to the SR Ca^{2+} -release channel complex (Pessah et al, 1985; Smith et al, 1985; Inui et al, 1987; Lai et al, 1987) and converting the \sim 96 pS Ca^{2+} -release channel into an open subconducting 56 pS state (Rousseau et al, 1987; Ashley and Williams, 1990). The effect of this cascade on intact muscle performance is to effectively by-pass the relatively slow rate of recirculation, making Ca^{2+} available for release in response to rhythmic stimulation in a manner inversely related to the interval between beats (Sutko and Willerson, 1980; Bers et al, 1987; 1989; Bose et al; 1988a; Bouchard et al, 1989). On the other hand, caffeine significantly inhibits uptake of Ca^{2+} by the SR (Weber and Herz, 1968; Fuchs, 1969) in addition to converting the Ca^{2+} -release channel complex into a fully open conducting state (Rousseau and Meissner, 1989). As a result, buffering of intracellular [free Ca^{2+}] is effectively abolished in the presence of caffeine (Bridge, 1986; Bers et al, 1989; Hryshko et al, 1989a,c; Bouchard et al, 1989), which may explain the rate-dependent increase of resting tension in the caffeine, but not ryanodine, experiments. As discussed by Bers (1987) an increase of resting intracellular [free Ca^{2+}] above a basal level of 50-100 nM, when added to a constant increment of Ca^{2+} during stimulation, can contribute significantly to the rise of intracellular [free Ca^{2+}] during contraction. This effect would be substantially enhanced during high-frequency stimulation in the presence of either caffeine or ryanodine due to (a) the reduced ability of the SR to retain its load of Ca^{2+} during

the diastolic interval, (b) the inhibition of SR Ca^{2+} uptake in the case of caffeine, or (c) the inhibition of myoplasmic Ca^{2+} extrusion by the Na^+ - Ca^{2+} exchange process. The gradual increase in the strength of contraction as the rate of stimulation is enhanced in this setting may thus reflect the amount of Ca^{2+} normally sequestered into the SR during the diastolic interval, particularly so in the presence of caffeine which abolishes steady-state rapid cooling contractures.

Contribution of sarcolemmal Na^+ - Ca^{2+} exchange: The data reported here suggest that, in addition to alterations in the amount of Ca^{2+} released from intracellular stores (e.g., Figs. 24 and 25), that a sarcolemmal-related $[\text{Na}^+]_o$ -dependent process is responsible for a large portion of the rise of intracellular [free Ca^{2+}] during high-frequency stimulation. Uncoupling of contraction amplitude from the level of SR Ca^{2+} loading, such as that induced by lowering $[\text{Na}^+]_o$ in canine ventricle (Figs. 28 and 29), has been demonstrated previously only in cells exposed to conditions favouring intracellular Ca^{2+} -overload (Fabiato, 1972; 1975; Stern et al, 1983; Allen et al, 1985; Bers and Bridge, 1988; Kort and Lakatta, 1988b), or in rat ventricular muscle bathed in normal physiological solutions (Bouchard and Bose, 1989). To fully understand the mechanism by which Na^+ - Ca^{2+} exchange helps mediate the rise of intracellular [free Ca^{2+}] during contraction, it is necessary to understand the apparent uncoupling of contractile strength from SR Ca^{2+} availability in low- $[\text{Na}^+]_o$ solution. The purpose of the experiments described in Figs. 31 through 40 was to determine whether the exchanger might be affecting contraction during alterations of stimulus frequency either in a direct fashion (Rich et al, 1989; Langer et al, 1989), or indirectly, through an intermediary cellular compartment such as the SR (Fabiato, 1985c; Leblanc and Hume, 1990).

Data from experiments designed to estimate the recirculating volume of Ca^{2+} available for release in perfusion solutions with different ionic composition indicate clearly that a slowed rate constant for Ca^{2+} transport within the SR is not limiting contraction at higher rates of stimulation. Were this the case, postrest contractions would have restituted either more slowly or to a smaller peak (Allen et al, 1976; Schouten et al, 1987; Stern et al, 1988). As discussed above, enhanced restitution of contraction at higher rates of stimulation in the absence of accompanying alterations

of SR Ca^{2+} (e.g., Fig. 28) probably reflects the added component of Ca^{2+} uptake due to inhibition of the outward mode of the exchanger during the extended diastolic interval.

Comparative data obtained from experiments on Type I and Type II muscles suggests that although contraction amplitude is proportional to the release of Ca^{2+} from intracellular stores in normal (Figs. 24 and 25) or low $[\text{Na}^+]_o$ solutions (Fig. 40), the negative staircase of contraction elicited in 70 mM $[\text{Na}^+]_o$ does not reflect the absolute buffering capacity of the SR for Ca^{2+} . Otherwise, those muscles displaying poor postrest contractions (Type IIb) should have produced a contractile staircase with a slope which was greater than those preparations yielding strong postrest contractions (Type I, Type IIa). This did not occur, however, as all three muscle subtypes produced contraction staircases with nearly identical slopes following partial replacement of $[\text{Na}^+]_o$, as well as steady-state cooling contractures which were uniformly insensitive to stimulus frequency, and mechanical restitution curves which were incompatible with Ca^{2+} transport rates limiting steady-state contraction.

The final possibility tested for the rate-dependent uncoupling of steady-state contraction from apparent SR Ca^{2+} availability was an inhibition of Ca^{2+} release due to Ca^{2+} -induced inactivation of the Ca^{2+} -release channel. This was a concern in the present experiments for two reasons. First, because of the previous demonstration in skinned canine cardiac Purkinje fibres by Fabiato (1985b) that an increase of bulk $[\text{free Ca}^{2+}]$ surrounding the SR beyond a pCa of 5.6 (5-10 ms integration time) clearly inhibited the magnitude of Ca^{2+} -induced release of Ca^{2+} . Secondly, we have found in past work on canine ventricular muscle that reducing the transmembrane driving force for Na^+ by either 50% reduction of $[\text{Na}^+]_o$ with equimolar sucrose substitution or application of cardioactive steroids results in increased diastolic myofilament oscillations in the 0.3-10 Hz range (Bose et al, 1987; 1988a,c), due to an increase in the frequency of spontaneous release of Ca^{2+} from the SR (Lappe and Lakatta, 1980; Lakatta and Lappe, 1981; Stern et al, 1983). Similar results have been obtained in rat ventricle with Li^+ substituting for Na^+ (Stern et al, 1983). We attempted to address this concern in the intact preparation by following the pattern of recovery for extrasystolic contractions under conditions expected to increase or decrease diastolic intracellular

[free Ca^{2+}]. Table 5 shows that the amplitudes of both steady-state contraction and steady-state cooling contracture were increased in a graded fashion as first $[\text{Ca}^{2+}]_o$ was increased from 1.25 to 2.5 mM, and then $[\text{Na}^+]_o$ reduced from 140 to 70 mM. As was illustrated in Figs. 32 and 37 for 140mM $[\text{Na}^+]_o/2.5\text{mM } [\text{Ca}^{2+}]_o$ solutions, the rate of Ca^{2+} recirculation within the longitudinal tubules of the SR (and hence, the leak of Ca^{2+} from the release compartment into the myoplasm) is increased in response to those conditions which augment steady-state cooling contracture amplitude. Because the magnitude of steady-state cooling contracture and accompanying contractions were enhanced moving from one test solution to the next, the intracellular free $[\text{Ca}^{2+}]$ within the relatively restricted volume of the t-tubule would also be expected to increase to some degree. However, with the exception of 140mM $[\text{Na}^+]_o/2.5\text{mM } [\text{Ca}^{2+}]_o$ solution at 0.5 Hz stimulation, increasing the intracellular Ca^{2+} load within a given basic cycle length reduced the time constant for the exponential recovery of ES contractions, opposite to the result predicted by the Ca^{2+} -inactivation hypothesis. Alternatively, increasing the rate of stimulation within a given treatment solution resulted in a graded decrease in the recovery constant in all three perfusion solutions, regardless of the directional change of steady-state contraction amplitude. Evidence in favour of the Ca^{2+} -inactivation hypothesis comes from the loss of significance of this trend after switching from the 140mM $[\text{Na}^+]_o/2.5\text{mM } [\text{Ca}^{2+}]_o$ solution to one containing 70mM $[\text{Na}^+]_o/2.5\text{mM } [\text{Ca}^{2+}]_o$. There remains the possibility that this trend was affected by the significant reduction of the excitation threshold for contraction in low- $[\text{Na}^+]_o$ solution (200 ms), compared with the 140mM $[\text{Na}^+]_o/2.5\text{mM } [\text{Ca}^{2+}]_o$ solution (150 ms). If so, this would be expected to significantly slow the recovery of contraction throughout the range of test intervals studied. That significance was demonstrated only for the 200 ms coupling interval (Fig. 34) suggests that the small (~ 5 mV) hyperpolarization of the membrane (Sheu and Fozzard, 1982) and depressed overshoot and upstroke velocity of the action potential (Hume and Uehara, 1985) expected with partial replacement of $[\text{Na}^+]_o$ may have combined to depress membrane excitability enough to account for this shift. Studies of the envelope of I_{Ca} -overshoot during premature excitations in canine ventricular myocytes at similar coupling intervals to those used in our experiments (Tseng,

1988) indicate that overshoot of trigger I_{Ca} peaks at ~ 100 ms following repolarization to -80 mV. Thus, a sufficient delay may have passed by 200 ms in the intact preparation bathed in 70 mM $[Na^+]_o$ for the increase of peak I_{Ca} to overcome depressed membrane excitability. This proposal is supported by the trend towards faster recovery of ES contractions in 70mM $[Na^+]_o/2.5$ mM $[Ca^{2+}]_o$ solution at basic cycle lengths of 1000 and 668 ms, despite the significant decrease of contraction amplitude at all three cycle lengths at the 200 ms coupling interval. Although we can not rule out for certain whether or not Ca^{2+} release is inhibited by a build-up of Ca^{2+} within the restricted cell volume of the t-tubule with this particular stimulation protocol, the bulk of evidence suggests that this may not be the case.

In the absence of strong evidence in support of reduced or inhibited Ca^{2+} release from the SR in Na^+ -deficient solutions, possible alterations of E_{NaCa} during the action potential may help to explain the differential sensitivity of steady-state contraction and rapid cooling contracture during alterations of stimulation frequency. As discussed above, calculation of E_{NaCa} ($E_{NaCa} = 3E_{Na} - 2E_{Ca}$) for muscles bathed in 140 mM $[Na^+]_o$ is -12.4 mV, when a_{Na}^i , a_{Ca}^i , a_{Na}^o and a_{Ca}^o are 6 mM, 150 nM, 110 mM and 1.45 mM, respectively. Reducing $[Na^+]_o$ to 70 mM was calculated to hyperpolarize E_{NaCa} by ~ 15 mV to -37 mV. To explain the lack of correlation between SR Ca^{2+} availability and contractile strength it is necessary to invoke a second, independent, cellular compartment from which Ca^{2+} is either derived for contraction, or can otherwise influence the release of Ca^{2+} from the SR. Leblanc and Hume (1990) have demonstrated that the kinetics of the outward mode of the exchanger ($E_{NaCa} > E_m$) are indeed fast enough to significantly influence the magnitude of Ca^{2+} release from the SR in guinea-pig ventricular myocytes. This observation may have important implications to our results, due to the strong dependence of canine ventricular muscle on intracellular release of Ca^{2+} for contraction compared with other mammalian species (Fabiato, 1981; 1982; Bers, 1985; Stern et al, 1988; Bers, 1989). Accordingly, increasing the frequency of stimulation in 140 mM $[Na^+]_o$ results in a graded increase of both steady-state contraction and cooling contracture, which would be expected to elevate the level of diastolic intracellular [free Ca^{2+}] (Lee and Clusin, 1987; Lee et al, 1987). Assuming a minimal increase of

diastolic a_{Ca}^i from 150 to 200 nM at 1.5 Hz stimulation, and an accompanying 30-35% increase of a_{Na}^i to ~8 mM (Cohen et al, 1982; Boyett et al, 1987; Wang et al, 1988), the value calculated for E_{NaCa} is equal to -26.7 mV. In the absence of stimulation, this constitutes a 115% increase of E_{NaCa} . In comparison, enhancing the rate of stimulation from 0.5 to 1.5 Hz in 70 mM $[Na^+]_o$ would result in a modest hyperpolarization of E_{NaCa} ; the degree to which would depend on the value chosen for a_{Ca}^i . If diastolic intracellular [free Ca^{2+}] does not change following an increase in frequency from 0.5 to 1.5 Hz (e.g., steady-state contraction decreases and cooling contracture remains unaltered) and a_{Na}^i is increased by 25% to 6.25 mM, E_{NaCa} will be equal to -55 mV. Alternatively, if a_{Ca}^i is increased by as much as 25%, E_{NaCa} will increase only 30% to -50 mV. Thus, increasing the rate of stimulation in solutions containing 70 mM $[Na^+]_o$ results in a 30-45% increase in the calculated E_{NaCa} value, compared to the 115% increase of this term in 140 mM $[Na^+]_o$. A problem with this interpretation is that opposite changes qualitatively occur in the calculated reversal potential for the exchanger and the strength of contraction in low- $[Na^+]_o$ solution, compared with the similar directional changes either going from 140 mM to 70 mM $[Na^+]_o$ or increasing the frequency of stimulation in the presence of 140 mM $[Na^+]_o$. As demonstrated both mathematically (Mullins, 1979; 1981; Noble, 1986; Difrancesco and Noble, 1985; Eisner and Lederer, 1985; Hilgemann and Noble, 1987) and experimentally (Kimura et al, 1986; Mechmann and Pott, 1986; Hume and Uehara, 1986a,b; Beukelmann and Wier, 1989; Egan et al, 1989; Miura and Kimura, 1989), the direction of thermodynamic equilibrium for the exchange process described by the reversal potential depends on the distribution of both ions across the cell membrane, and not the exchange rate, nor its kinetics at a given time or voltage. Hence, shifts in the reversal potential can not be compared quantitatively with the accompanying alterations of contractile strength. Despite this, however, theoretical considerations of this nature do raise the possibility that quantitative differences in transsarcolemmal Na^+ and Ca^{2+} movements during the action potential may affect contraction either by directly altering the release of Ca^{2+} from intracellular stores as shown by Leblanc and Hume (1990), or indirectly, perhaps by Ca^{2+}

accumulating at the inner surface of the membrane and effecting a back gradient in the direction of the myofilaments.

A final note regarding the applicability of results obtained with the ES/PES protocol described in Figs. 32-34 to the differential effect of $[\text{Na}^+]_o$ -withdrawal on steady-state contraction and steady-state cooling contracture, is the possibility that lowering $[\text{Na}^+]_o$ with Li^+ exerts an influence on cell function separate from that observed when external Na^+ is replaced with sucrose. The data shown in Fig. 40 demonstrates that reduction of $[\text{Na}^+]_o$ with sucrose (or choline chloride), while still abolishing the negative staircase, does not convert it into a negative direction as was observed with Li^+ substitution. One possibility is that Ca^{2+} binding to intracellular sites may be favoured by sucrose-compared with Li^+ -substitution, as demonstrated previously in guinea-pig taenia coli (Brading et al, 1980). An increase of intracellular Ca^{2+} in this manner may help to overcome, or perhaps even oppose, the result of inhibited sarcolemmal $\text{Na}^+ \text{-Ca}^{2+}$ exchange. An alternate possibility, is that Li^+ may act in some unknown fashion to depress contraction in a frequency-dependent manner. Matsuoka, Noma and Powell (1990) have shown that, by itself, complete removal of $[\text{Na}^+]_o$ by replacement with $[\text{Li}^+]_o$ did not significantly affect peak I_{Ca} in guinea-pig ventricular cells. However, they also found evidence to suggest that the potentiating effect of intracellular cAMP on both I_{Ca} and epinephrine-induced inward creep (chloride) currents could be markedly reduced by replacing $[\text{Na}^+]_o$ with $[\text{Li}^+]_o$, which the authors suggested may have been related to Li^+ -inhibition of adenylate cyclase or GTP-binding protein. Similar observations have been made with respect to the frequency-dependence of I_{Ca} in frog ventricular myocytes, where increasing the rate of stimulation from a holding potential of -40 mV markedly reduces the amplitude of peak I_{Ca} (Schouten and Morad, 1990; see also Noble and Shimoni, 1981). This effect was abolished by increasing the holding potential to -90 mV, and reversed by microinjection of cAMP (25 μM) into the cell. The observation that this effect could be mimicked by inhibition of phosphodiesterase activity (theophylline 0.5 mM) suggests that gating of the Ca^{2+} channel may be regulated during variation of stimulus frequency by accompanying alterations of cellular cAMP content and breakdown. Although it is unknown to what degree such a mechanism may be

involved in intact tissues, these results do offer a possible explanation for the discrepancy between the amount of Ca^{2+} presumably available for release and the graded decrease of contractile strength at higher rates of stimulation in 70 mM $[\text{Na}^+]_o$.

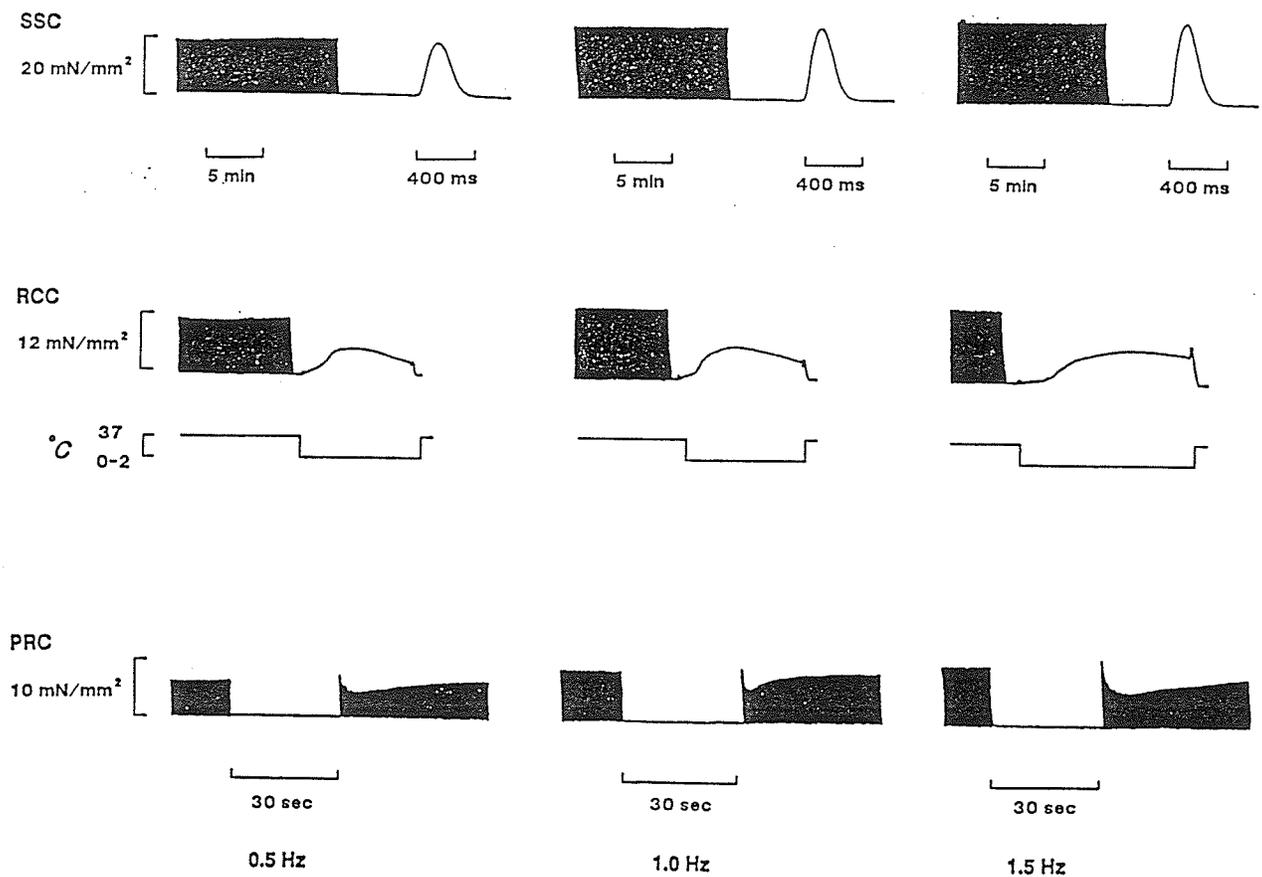


Figure 24. Effect of stimulation frequency on steady-state contraction (SSC), steady-state rapid cooling contracture (RCC) and postrest contraction (PRC) in canine ventricular muscle. Recordings are from 3 different muscles perfused with solutions containing 140 mM $[Na^+]_o$ /2.5 mM $[Ca^{2+}]_o$.

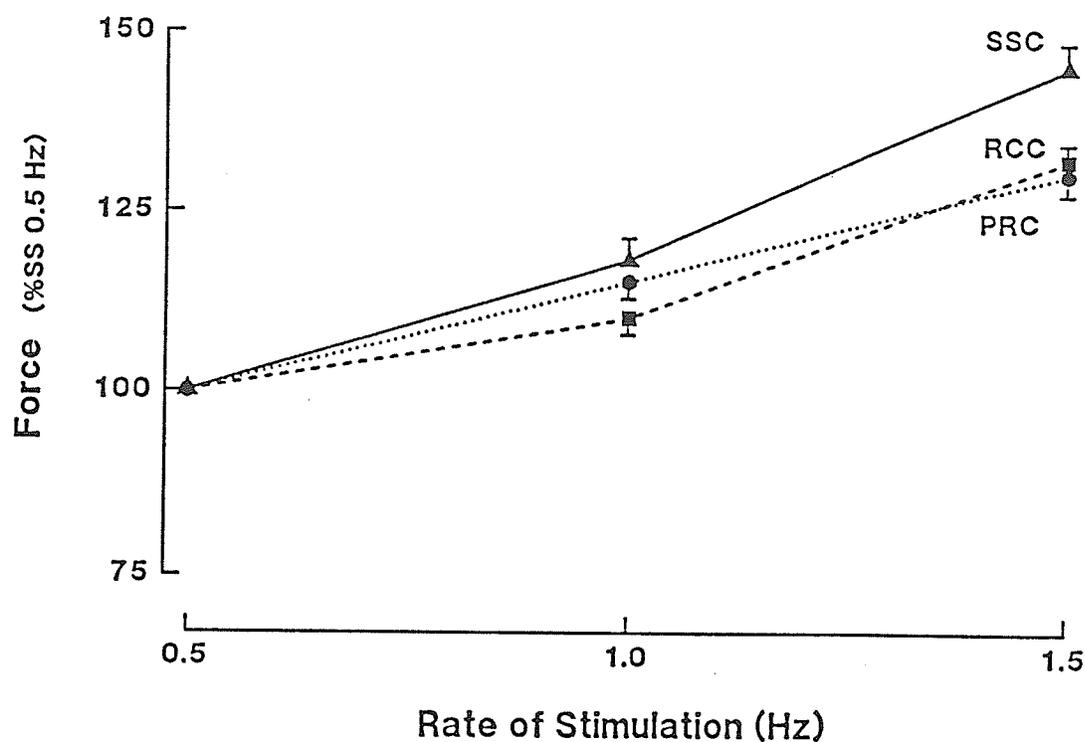


Figure 25. Averaged data from 10 preparations illustrating the frequency-dependence of steady-state contraction, steady-state rapid cooling contracture and postrest contraction in canine ventricle. Following an increase in the rate of stimulation from 0.5 Hz to 1.0 and 1.5 Hz, steady-state contraction was increased to $18 \pm 4\%$ and $44 \pm 8\%$, while the equivalent steady-state cooling contractures and postrest contractions were increased to $10 \pm 3\%$ and $32 \pm 5\%$, and $15 \pm 8\%$ and $30 \pm 5\%$ at 1.0 and 1.5 Hz, respectively. Analysis of variance (ANOVA) indicated that the increase of steady-state contraction was significantly greater ($P < 0.05$) than the accompanying increase of either steady-state cooling contracture or postrest contraction.

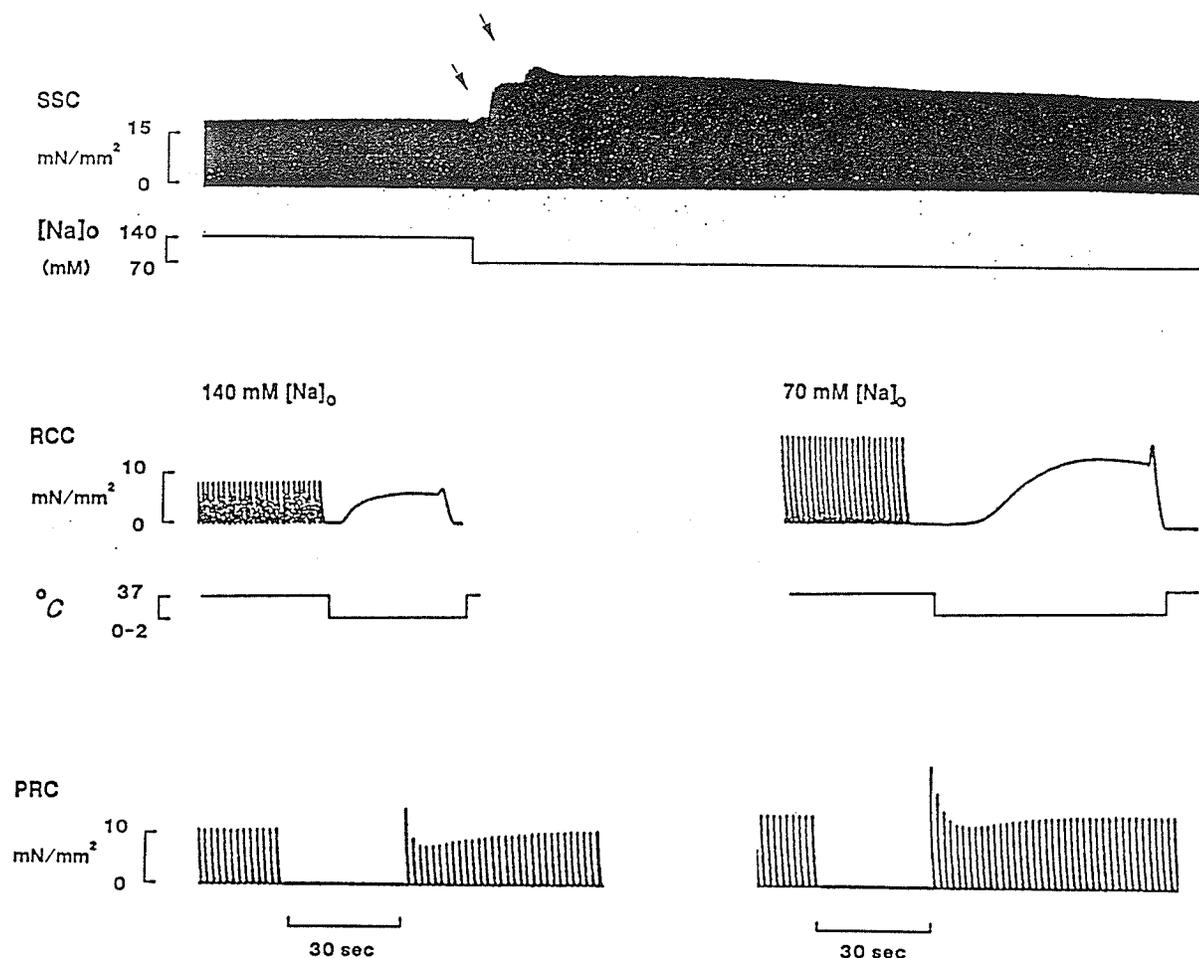


Figure 26. Effect of lowering $[\text{Na}^+]_o$ from 140 to 70 mM on developed tension in response to electrical stimulation and rapid cooling in canine ventricular muscle. Top panel illustrates the effect of $[\text{Na}^+]_o$ -depletion on peak developed and resting tension for a preparation stimulated at 0.5 Hz. Arrows indicate the times at which the perfusion solution was replaced with fresh Li^+ -containing solution (50% substitution). The effect of $[\text{Na}^+]_o$ -depletion on peak rapid cooling contracture (middle panel) and postrest contraction amplitude (bottom panel) are also shown. The mean increase of steady-state contraction, steady-state cooling contracture and postrest contraction was $91.5 \pm 4\%$, $90 \pm 7\%$ and $110 \pm 8\%$, respectively.

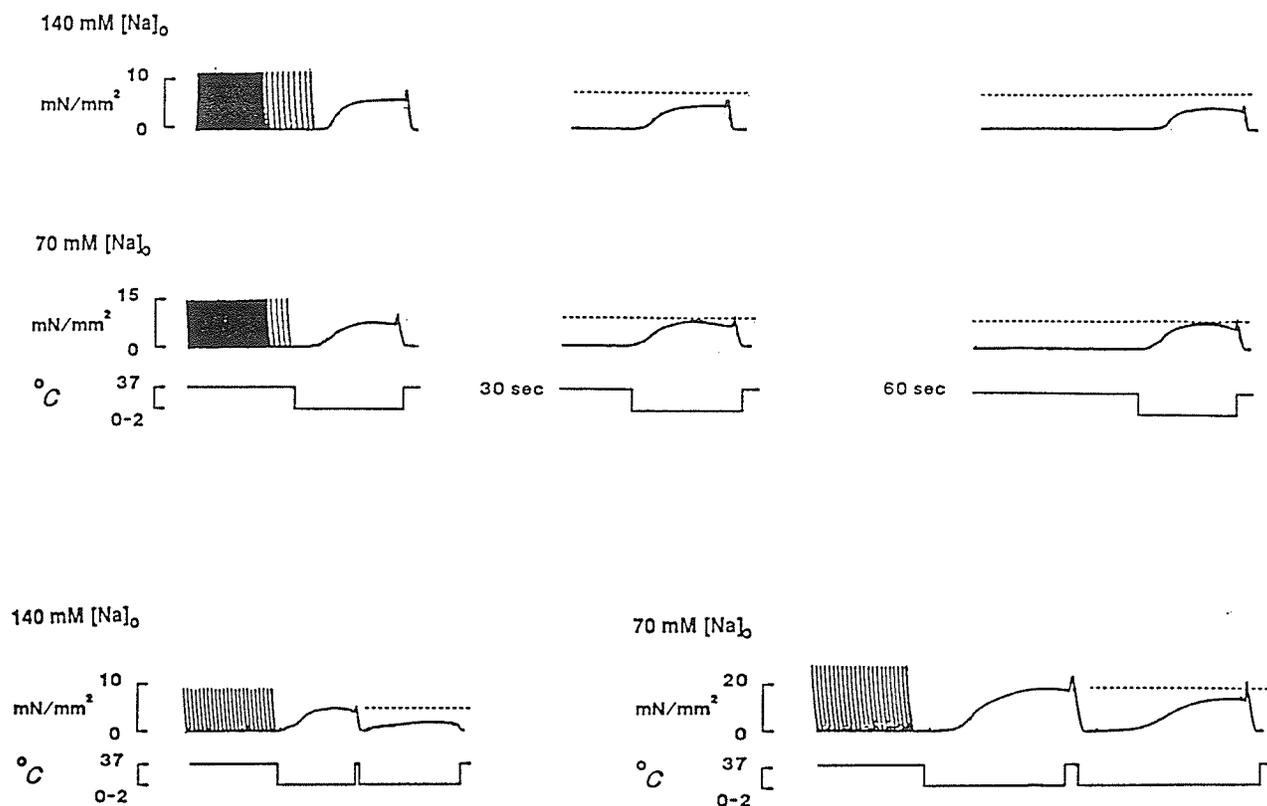


Figure 27. Effect of lowering $[Na^+]_o$ from 140 to 70 mM on myoplasmic Ca^{2+} extrusion by sarcolemmal Na^+-Ca^{2+} exchange in canine ventricular muscle. *A)* Upper Panel: termination of rhythmic stimulation at 0.5 Hz in 140 mM $[Na^+]_o$ leads to a time-dependent loss of cell Ca^{2+} seen as a decay of peak cooling contracture amplitude. Lower Panel: Inhibition of rest-decay following reduction of $[Na^+]_o$ to 70 mM. *B)* Paired cooling protocol. Lowering $[Na^+]_o$ to 70 mM results in a decrease in the ability of the preparation to extrude Ca^{2+} during the process of rewarming from 0-2°C back to 37°C.

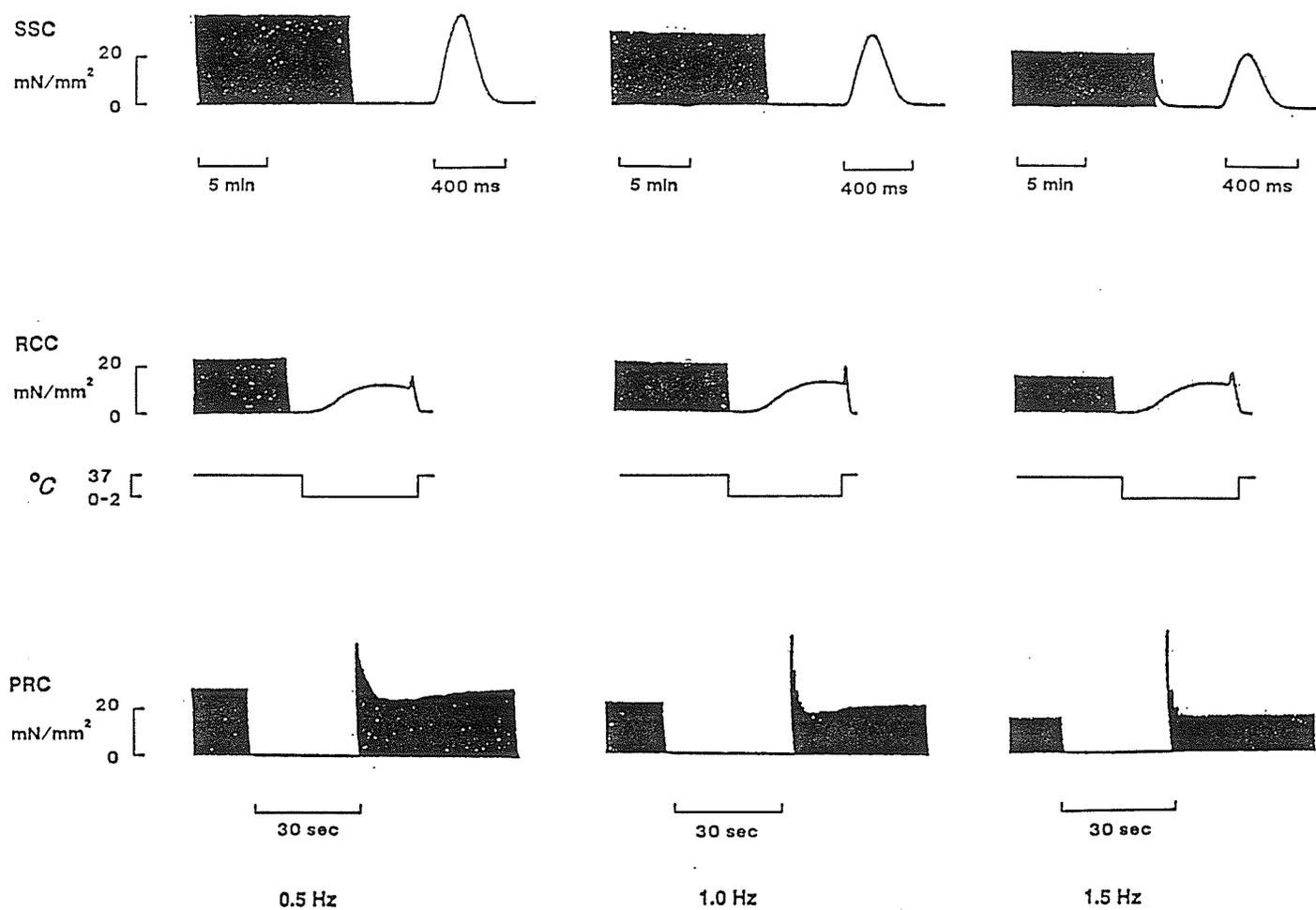


Figure 28. Effect of stimulation frequency on steady-state contraction (SSC), steady-state rapid cooling contracture (RCC) and postrest contraction (PRC) in canine ventricular muscle following the reduction of $[Na^+]_o$ from 140 mM to 70 mM. Recordings were obtained from 3 different muscles perfused with solutions containing 2.5 mM $[Ca^{2+}]_o$.

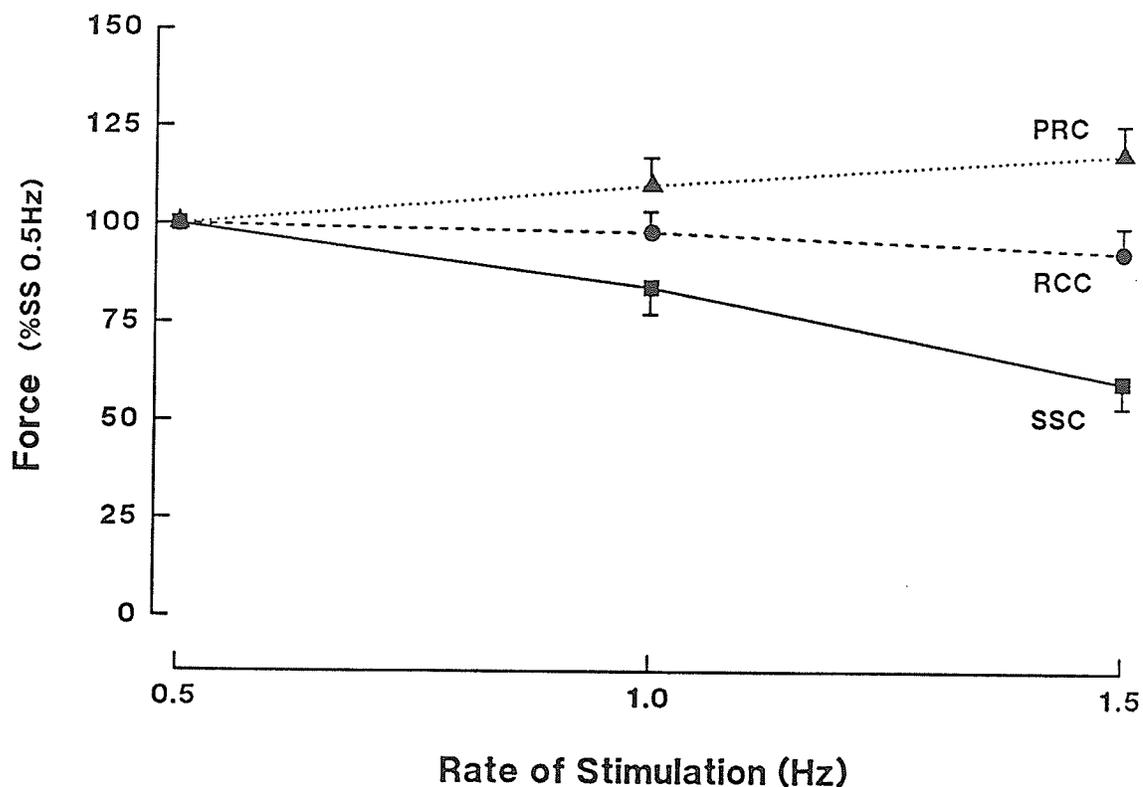
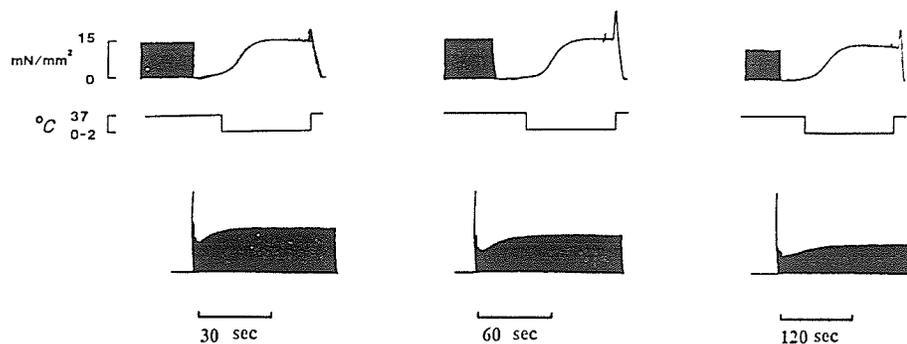


Figure 29. Averaged data from 10 canine ventricular preparations illustrating the frequency-dependence of steady-state contraction, steady-state rapid cooling contracture and postrest contraction in 70 mM $[Na^+]_o$. Increasing the frequency of stimulation from 0.5 to 1.0 and 1.5 Hz significantly depressed the strength of steady-state contraction by $16 \pm 1.5\%$ and $36 \pm 3\%$. Steady-state cooling contracture was reduced by $2 \pm 5\%$ and $7 \pm 10\%$ at similar rates of stimulation, while postrest contraction was increased significantly by $10 \pm 6\%$ and $18 \pm 2\%$, respectively.

TYPE IIa



TYPE IIb

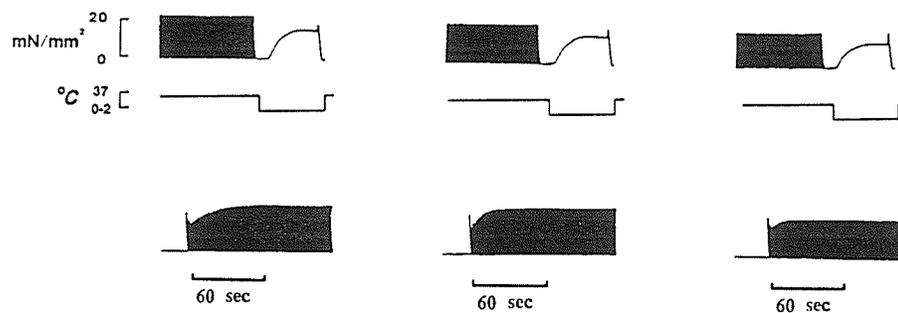


Figure 30. Tissue heterogeneity in the contractile properties of isolated canine ventricle bathed in 140 mM $[Na^+]_o$. Top Panel: Example of a Type IIa control muscle displaying strong postrest contraction and a negative staircase in the presence of 140 mM $[Na^+]_o$. Bottom Panel: Representative Type IIb control muscle displaying poor postrest contraction and a negative staircase in 140 mM $[Na^+]_o$.

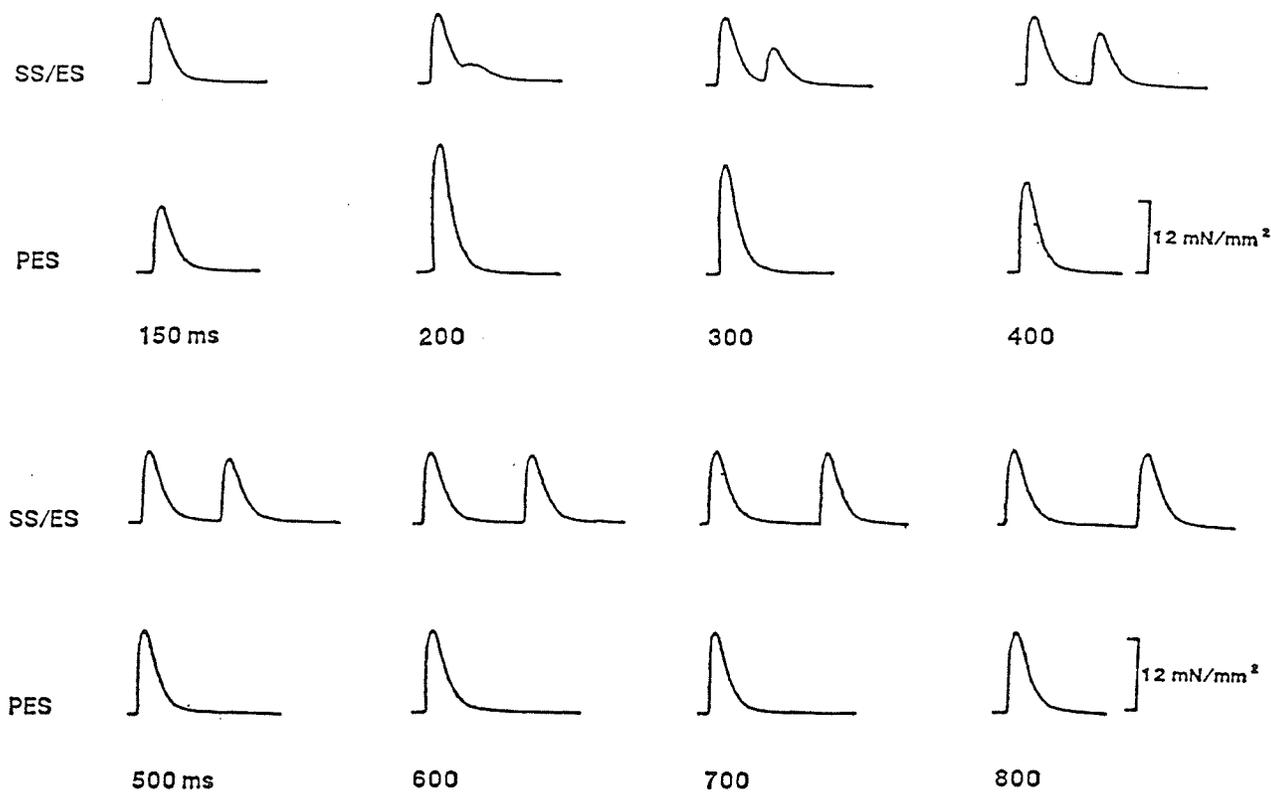


Figure 31. Effect of premature stimulation on contraction of canine ventricular muscle. Typical response of muscles bathed in solutions containing 140 mM $[Na^+]_o$ /2.5 mM $[Ca^{2+}]_o$ to premature stimulation at coupling intervals ranging from 150-800 ms. Basic cycle length of contractions preceding the ES/PES pair was 2000 ms, and the time constant for full recovery of the extrasystolic contraction was 550 ms.

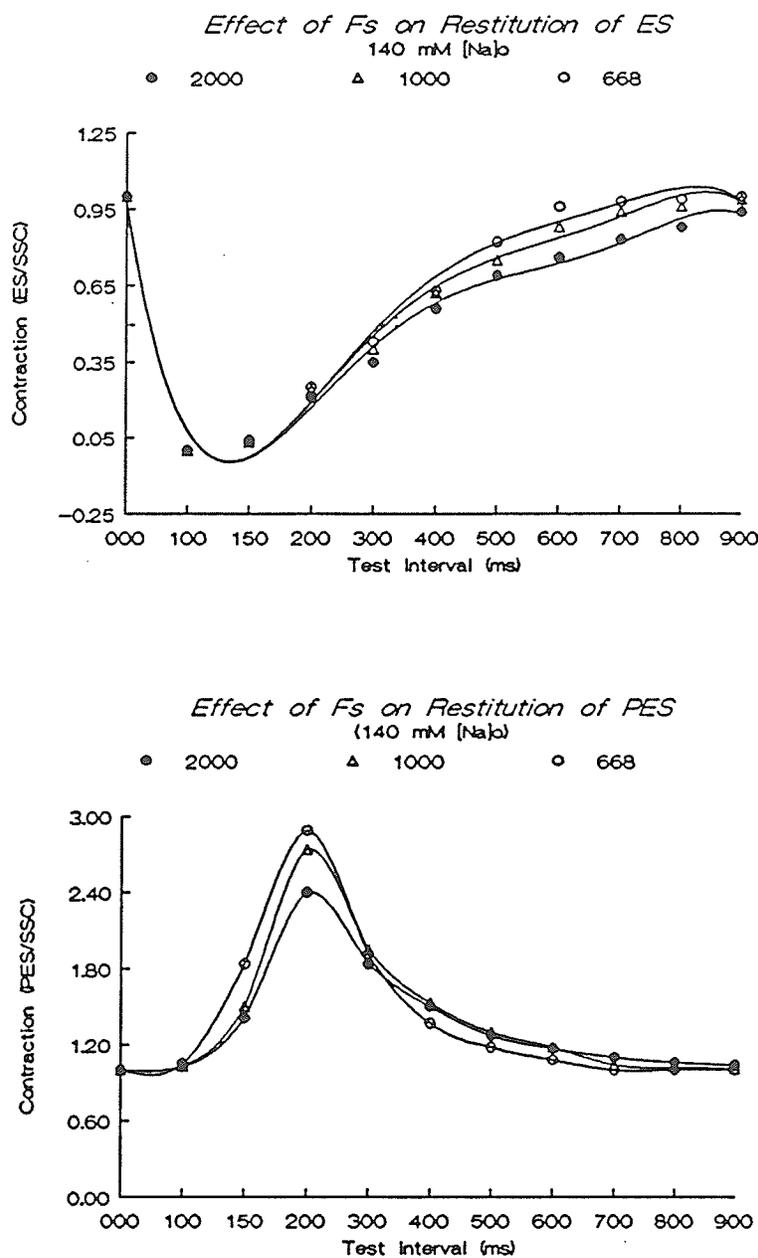


Figure 32. Averaged effect of altered frequency of stimulation on extrasystolic and post-extrasystolic contraction in 140 mM $[Na^+]_o/2.5$ mM $[Ca^{2+}]_o$. Top Panel: Recovery curves for extrasystolic contractions obtained at basic cycle lengths corresponding to steady-state stimulation at 0.5 Hz (2000 ms), 1.0 Hz (1000 ms) and 1.5 Hz (668 ms). Each point was obtained by dividing the amplitude of the ES contraction by the preceding steady-state contraction at individual coupling intervals. Bottom Panel: Effect of altering the basic cycle length on the restitution of the following post-extrasystolic contraction. Points were obtained by dividing the amplitude of the PES contraction by the amplitude of the final steady-state contraction preceding the ES/PES pair.

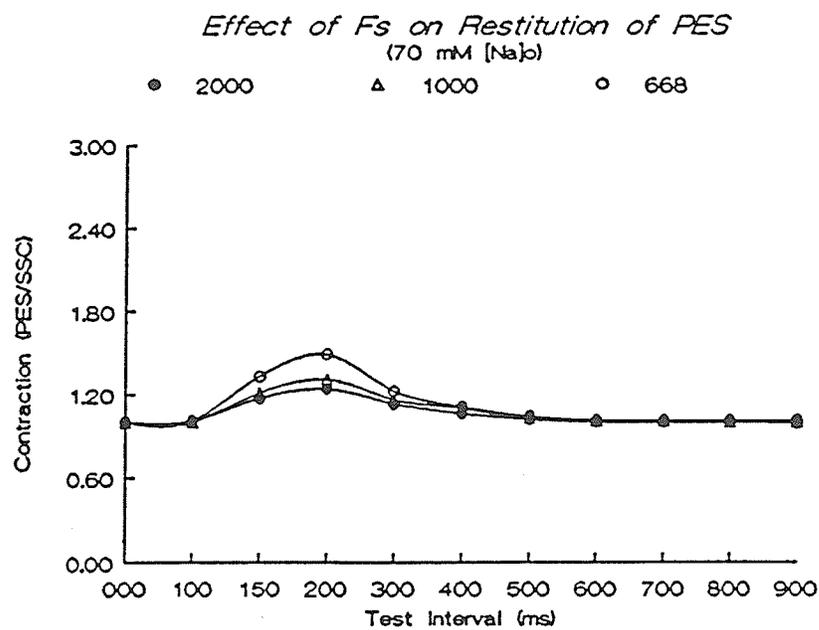
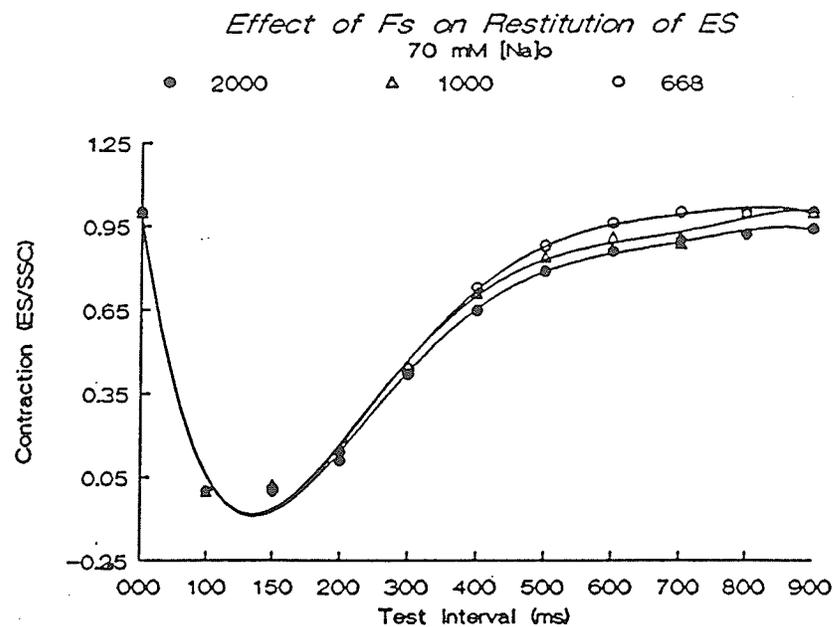


Figure 33. Averaged effect of altered frequency of stimulation on extrasystolic and post-extrasystolic contraction in 70 mM $[Na^+]_o/2.5$ mM $[Ca^{2+}]_o$. See Fig. 31 for details of stimulation protocol and analysis.

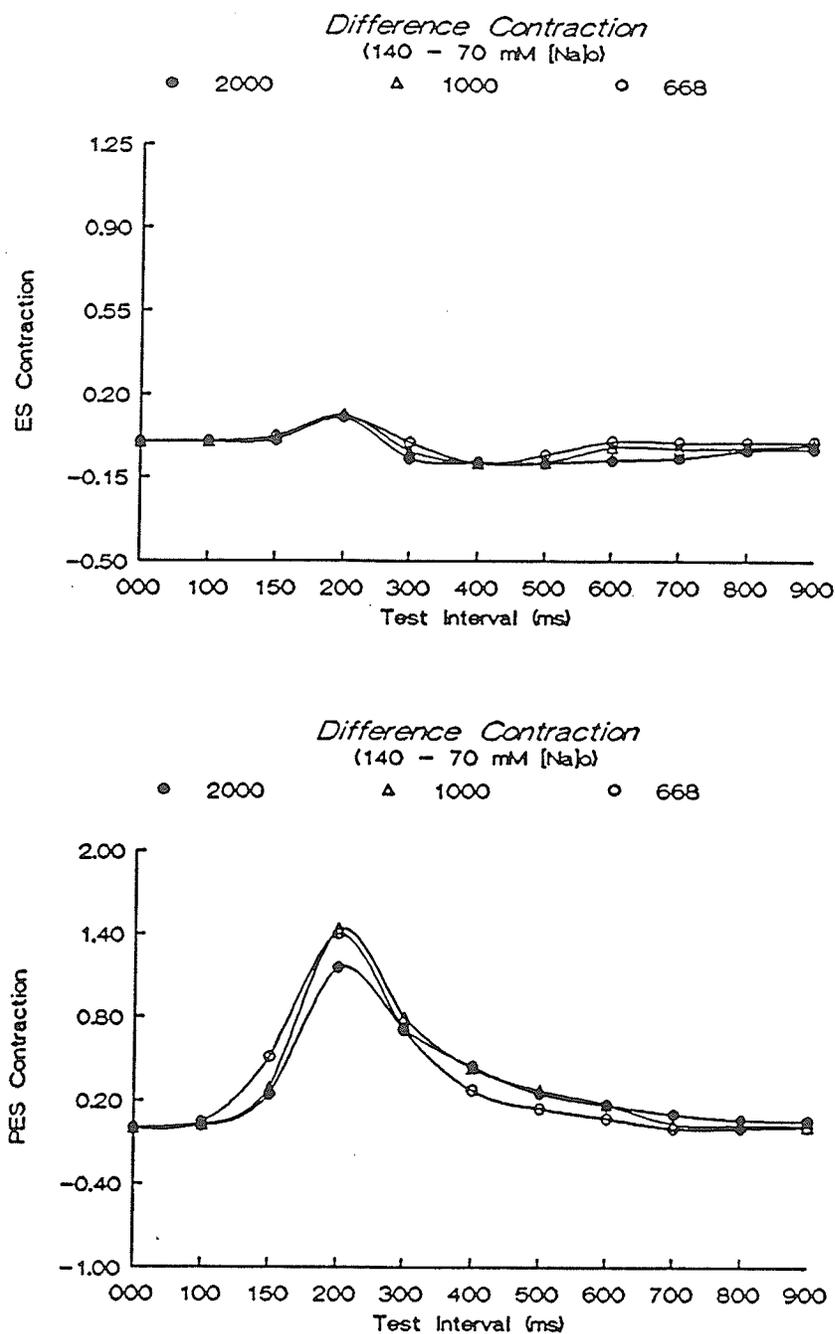


Figure 34. $[\text{Na}^+]_o$ -dependence of extrasystolic and post-extrasystolic contraction in response to altered stimulation frequency at constant $[\text{Ca}^{2+}]_o$. Difference contractions for ES (top panel) and PES contractions (bottom panel) were calculated by subtracting the response in 70 mM $[\text{Na}^+]_o$ from the equivalent response in 140 mM $[\text{Na}^+]_o$. Paired t-tests were then conducted at each coupling interval and basic cycle length to determine the points significantly different from unity ($P < 0.05$). See text for results of the statistical analysis.

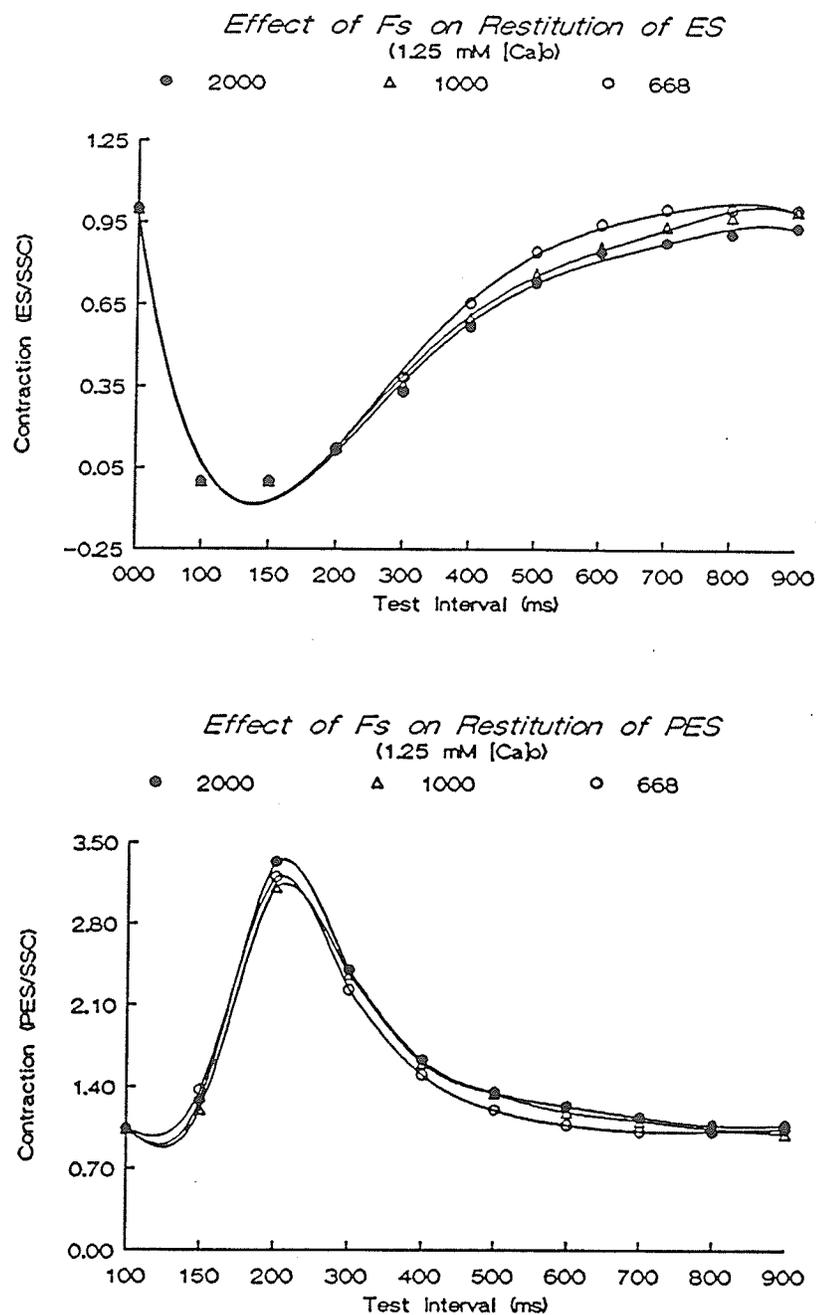


Figure 35. Effect of altered frequency of stimulation on extrasystolic and post-extrasystolic contraction in 140 mM $[Na^+]_o/1.25$ mM $[Ca^{2+}]_o$. Each point was obtained by dividing the amplitude of the ES contraction by the preceding steady-state contraction at individual coupling intervals. Bottom Panel: Effect of altering the basic cycle length on the restitution of the following post-extrasystolic contractions. Points were obtained by dividing the amplitude of the PES contraction by the amplitude of the final steady-state contraction preceding the ES/PES pair.

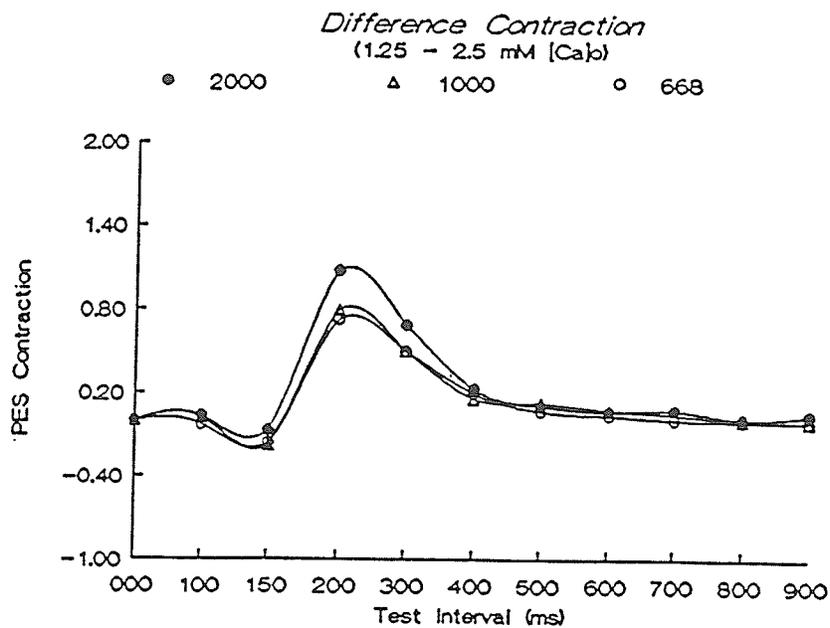
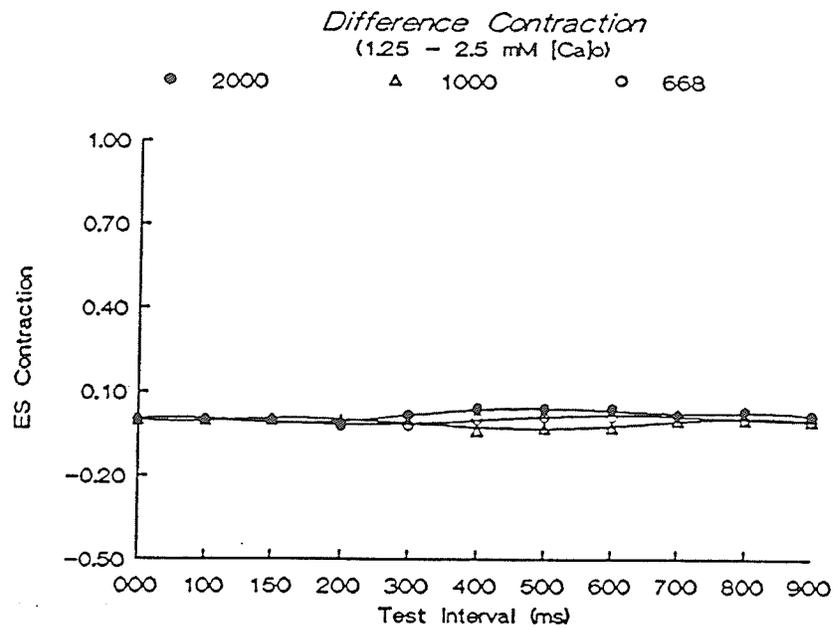


Figure 36. $[Ca^{2+}]_o$ -dependence of extrasystolic and post-extrasystolic contractions in response to altered stimulation frequency at constant $[Na^+]_o$. Protocols for data acquisition and analyses were identical to those in Fig. 33. See text for results of the statistical analysis.

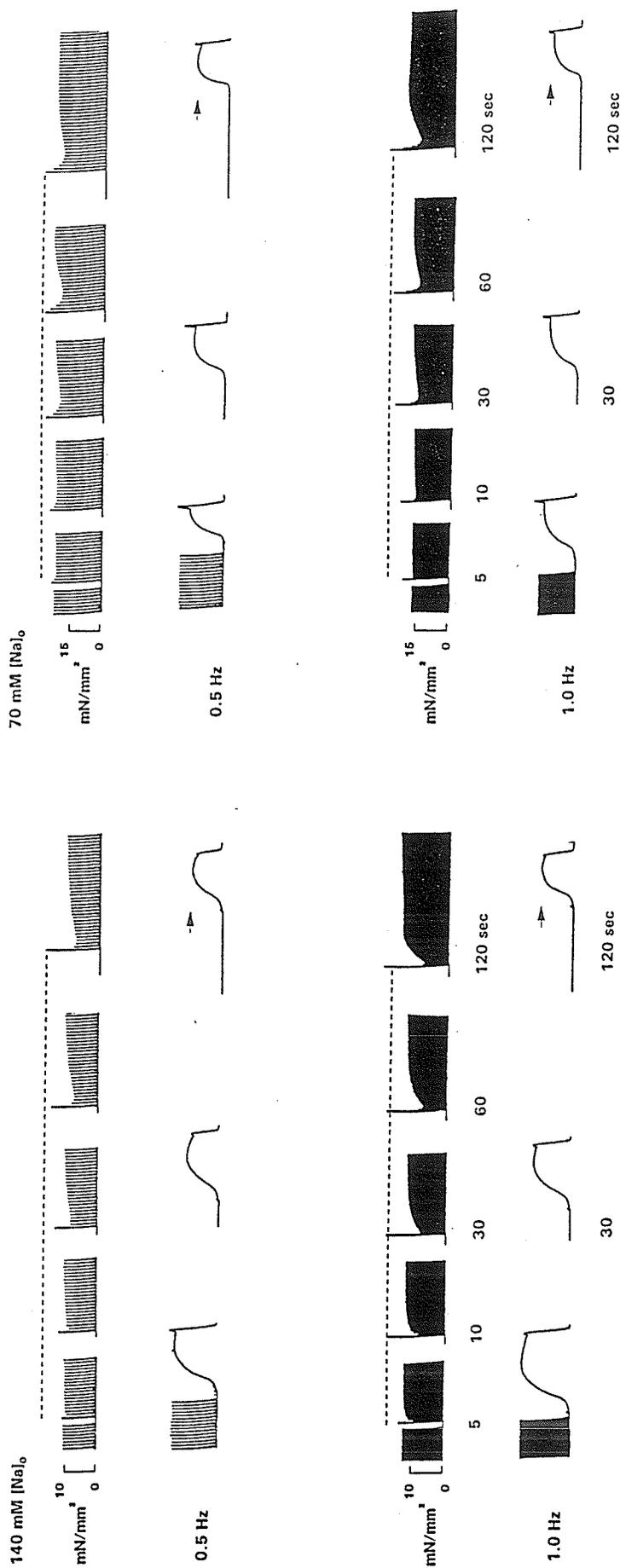


Figure 37. Frequency- and time-dependence of postrest contraction and postrest rapid cooling contracture in 140 and 70 mM [Na⁺]_o. Left Panel: Estimation of the recirculating fraction of Ca²⁺ available for release following stimulation at 0.5 Hz (top pair of recordings) and 1.0 Hz (bottom pair of recordings) in 140 mM [Na⁺]_o. Right Panel: Equivalent response after lowering [Na⁺]_o to 70 mM. Dashed lines in each panel indicate the peak of the restitution process within a given perfusion solution at 0.5 Hz. Arrows represent peak cooling contracture amplitude after 120-sec rest following stimulation at 0.5 Hz.

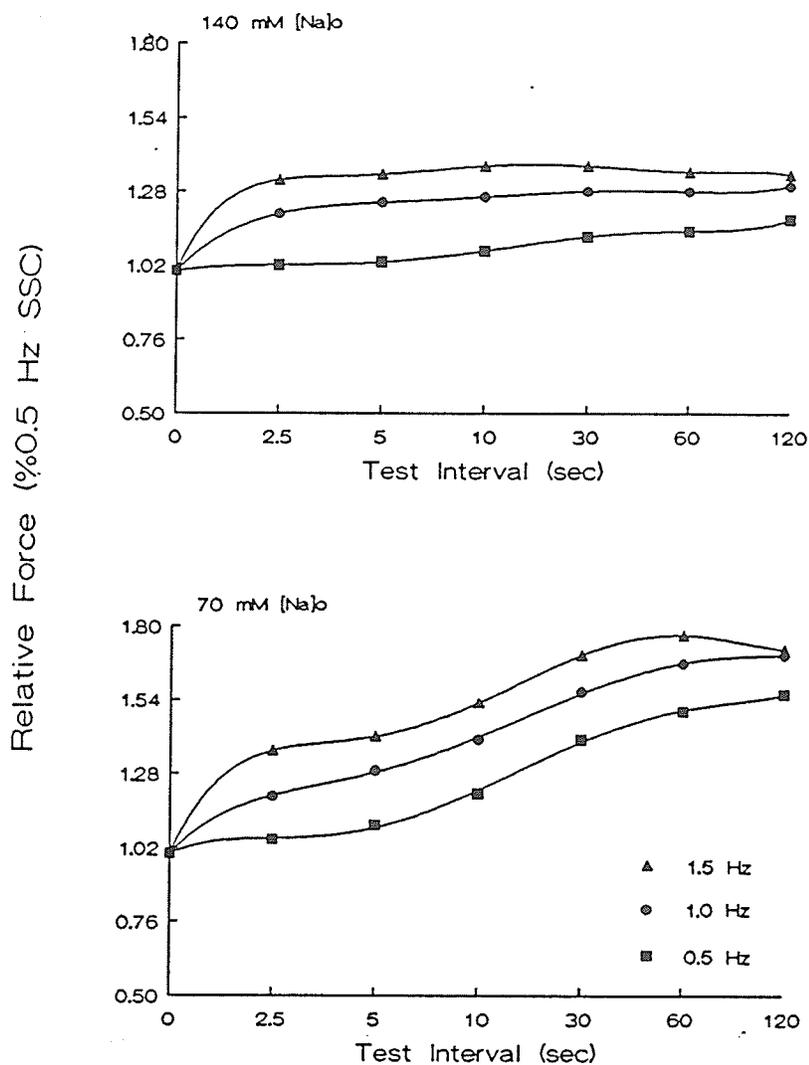
Restitution of Postrest Contraction

Figure 38. Effect of stimulation frequency on mechanical restitution in canine ventricular muscle bathed in 140 and 70 mM [Na]⁺_o. Restitution curves were constructed by plotting the amplitude of postrest contractions elicited at different rates of stimulation against steady-state contraction amplitude at 0.5 Hz in the presence of 140 mM [Na]⁺_o (top panel) and following reduction of [Na]⁺_o to 70 mM (bottom panel).

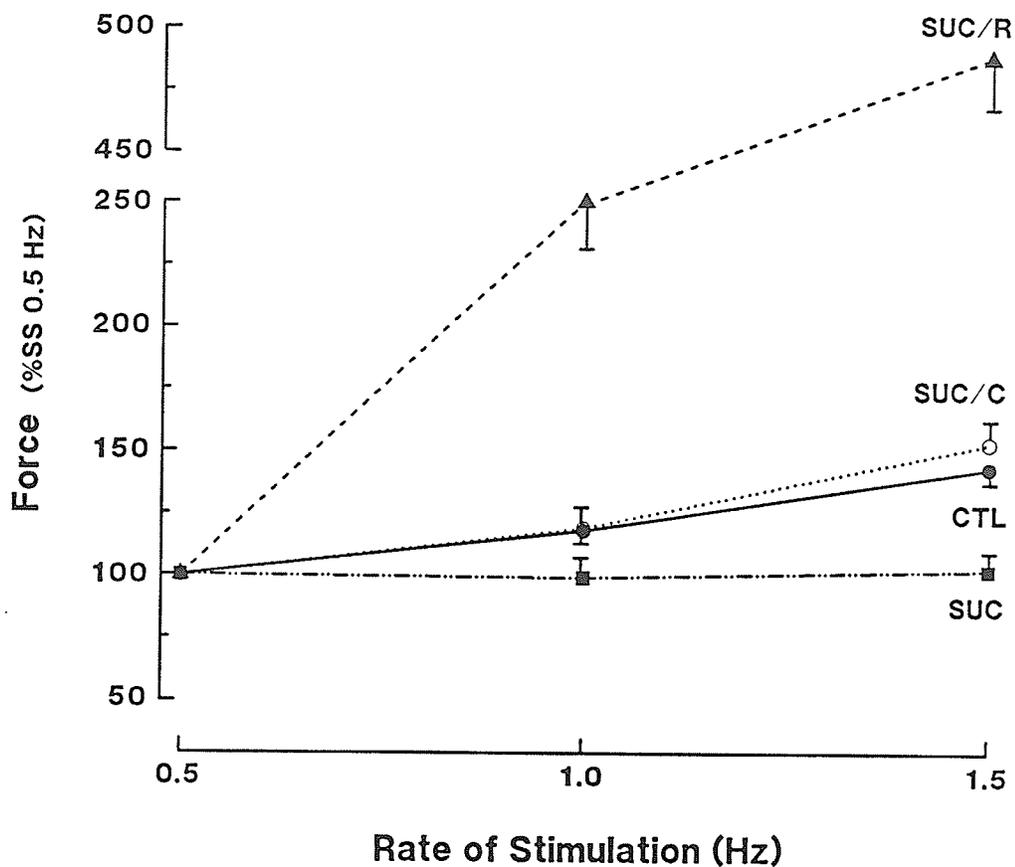


Figure 39. Effect of ryanodine and caffeine on the negative force staircase in $[\text{Na}^+]_o$ -depleted solutions. Following reduction of $[\text{Na}^+]_o$ to 70 mM by equimolar sucrose-substitution (SUC), subsequent addition of 3 mM caffeine (SUC/C) or 10 nM ryanodine (SUC/R) resulted in the restoration of the positive staircase of steady-state contraction.

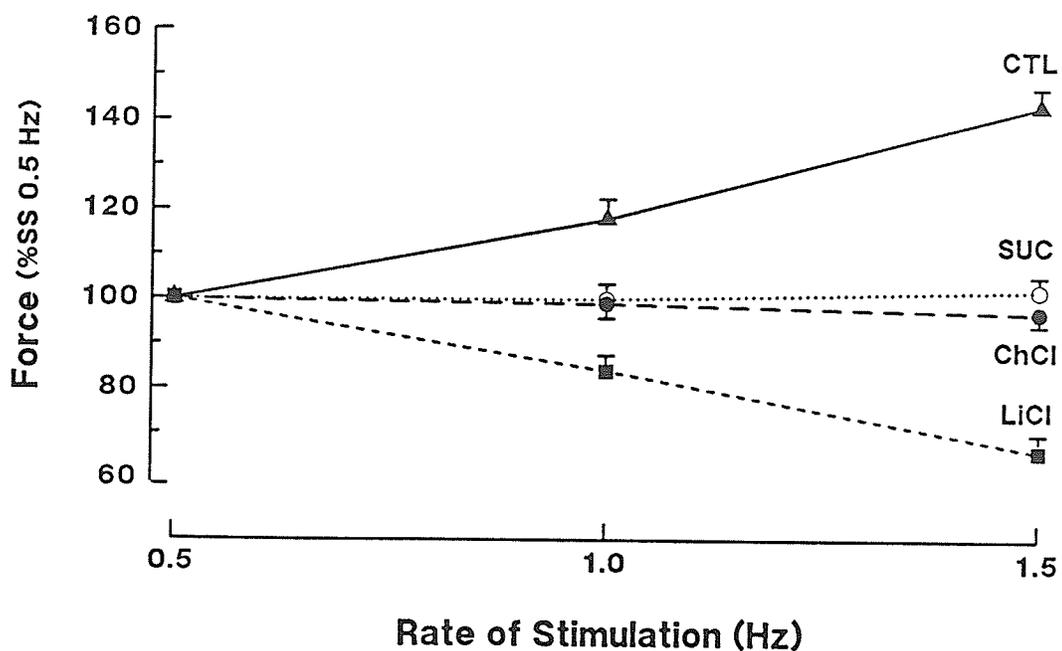


Figure 40. Differential effect on frequency-dependent contraction following reduction of $[\text{Na}^+]_o$ with equimolar substitution of $[\text{Li}^+]_o$, [choline chloride] $_o$ or [sucrose] $_o$. Staircase responses for steady-state contraction in solutions containing 140 mM $[\text{Na}^+]_o$ and 70 mM $[\text{Na}^+]_o$ (Li^+ -substitution) were identical to those shown in Figs. 24 and 28. Reduction of $[\text{Na}^+]_o$ with either [sucrose] $_o$ or [choline chloride] $_o$ rendered the preparations virtually insensitive to alterations in the frequency of stimulation.

Table 5. Effect of ionic composition on the inotropic state and rate constant for recovery of extrasystolic contraction in canine ventricular muscle.

Treatment	C_{ss} (mN/mm ²)	RCC_{ss} (mN/mm ²)	tau (ms)	<i>P</i>
<i>BCL 2000 ms</i>				
2.50 [Ca]o/140 [Na]o	16±0.8	15±0.6	508±22	0.38
1.25 [Ca]o/140 [Na]o	7.0±0.7	9.6±0.8	480±14	
2.50 [Ca]o/140 [Na]o	16±0.8	15±0.6	551±42	0.01
2.50 [Ca]o/ 70 [Na]o	33±2.3	26±0.5	423±20	
<i>BCL 1000 ms</i>				
2.50 [Ca]o/140 [Na]o	19±0.8	16.5±0.5	450±22	0.5
1.25 [Ca]o/140 [Na]o	7.7±0.5	10.5±0.1	460±18	
2.50 [Ca]o/140 [Na]o	19±0.8	16.5±0.5	448±19	0.02
2.50 [Ca]o/ 70 [Na]o	28±0.2	25.8±0.8	390±13	
<i>BCL 668 ms</i>				
2.50 [Ca]o/140 [Na]o	23±2.0	19.8±1.0	424±21	0.9
1.25 [Ca]o/140 [Na]o	8.4±0.4	11.5±1.0	427±16	
2.50 [Ca]o/140 [Na]o	23±2.0	19.8±1.0	442±20	0.06
2.50 [Ca]o/ 70 [Na]o	21±0.8	24.5±2.0	386±12	

BCL = Basic cycle length for steady-state stimulation preceding ES/PES pair; C_{ss} = peak steady-state contraction; RCC_{ss} = peak steady-state rapid cooling contracture; tau = rate constant of recovery (66.6%) for extrasystolic contraction; *P* = degree of significance from paired-t test obtained by comparing tau values going from one test solution to the next within a given *BCL*.

**INFLUENCE OF EXPERIMENTAL DIABETES ON SARCOPLASMIC
RETICULUM FUNCTION IN RAT VENTRICULAR MUSCLE**

SECTION 4

SUMMARY

We examined whether the decrease in contractility in streptozotocin-induced diabetes in the rat is accompanied by reduced or excessive loading of the sarcoplasmic reticulum (SR) with Ca^{2+} . Pooled SR Ca^{2+} content and fractional release on stimulation were estimated with rapid cooling contracture (RCC) and twitch height measurements, respectively. Interval-force relations were studied to assess the ability of diabetic tissue to alter the relative contribution of SR Ca^{2+} for contraction. Two months after injection with streptozotocin, peak isometric contraction and steady-state RCC decreased in parallel to ~50% of control values. The time to peak force development during contraction as well as that needed for complete relaxation was prolonged to 156 and 161% in diabetes. Mechanical perturbations of this nature were observed in the presence of both 1.25 and 2.5 mM $[\text{Ca}^{2+}]_o$. A stepwise increase in the rate of stimulation from 0.2 to 0.5 and 1.0 Hz resulted in a negative force staircase, the slope of which was identical in control and diabetic animals in each $[\text{Ca}^{2+}]_o$ tested. Postrest contractions and RCC elicited after variable test intervals were significantly depressed following 0.2 and 0.5 Hz stimulation in diabetic muscles perfused with 1.25 mM $[\text{Ca}^{2+}]_o$. This defect of SR Ca^{2+} availability was reversed by increasing the stimulation frequency to 1.0 Hz or by elevating $[\text{Ca}^{2+}]_o$ to 2.5 mM. The results suggest that the marked reduction of developed tension in diabetic tissues was a consequence of depleted SR Ca^{2+} stores, rather than a result of chronic SR Ca^{2+} overloading. The maintained integrity of the interval-force relation in the presence of diabetes, implies that the cellular mechanisms responsible for frequency- and time-dependent alterations in SR Ca^{2+} availability are not disturbed at this stage of disease.

INTRODUCTION

Numerous investigations have been performed in an attempt to reveal possible cellular (Hamby et al, 1974; Haider et al, 1977; Regan et al, 1981) and subcellular (Fein et al, 1980; Onji and Liu, 1980; Penpargkul et al, 1981; Nordin et al, 1985; Horackova and Murphy, 1988) derangements in streptozotocin-induced cardiomyopathy. Results from work on animal models of diabetes have been complemented to a large degree by similar results obtained from diabetic patients exhibiting signs of cardiovascular dysfunction (for review, see: Gotzsche, 1986; Dhalla et al, 1985; Tahiliani and McNeill, 1986). Although of a different nature, these studies have one thing in common in that they point to a specific defect which appears to be related to Ca^{2+} metabolism within the myocardial cells themselves. Reports of specific biochemical (Ingebretsen et al, 1981; Lopaschuck et al, 1983; Heyliger et al, 1987), mechanical (Fein et al, 1980; Penpargkul et al, 1981; Ingebretsen et al, 1981) and electrophysiological (Fein et al, 1983; Nordin et al, 1985) abnormalities have further indicated that Ca^{2+} movements across sarcolemmal and sarcoplasmic reticulum (SR) membranes are altered. One important point raised by these studies, is the issue of whether depressed contractility associated with diabetes might be a result of altered loading of the SR with Ca^{2+} .

Excessive accumulation of Ca^{2+} within the SR has been shown previously to reduce contractility of multicellular (Fabiato, 1981; Allen et al, 1985; Bers and Bridge, 1988; Bose et al, 1988b) and single cell preparations (Fabiato, 1981; Capogrossi et al, 1988; Stern et al, 1988) of cardiac muscle by increasing the probability and frequency of spontaneous Ca^{2+} release from the SR during diastole (Stern et al, 1985; Bose et al, 1988b; Capogrossi et al, 1988; Stern et al, 1988). This is pertinent to most *in vitro* studies of diabetes, as the rat has been reported to be the species most dependent on SR Ca^{2+} stores for contraction (Fabiato, 1982; 1983; Bers, 1985) as well as that most susceptible to spontaneous SR diastolic Ca^{2+} release (Stern et al, 1988). Certain mechanical and electrophysiological features associated with diabetes correlate well with conditions of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) overload, most notably an increased predisposition to ouabain-induced toxicity (Fein et al, 1983), enhanced probability of triggered electrical activity and

aftercontractions (Nordin et al, 1985) and increased series compliance in the contractile apparatus (Regan et al, 1974; 1981; Schaffer et al, 1989). It is also possible that prolonged action potentials recorded from diabetic ventricle (Fein et al, 1983; Nordin et al, 1985) may have a significant impact on cellular Ca^{2+} homeostasis. This is suggested by recent work on voltage clamped cardiac tissues which has shown that increasing the duration of membrane depolarization can under certain circumstances reduce $[\text{Na}^+]_o$ -dependent cytosolic Ca^{2+} efflux and consequently affect beat-dependent changes of a_{Na}^i (Brill et al, 1986; Boyett et al, 1987; Terrar and White, 1989), force of contraction (Boyett et al, 1987; duBell and Houser, 1989), and the cytosolic Ca^{2+} transient (duBell and Houser, 1989).

Despite the potential for intracellular Ca^{2+} overload in the presence of diabetes, a large body of data does not support this hypothesis. Even though many preparations of intact diabetic ventricle show reduced contractility (Ingebretsen et al, 1980; Penpargkul et al, 1980) or sensitivity to various inotropic agents (Horackova and Murphy, 1988), others have described little change in resting (Fein et al, 1980) and active tension (Fein et al, 1980; Takeda et al, 1988; Muret et al, 1989; Lagidac-Gossmann and Feuvray, 1990). Similar conflicting results have been reported for measurements of resting membrane potential and action potential duration (Fein et al, 1983; Nordin et al, 1985; Horackova and Murphy, 1988), although these differences are likely a reflection of the different experimental protocols used to study them. The reduced rate of rise and maximal amplitude of action potentials recorded from multicellular diabetic preparations (Fein et al, 1983; Nordin et al, 1985) suggests that membrane Ca^{2+} conductance may in fact be reduced. This is supported by the reduction in total ^{45}Ca content of diabetic ventricular myocytes (Horackova and Murphy, 1988) and the observation that net cellular Ca^{2+} influx in a wide variety of experimental conditions is markedly reduced in both acute and chronic diabetes (Bergh et al, 1988; Tani and Neely, 1988; Horackova and Murphy, 1988). Finally, important biochemical alterations in the membrane of the SR coincident with diabetes, such as reduced activity of Ca^{2+} -ATPase (Penpargkul et al, 1980; 1981; Lopaschuck et al, 1983; Heyliger et al, 1987) and altered lipid

composition (Lopaschuck et al, 1983), may in fact render this organelle ineffective as an intracellular Ca^{2+} sink, even in the presence of normal concentrations of cytosolic Ca^{2+} .

Quantitative changes in the strength of contraction which result from modification of the interval between beats have been used extensively to model the subcellular mechanisms involved in force production (for review, see Koch-Weser and Blinks, 1963; Morad and Goldman, 1973; Wohlfart and Noble, 1982). These include changes in both the rate and rhythm of stimulation. In particular, postrest contraction has proven to be a reliable and highly sensitive assay for agents which perturb SR Ca^{2+} release (Bers, 1985; Bose et al, 1988a; Kort and Lakatta, 1988b; Bouchard and Bose, 1989), while modification in the rate of stimulation is associated with changes in contraction which are sensitive to both transsarcolemmal Ca^{2+} entry as well as SR Ca^{2+} loading *per se* (Gibbons and Fozzard, 1971; Kurihara and Sakai, 1985; Bouchard and Bose, 1989). We have used both these approaches to examine the functional ability of the SR to store and release Ca^{2+} following the induction of experimental diabetes with streptozotocin. Total SR Ca^{2+} has been estimated in these preparations by the magnitude of rapid cooling contractures (Kurihara and Sakai, 1985; Bridge, 1986; Bers et al, 1987; 1989). The results suggest that both pooled SR Ca^{2+} stores and fractional release into the myofilaments in response to an action potential are depressed in diabetic ventricular muscle.

MATERIALS AND METHODS

Induction of Diabetes

Experimental diabetes was produced by injection of 65 mg/kg streptozotocin (Sigma Chemical Co.) dissolved in citrate buffer (0.1 M) into the tail vein of 3-4 week old male Sprague Dawley rats weighing 125-175 grams. Less than 1 ml of the streptozotocin solution was injected into each rat within twenty minutes of its preparation, in a manner similar to that described by Like and Rossini (1976). All animals had continuous access to rat chow and were given water ad libitum. Onset and progression of diabetes was estimated in the first few weeks after injection by overt physical signs including markedly enhanced water consumption and urine output, and reduced body weight. Disease was confirmed on the basis of blood glucose measurement, which was determined by injecting 25 μ l of heparinized plasma into a glucose analyzer (Yellow Springs Instruments) which was calibrated prior to each experiment. Plasma glucose levels were markedly elevated in the animals treated with streptozotocin in this study (17 ± 0.75 mM compared to 3.1 ± 0.25 mM for controls). Pairs of age-matched control and diabetic animals were used in the various experimental protocols.

General Preparation

All animals in this study were killed by decapitation under light ether anaesthesia. Hearts were quickly removed through a thoracotomy, perfused retrogradely through the aorta with ice-cold Krebs-Henseleit (KH) solution, and pinned to the bottom of a dissection chamber containing fresh KH solution. The KH solution had the following composition in mM: NaCl, 118; KCl, 4.7; CaCl₂, 2.5; MgSO₄, 1.2; KH₂PO₄, 1.4; NaHCO₃, 25; glucose, 11. The right ventricular lumen was exposed, and thin free-running trabeculae (0.25-0.5 mm diameter) tied under a dissecting microscope with 8-0 silk thread (Matuda). After removal from the heart, trabeculae were transferred to a muscle chamber forming part of a two circuit parallel by-pass apparatus containing both 50 ml warmed

(37°C) and 60 ml cooled (0-2°C) recirculating HEPES buffer. The ionic composition of the HEPES buffer was (in mM): NaCl, 140; KCl, 4.7; MgCl, 1.0; HEPES ([4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid]), 5; and glucose 10. Depending on the experimental protocol, $[Ca^{2+}]_o$ in the bathing solution was either 1.25 or 2.5 mM. The preparation was equilibrated for 45-60 minutes, during which time the muscles were gradually stretched to the length permitting maximal isometric force production (L_{max}). Field stimulation was provided by a stimulator (Grass model SD44) connected to two platinum electrodes, at a voltage 50-75% greater than that needed for threshold stimulation. HEPES buffer was chosen over KH because of the greater stability of $[pH]_o$ during changes in temperature of the bathing medium in the recirculating apparatus. Solution pH determined during recirculation by either standard glass pH electrode (Radiometer, Copenhagen, Denmark) or pH/blood gas analyzer (Instrumentation Laboratories, Lexington, Mass, USA) indicated that the pH of the solutions at the two temperatures were within 0.2 pH (pH 7.3-7.5) units of each other. In three separate experiments, we observed no difference in the interval-force relation in HEPES compared to KH buffer ($P < 0.05$).

Contraction Measurements

Force development in response to changes in the pattern of electrical stimulation was measured with a force transducer (Grass model FT 03C) and recorded on a 4 channel pen recorder (Grass Model 7). Frequency-force relations were obtained by comparing twitch amplitudes in response to varying the frequency of rhythmic electrical stimulation in the range of 0.2 to 1.0 Hz. It has been observed previously, that contractions elicited after brief periods of rest are highly dependent on intracellular Ca^{2+} stores, rather than on the entry of extracellular Ca^{2+} during muscle activation (Bers, 1985). The amplitude of the first postrest beat and the ensuing recovery of steady-state contraction are thus preferentially blocked by agents which inhibit SR Ca^{2+} storage or release such as ryanodine, caffeine, or Sr^{2+} ions (Bers, 1985; Kort and Lakatta, 1988b; Hryshko et al, 1989a,c; Bouchard and Bose, 1989) as opposed to drugs which alter steady-state Ca^{2+} influx during stimulation such as Co^{2+} or La^{3+} (Bers, 1985). The postrest response was

determined in the present experiments by comparing twitch amplitudes following resumption of stimulation after brief rest intervals ranging from 30 to 240 seconds with those present prior to rest. Once the effect of a given frequency of stimulation was established the entire range of rest intervals was imposed intermittently on the muscle, yielding a family of rest response curves at the different drive rates. A minimum of 10 minutes was allowed for equilibration of the preparation at each new frequency of stimulation prior to initiating a given set of rest intervals. Changes in the amplitude of twitch contractions following modification of the stimulus interval have been expressed in two ways throughout this study. Contractions were expressed in units of force normalized for muscle cross-sectional area (mN/mm^2) to permit an estimate of muscle contractility in the presence of diabetes. In addition, twitch amplitudes were also expressed as a percentage of those immediately preceding or following a given test beat, thus forming part of the interval-force relation.

Rapid Cooling Contractures

For these experiments, two sets of physiological solutions (37°C and $0\text{-}2^\circ\text{C}$) were maintained in a two-circuit parallel by-pass system. Manual diversion of solutions from one parallel circuit to the other allowed very rapid (< 1 sec) switching of bathing media at a flow rate of ~ 40 ml/min. Rapid cooling contractures were elicited both in the place of a regularly driven beat (e.g., steady-state cooling contracture), and after variable periods of rest. The protocol for obtaining cooling contractures was identical to that described above for twitch contractions, including the two methods of data analysis. Previous studies by Kurihara and Sakai (1985), and Bridge (1986) have indicated that rapid cooling of mammalian heart muscle results in contractures which presumably reflect the degree of intracellular Ca^{2+} loading. Contractures of this nature are thought to indicate the amount of Ca^{2+} present within the SR, since (a) they are strongly affected by pharmacological manipulations which alter SR Ca^{2+} loading and release (Bridge, 1986; Bers et al, 1989; Bouchard et al, 1989), (b) remain unaffected by perturbations designed to alter Ca^{2+} influx across the sarcolemma during cooling (Kurihara and Sakai, 1985; Bridge, 1986), and (c) have a similar species-dependence to that shown for postrest contraction (Bers, 1985) and spontaneous diastolic

SR Ca^{2+} release (Stern et al, 1988). The large drop in sensitivity of the myofilaments for cytosolic Ca^{2+} during cooling to 0-2°C (Harrison and Bers, 1989; 1990) suggests that a mass release of Ca^{2+} occurs from the SR, which probably reflects the majority of Ca^{2+} present within pooled SR stores prior to cooling the preparation (Bers et al, 1989).

Statistics

Data are shown as the mean \pm S.E.M. Data analysis was performed with a two-way repeated measures ANOVA to control for both the presence and duration of diabetes. The level of significance used for all blocked experiments was $P < 0.05$. When statistical significance was demonstrated by ANOVA, Duncan's multiple range test was used to determine the source and degree of significance within a given experimental treatment.

RESULTS

Altered contractility of diabetic ventricular muscle

Peak contraction amplitude: Peak isometric force of contractions in response to steady-state stimulation at 0.2 Hz was used to quantitate changes in myocardial contractility in the present streptozotocin-induced model of diabetes. Figure 41 illustrates the effect of four and eight weeks diabetes on isometric contraction. The top panel shows superimposed contractions recorded from muscles obtained from an eight week diabetic rat and a corresponding age-matched control animal and illustrates the marked reduction in contractile force associated with diabetes. $[Ca^{2+}]_o$ in both series of experiments was 1.25 mM. The degree to which contractions were depressed in the presence of diabetes depended on the duration of disease. The results of ten such experiments are shown in Table 6. Although contractions were significantly depressed following both four and eight weeks disease, the reduction in contractility was significantly greater in animals subjected to the longer period of diabetes, as indicated in Tables 6 and 7.

The possibility that the difference in contractility between diabetic and control preparations was related to $[Ca^{2+}]_i$ -overload was investigated by repeating this and later protocols in both 1.25 and 2.5 mM $[Ca^{2+}]_o$. Previous studies have shown that the SR of rat ventricular muscle accumulates enough Ca^{2+} to be at or near the point of saturation at 2.5 mM $[Ca^{2+}]_o$ (Fabiato, 1981; Schouten et al, 1987) and thus more likely to manifest signs of spontaneous diastolic SR Ca^{2+} release (Capogrossi et al, 1988; Stern et al, 1988). As indicated by the data given in Table 6, increasing $[Ca^{2+}]_o$ from 1.25 to 2.5 mM had no effect on the degree of contractile depression at either stage of disease. That is, contractions were diminished by the diabetic process to equal percentages in 1.25 and 2.5 mM $[Ca^{2+}]_o$, with a greater depression after eight weeks than four weeks within a given $[Ca^{2+}]_o$.

Twitch mechanics: Developmental effects of diabetes on time to peak tension and time for complete relaxation during contractions are illustrated in Panel B of Fig. 41. We have found in previous experiments that the time course of contraction is sensitive to changes in the duration of both SR Ca^{2+} uptake and release (Bouchard et al, 1989). Thus, calculations here were made from experiments performed in 1.25 mM $[\text{Ca}^{2+}]_o$ to avoid precipitation of mechanical oscillations due to localized Ca^{2+} release from the SR at higher perfusate Ca^{2+} . Although it appears that these do not occur in untreated rat ventricle at rates of stimulation greater than 0.2 Hz (Kort and Lakatta, 1989b), this effort was warranted due to the increased incidence of triggered activity and aftercontractions reported in similar preparations perfused with solutions containing 2.5 mM Ca^{2+} (Nordin et al, 1985). The progression of diabetes through weeks four to eight was associated with a gradual slowing of contraction and relaxation phases of the twitch. The top trace in Panel B shows the contractile response of a muscle obtained from a four week control rat to rhythmic stimulation at 0.2 Hz. The chart speed was increased after the first few contractions to highlight slight differences in terminal relaxation in each trace. The middle and bottom recordings show representative responses of muscles obtained after four and eight weeks diabetes, respectively. Arrows illustrate the point at which relaxation was completed for each of the contractions. Compared with changes in the time course of relaxation, the increase in time to peak tension took place more gradually and was not readily apparent until eight weeks after the induction of diabetes. Averaged data illustrating the dependence of these changes on the duration of diabetes in ten such experiments has been provided in Table 6.

These observations are in good agreement with the previous description of slowed contraction in an established model of diabetes at varying $[\text{Ca}^{2+}]_o$ (Fein et al, 1980; 1983), and appear to be related to similar time-dependent shifts in myosin isozyme distribution (Dillman, 1980; Takeda et al, 1988; Schaffer et al, 1989) and Ca^{2+} uptake inferred from preparations of SR vesicles (Penpargkul et al, 1981; Lopaschuck et al, 1983). The strong dependence of rat ventricular muscle on SR Ca^{2+} for contraction (Fabiato, 1981; 1983; Bers, 1985) combined with the altered sensitivity of diabetic ventricular preparations to inotropic agents exerting their effects by altering SR Ca^{2+} (Horackova

and Murphy, 1988), suggest that pooled SR Ca^{2+} stores may be altered in diabetes. As past work has suggested that rapid cooling contractures do not discriminate against different functional pools of Ca^{2+} located within the SR (Kurihara and Sakai, 1985; Bridge, 1986; Bers et al, 1987; 1989), these were employed as a probe to test for time-dependent changes in total SR Ca^{2+} content accompanying diabetes.

Rapid cooling contractures: Representative responses of control and diabetic muscles to rapid cooling are shown in Fig. 42. The control response to cooling illustrated in the top panel is that of a four week control animal exposed to 1.25 mM $[\text{Ca}^{2+}]_o$. This age group was chosen for comparison with four as well as eight week diabetic muscles because changes in force associated with cooling in the older diabetic animals would be less likely to be different from younger age-matched controls. Rapid cooling contractures were obtained in this series of experiments by terminating rhythmic stimulation and eliciting a cooling contracture in place of a regularly driven beat (steady-state cooling contracture). The top panel shows that rapid cooling induces a prompt contractile response, which in the rat is greater than steady-state contraction. After reaching a peak, force of contracture normally declines over the course of 30-40 sec, eventually returning to the level of resting tension found prior to cooling. Upon rewarming, steady-state contraction is gradually re-established over the next 60-90 sec. Similar results were obtained with nine other muscles.

The amplitude of cooling contratures associated with diabetes is shown in the bottom panel of Fig. 42. Pooled data for these and nine other sets of cooling experiments for this age group are also presented in Table 7. Peak cooling contracture magnitude was reduced by 50% in trabeculae obtained from animals subjected to four weeks diabetes. This represents a slightly greater depressant effect of diabetes on peak steady-state cooling contracture, compared with the corresponding decrease in steady-state contraction. When $[\text{Ca}^{2+}]_o$ was elevated to 2.5 mM during these experiments, the depressant effect of diabetes on steady-state cooling contracture was significantly reduced. Values averaged from 10 experiments are shown in Table 7 and indicate that

elevating perfusate Ca^{2+} antagonizes the depressant effect of diabetes on SR Ca^{2+} loading, and that this effect occurs independent of changes in steady-state contraction. A further reduction in peak cooling contracture amplitude was observed when the duration of diabetes was extended to eight weeks. This is illustrated in the bottom Panel of Fig. 42, which also shows the lack of effect of diabetes on the rate of rise of cooling contracture. The most noticeable differences in contracture profile following eight weeks diabetes were the marked slowing of relaxation, and the reduction of peak contracture amplitude in both 1.25 and 2.5 mM $[\text{Ca}^{2+}]_o$. These values correlate well with the corresponding decrease of steady-state contraction at the same stage of disease (53 and 56 percent for steady-state contraction and cooling contracture in 1.25 mM $[\text{Ca}^{2+}]_o$; 66 and 60 percent in 2.5 mM $[\text{Ca}^{2+}]_o$).

RCC mechanics: In addition to changes of cooling contracture peak, Fig. 42 also shows the influence of diabetes on the time course of relaxation of steady-state cooling contractures. The responses of all three muscles shown here were obtained at a stimulus frequency of 1.0 Hz. Although as will be shown later, a range of frequencies was tested, recording rapid cooling contractures at this frequency facilitates visual inspection of small changes in the rate of contracture relaxation. In the control muscles, relaxation of cooling contracture amplitude to 50% peak contracture required 10.3 ± 1.8 and 11.7 ± 2.0 sec in 1.25 and 2.5 mM $[\text{Ca}^{2+}]_o$ respectively. Four weeks of diabetes resulted in a significant increase in the time taken for relaxation, to 14.4 ± 3.3 ($P < 0.05$) and 16.9 ± 1.9 ($P < 0.05$) sec in 1.25 and 2.5 mM $[\text{Ca}^{2+}]_o$. A comparison of relaxation times associated with different rates of stimulation (0.2 to 0.5 and 1.0 Hz) revealed no difference in the time course of relaxation in either the control or diabetic muscles. An analysis of the time course for 75% relaxation yielded identical results. When the duration of disease was prolonged to eight weeks, a further slowing of relaxation was observed. Relaxation to 50% peak cooling contracture amplitude required 19.8 ± 7.0 seconds in 1.25 mM and 23.4 ± 6.8 sec in 2.5 mM $[\text{Ca}^{2+}]_o$ (eight week control times were 11.0 ± 1.1 ($P < 0.05$) and 11.9 ± 1.4 ($P < 0.05$) sec). Similar to

the results obtained after four weeks, relaxation times were not influenced by stimulus frequency in either group.

Interval-force relationship in diabetes

Frequency-force relations: The typical response of untreated rat ventricular trabeculae to a progressive increase in the frequency of rhythmic stimulation from 0.2 to 0.5 and 1.0 Hz is depicted in the top panel of Fig. 43., and in Figs. 44, 46, and 47 during later experiments. There was an inverse correlation between peak isometric force development and the frequency of rhythmic stimulation at a $[Ca^{2+}]_o$ of 1.25 mM in four and eight week control animals. Changes in contraction were not accompanied by a significant shift in either the time to peak tension or time for complete relaxation, as shown in the inset of Fig. 43A. The negative frequency-force relationship persisted when the $[Ca^{2+}]_o$ was elevated to 2.5 mM. We found no significant differences in the contractile response of four (n=10) and eight (n=10) week control rats to increasing frequency of stimulation in the presence of either 1.25 or 2.5 mM $[Ca^{2+}]_o$.

The effect of diabetes on staircase responses averaged from 10 experiments in each age group are shown in panels B and C of Fig. 43. Although peak isometric force was progressively impaired with increasing duration of diabetes (Table 6), no effect of disease was observed on the frequency-force relation after four weeks (middle panel; n=10) and eight weeks (bottom panel; n=10) of diabetes. Similar to the control response, the negative inotropic effect of stimulation was not accompanied by a change in the time to peak of contraction or that required for complete relaxation. This was also true when $[Ca^{2+}]_o$ was elevated to 2.5 mM. If the reduced contractility of diabetic preparations described above were indeed a manifestation of enhanced spatial summation of focal regions of SR Ca^{2+} release within stacked myocytes (Stern et al, 1983; 1988; Bose et al, 1988b), then a decrease in the interval between evoked contractions would have been expected to reduce the incidence of spontaneous contractile waves (Capogrossi et al, 1988). This did not occur, however, as the slope of the frequency-force relation was not altered in the

presence of four or eight weeks of diabetes. The equal depressant effect of increasing the rate of electrical stimulation in control and diabetic preparations suggests that either the basal frequency or the statistical probability of spontaneous contractile waves during diastole are similar in the two groups.

After obtaining frequency-force relations for steady-state contraction, the protocol was then repeated with steady-state rapid cooling contractures. Representative responses of a four week control muscle and those for muscles obtained after four and eight weeks diabetes are shown in Fig. 44. For the control muscle bathed in 1.25 mM $[Ca^{2+}]_o$, the amplitude of peak steady-state cooling contracture remained unaffected by increasing frequency of stimulation. The inability of rapid stimulation to alter pooled SR Ca^{2+} occurred even though the size of preceding steady-state contraction was progressively diminished and was consistently observed in all ten control muscles examined in each age group. This rather surprising discrepancy between pooled SR Ca^{2+} content and fractional release of Ca^{2+} into the myofilaments is not observed in the vast majority of mammalian species (Gibbons and Fozzard, 1971; Kurihara and Sakai, 1985; Bouchard and Bose, 1989) and appears to reflect the difference in intracellular Ca^{2+} handling in myocytes of the rat (Ragnarsdottir et al, 1982; Schouten et al, 1987; Kort and Lakatta, 1988b; Shattock and Bers, 1989). Similar to the change in steady-state contraction, elevating $[Ca^{2+}]_o$ to 2.5 mM had no effect on peak cooling contracture at increasing rates of stimulation.

The influence of four and eight weeks diabetes on steady-state cooling contracture at different rates of stimulation was similar to that observed for the twitch staircase response, as indicated by the corresponding traces in Fig. 44. Increasing the rate of stimulation in 1.25 mM $[Ca^{2+}]_o$ resulted in a decrease in steady-state contraction in both test groups, which again was not accompanied by a similar decline of peak steady-state cooling contracture. The data provided in Table 7 indicate that increments in the rate of stimulation exerted no effect on the amplitude of peak steady-state cooling contracture in all control and diabetic muscles studied and that this response was not affected by changes of $[Ca^{2+}]_o$. Upon closer scrutiny, however, a significant difference was revealed in the relationship between steady-state twitch and cooling contracture force at different

frequencies of stimulation. These subtle differences may be important as they suggest that the fraction of total cytoplasmic Ca^{2+} originating from the SR at different rates of stimulation may be altered in diabetes. This possibility is suggested by calculation of the apparent SR Ca^{2+} "release fraction" associated with a given conditioning interval. This was done by measuring the ratio of peak steady-state contraction to peak steady-state rapid cooling contracture ($\text{SSC mN/mm}^2:\text{SS RCC mN/mm}^2$). This analysis assumes that there is a single compartment of releasable Ca^{2+} within the SR and also, that this compartment represents a pool of Ca^{2+} that is distinctly separate from other stores present in the SR (Stern et al, 1988). Compartmentalization of SR Ca^{2+} pools of this nature is a feature of most recirculation models of intracellular Ca^{2+} handling (Morad and Goldman, 1973; Wohlfart and Noble, 1982; Bose et al, 1988a) and is supported by experimental measurements of time- and beat-dependent changes of pooled SR Ca^{2+} loading and fractional release in those species particularly dependent on SR stores for contraction (e.g., rat and dog; Bers, 1985; Fabiato, 1981; 1985c; Bouchard and Bose, 1989). The scale is an arbitrary one, where a value of 1.0 is equivalent to the maximal release of Ca^{2+} possible from pooled SR stores (100% total SR Ca^{2+} released into the myofilaments). An important limitation of this analysis is the decrease in myofilament Ca^{2+} -sensitivity reported to accompany rapid cooling of untreated ventricular muscle (Harrison and Bers, 1989). As a result, force associated with cooling cannot be compared directly with force of contraction (Bridge, 1986; Bers, 1989; Bers et al, 1989). However, as it is likely that the drop in contractility related to cooling is a constant factor from one stimulus frequency to the next, then scaling contraction in this manner may offer useful information with regards to that fraction of cytoplasmic Ca^{2+} for contraction which was released from SR stores in response to an action potential.

Figure 45 shows the result of calculations made from steady-state cooling contractures elicited at the three test frequencies in both 1.25 and 2.5 mM $[\text{Ca}^{2+}]_o$. Data have been pooled from six control muscles (solid lines) and six muscles obtained from rats subjected to eight weeks diabetes (broken lines). The stimulation protocol is illustrated in the inset. As expected, the calculated release fraction decreases with increasing frequency of stimulation in control muscles in the

presence of 1.25 mM $[Ca^{2+}]_o$. Along with reduced steady-state force production (Fig. 40; Table 6) this would imply that the quantity of Ca^{2+} released from the SR is reduced. The values calculated for fractional release at 0.2 Hz are very close to those predicted (0.75) by a mathematical model of releasable Ca^{2+} specific for rat ventricular muscle (Stern et al, 1988). Following eight weeks diabetes, the ratio between peak cooling contracture and steady-state contraction was significantly altered. This resulted in correspondingly lower values calculated for the apparent release fraction at 0.2 and 0.5 Hz. This decrement in presumed SR Ca^{2+} availability was overcome by stimulation at 1.0 Hz in 1.25 mM $[Ca^{2+}]_o$ (panel A) or at all 3 test frequencies by raising the perfusate Ca^{2+} to 2.5 mM (panel B).

Postrest response: Previous studies have shown that resuming electrical stimulation after a brief period of rest gives rise to contractions which are highly sensitive to drugs altering SR Ca^{2+} release (Bers, 1985). In rat ventricle specifically, potentiated postrest contractions appear to be a composite function of time- and beat-dependent recycling of Ca^{2+} within the SR (Orchard and Lakatta, 1985; Schouten et al, 1987) combined with a small net cellular Ca^{2+} gain through the activity of the Na^+-Ca^{2+} exchange process (Ragnarsdottir et al, 1982; Shattock and Bers, 1989). As a final method of determining the functional ability of the SR to store and release Ca^{2+} in the presence of diabetes, postrest contractions and rapid cooling contractures were elicited at the three test frequencies. Fig. 46 illustrates the response of an isolated trabecula obtained from a four week control animal to a progressive increase in the duration of rest in 1.25 mM $[Ca^{2+}]_o$. The top panel shows that gradual lengthening of the rest interval from 30 to 120 and 240 sec has no effect on the amplitude of the first postrest contraction. Increasing the rate of stimulation to 0.5 and 1.0 Hz gradually augmented the amount of postrest potentiation (expressed as a percentage of preceding steady-state contraction), but had no effect on the level of force development associated with these contractions (e.g., the augmented postrest potentiation was a reflection of decreasing steady-state contraction). Increasing $[Ca^{2+}]_o$ to 2.5 mM resulted in an increase in the contractility of all ten control preparations studied, as shown in Table 6. Despite the augmented

contractility, the pattern of tension change following changes in the rate of stimulation was quite similar to that observed in 1.25 mM $[Ca^{2+}]_o$ with respect to its frequency-dependence. Although postrest potentiation was enhanced with increasing frequency of stimulation in 2.5 mM $[Ca^{2+}]_o$, the magnitude of change was quantitatively greater in 1.25 mM $[Ca^{2+}]_o$, as illustrated in Fig. 48. A second example of this $[Ca^{2+}]_o$ -dependence is furnished by the absence of postrest potentiation at 0.2 Hz in 2.5 mM, compared with that present in 1.25 mM $[Ca^{2+}]_o$. These observations, combined with the relatively small change in steady-state cooling contracture following an increase in perfusate Ca^{2+} (see Table 6) are consistent with the proposal made earlier that intracellular stores are probably at or near saturation in the presence of 2.5 mM $[Ca^{2+}]_o$.

The influence of diabetes on postrest contraction is shown in Figs. 47 and 48. The degree of postrest potentiation associated with preparations of diabetic muscle decreased in parallel with the duration of disease. Although as shown in Table 6, postrest force (expressed in mN/mm^2) was not yet affected after four weeks diabetes ($n=10$), the degree of postrest potentiation was significantly depressed after 30 sec rest at 0.2 and 0.5 Hz steady-state stimulation in 1.25 mM $[Ca^{2+}]_o$. Increasing the rate of stimulation to 1.0 Hz, or elevating $[Ca^{2+}]_o$ to 2.5 mM abolished the apparent defect in SR Ca^{2+} loading. When diabetes was extended to eight weeks ($n=10$), both postrest potentiation (Fig. 47) and postrest force (Table 6) were markedly depressed.

The data presented so far imply that even though pooled SR stores appear to be severely depleted, the ability of diabetic preparations to retain Ca^{2+} during mechanical quiescence may remain unaffected. In this final series of experiments, rapid cooling contractures were elicited after identical rest intervals to those just described to determine if the changes in postrest contraction accompanying diabetes were related to similar alterations in pooled SR Ca^{2+} content. The protocol for this experiment is shown in the top of Fig. 49. In muscles obtained from four week control animals, no difference was observed in the amplitude of cooling contractures elicited immediately following steady-state stimulation and those elicited after 30, 120 and 240 sec rest in the presence of 1.25 mM $[Ca^{2+}]_o$. The similarity in peak steady-state and postrest cooling contracture amplitude persisted when the rate of stimulation was increased from 0.2 to 0.5 and 1.0

Hz. Similar observations were made in all ten experiments when the perfusate Ca^{2+} was increased to 2.5 mM or muscles from eight week control animals were examined (see also Table 7).

In muscles obtained from 10 four week diabetic animals, peak force associated with rapid cooling following 30 sec rest was significantly depressed in 1.25 mM $[\text{Ca}^{2+}]_o$, as described in Table 7. Moreover, as was observed for postrest contraction, this depression was again overcome by increasing $[\text{Ca}^{2+}]_o$ to 2.5 mM. A gradual increase in the rest interval prior to cooling had no effect on the amplitude of postrest rapid cooling contracture for any combination of stimulus frequency or $[\text{Ca}^{2+}]_o$ investigated. When postrest cooling contracture was re-examined after eight weeks diabetes ($n=10$), no change was observed in the amplitude of peak contracture in response to changes in rest duration, rate of stimulation or $[\text{Ca}^{2+}]_o$. The only exception was that the marked reduction of force development associated with postrest cooling contracture was present in both 1.25 and 2.5 mM $[\text{Ca}^{2+}]_o$. As was noted in earlier experiments on steady-state cooling contractures, a significant interaction between steady-state contraction and postrest cooling contracture existed. The degree of interaction was almost identical to that described in Fig. 45 for both 1.25 and 2.5 mM $[\text{Ca}^{2+}]_o$. Hence, the calculated release fractions for muscles obtained from six control and six diabetic animals, illustrated in Fig. 49, were virtually superimposable over those shown earlier. Thus, the results of the postrest experiments are very similar to those obtained by altering the frequency of rhythmic stimulation in all preparations of diabetic ventricle investigated. Taken together, these observations suggest that the reduced contractility associated with eight weeks of diabetes is a result of diminished intracellular Ca^{2+} availability, as opposed to the end result of chronic intracellular Ca^{2+} overloading.

DISCUSSION

This work was aimed at providing basic information about the cellular mechanisms underlying the reduction in force development associated with certain models of diabetic cardiomyopathy. The parallel reduction of both steady-state contraction and steady-state rapid cooling contracture suggests that pooled SR Ca^{2+} content and release of this Ca^{2+} in response to an action potential are progressively depressed following the induction of diabetes with streptozotocin. Changes in the interval-force relationship, and the corresponding sensitivity of these contractile alterations to $[\text{Ca}^{2+}]_o$, support this conclusion and would further suggest that modifications in the source of activator Ca^{2+} for contraction may occur to compensate for the general decline in force producing ability of the myocardium in diabetes.

Possible mechanisms underlying depressed force production

Underloading vs overloading of intracellular Ca^{2+} stores: One of the purposes of this paper was to examine if the negative inotropic effect of diabetes was due to chronic intracellular Ca^{2+} -overload. A general observation related to each series of experiments was that elevating $[\text{Ca}^{2+}]_o$ from 1.25 to 2.5 mM did not produce mechanical alterations in diabetic preparations which are usually associated with conditions of $[\text{Ca}^{2+}]_i$ -overloading (e.g., aftercontractions, mechanical alternans, or elevated resting tension). This included the insensitivity of the decrease in steady-state contraction and rapid cooling contracture (Table 6 and 7), postrest contraction and cooling contracture (Figs. 47 and 49), and slope of the frequency-force relation (Fig. 43) to changes in perfusate Ca^{2+} . Moreover, increasing $[\text{Ca}^{2+}]_o$ from 1.25 mM to 2.5 mM uniformly *reversed* the depressed contractility associated with diabetes at varying stages of disease. This may be an important observation, as threshold $[\text{Ca}^{2+}]_o$ for the occurrence of spontaneous SR Ca^{2+} release at a steady-state frequency of 0.2 Hz in isolated rat ventricular muscle is 1.9 ± 0.03 mM (Capogrossi et al, 1988). It has been observed previously that an increase in spontaneous Ca^{2+} release from the

SR results in impaired force development which is not accompanied by a similar decrease in the amplitude of the cytoplasmic Ca^{2+} transient (Allen et al, 1985) or steady-state rapid cooling contracture (Bers and Bridge, 1988). The observation that depressed steady-state contractions are almost always paralleled by a proportional decrease in steady-state cooling contracture is thus inconsistent with this portion of the $[\text{Ca}^{2+}]_i$ -overload hypothesis. In preliminary experiments performed on untreated rat papillary muscles, we have observed that inducing $[\text{Ca}^{2+}]_i$ -overload by either partial replacement of $[\text{Na}^+]_o$ with Li^+ , elevating $[\text{Ca}^{2+}]_o$, or administration of high doses of ouabagenin substantially reduces the amplitude of postrest contraction, renders the muscles insensitive to changes in the rate of stimulation, and consistently results in elevated resting tension (Bouchard and Bose, unpublished observations). Although these results are preliminary and need to be interpreted with caution, they are consistent with the finding that time-dependent restitution of postrest contraction after terminating stimulation parallels the increase in the average frequency of scattered light intensity fluctuations (SLIF) in resting rat ventricular muscle (Kort and Lakatta, 1988*b*; Shattock and Bers, 1989). Characteristic changes in the autocorrelation function (Stern et al, 1985; Kort and Lakatta, 1988*a,b*) or power spectrum (Bose et al, 1988*b*) of SLIF have been shown to result from cumulative myofilament oscillations due to asynchronous release of Ca^{2+} from SR stores during diastole. If the covariance in the restitution of SLIF and postrest contraction are indeed due to an increase in the amount of Ca^{2+} present within the release compartment (Allen et al, 1985; Capogrossi et al, 1988), then elevating $[\text{Ca}^{2+}]_o$ to 2.5 mM should be expected to worsen cumulative myofilament oscillations due to an increase in the frequency of spontaneous release (Capogrossi et al, 1988). This effect, however, was not observed as the amplitude of both postrest contraction and rapid cooling contracture was diminished more profoundly in 1.25 mM, than in 2.5 mM $[\text{Ca}^{2+}]_o$.

Earlier reports have indicated that impaired steady-state contraction in the presence of SR Ca^{2+} overloading (Allen et al, 1985; Bers and Bridge, 1988) may be due, in part, to an increase in the "refractoriness" of the SR Ca^{2+} release channel effected by the preceding spontaneous contractile wave (Fabiato and Fabiato, 1975; 1976; Fabiato, 1981). It has been suggested (Capogrossi et al,

1988), that one way to prevent spontaneous release from saturated intracellular stores is to reduce the interval between evoked contractions. This maneuver has been found to decrease both the incidence and frequency of spontaneous contractile waves in isolated rat ventricular myocytes while leaving unaffected the SR Ca^{2+} -dependent inotropic ceiling. If the negative inotropic effect of diabetes was due to an increase in the number of cells within a given muscle segment exhibiting spontaneous Ca^{2+} release, one might expect the slope of the frequency-force relation to be significantly reduced, or even reversed, in a given $[\text{Ca}^{2+}]_o$ (cf. Fig.1 of Capogrossi et al, 1988). The similarities in the slope of the frequency-force relation between control and diabetic muscles, combined with the insensitivity of the staircase response to $[\text{Ca}^{2+}]_o$ in diabetic preparations, provides additional evidence that the reduced force of contraction exhibited by muscles obtained from diabetic animals may be due primarily to a fundamental depletion of pooled SR Ca^{2+} stores, rather than a direct result of chronic $[\text{Ca}^{2+}]_i$ -overload. This conclusion is supported by the recent observation of Tani and Neely (1988) that hearts from diabetic rats are more resistant to ischemia and develop fewer signs of $[\text{Ca}^{2+}]_i$ -overload upon reperfusion than control hearts.

Changes external to the sarcoplasmic reticulum: In addition to perturbation of intracellular Ca^{2+} release, other mechanisms which are unrelated to Ca^{2+} handling by the SR may also be involved in the negative inotropic effect of diabetes. Previous work has indicated that tissue compliance is significantly elevated in response to chronic diabetes (Regan et al, 1974; 1981; Schaffer et al, 1989). While this might be expected to reduce or perhaps even slow the development of contraction in response to a given increase of myoplasmic Ca^{2+} , it seems reasonable to suggest that any existing alteration of the viscoelastic properties of the muscle fibres accompanying diabetes would remain relatively stable from one stimulation frequency to the next. This is supported by the fact that similar directional changes of baseline tension and twitch profile were observed during alterations in the rate and rhythm of stimulation in both groups. Numerous other aspects of the data support that proposal, including: (a) similarities in the slope of the frequency-force relations in control and diabetic muscles, (b) the insensitivity of the frequency-

force relation to preceding changes in the inotropic state of the preparation in either group following alteration of $[Ca^{2+}]_o$, and (c) the much greater effect of diabetes on the time required for complete relaxation of the twitch, compared with time to peak tension measurements. Although a more general role for altered tissue compliance in depressing contractility can not be excluded on the basis of the techniques employed in this study, the parallel reduction of steady-state contraction with postrest contraction and steady-state cooling contracture throughout the various manipulations of stimulation frequency and perfusate Ca^{2+} suggest that changes in developed tension accompanying diabetes in the present experiments are more strongly related to perturbations of intracellular Ca^{2+} loading and release.

It could also be argued that the depressant effect of diabetes on steady-state contraction and rapid cooling contracture amplitude may be secondary to a diabetes-related decrease in the responsiveness of the myofilaments to Ca^{2+} . This issue was recently addressed by Murat et al. (1989) who demonstrated that maximal myofilament Ca^{2+} sensitivity of skinned subendocardial ventricular fibres from diabetic rat hearts was modestly enhanced in a pCa range of 6.5-5.0, rather than reduced. Although these experiments were conducted at 22°C and peak developed force remained unaffected by 4-6 weeks of diabetes, this is the range in which the rise of intracellular free Ca^{2+} would be expected to deviate during either an electrically-stimulated twitch (Fabiato, 1981; Bers et al, 1989; duBell and Houser, 1989) or rapid cooling contracture (Bers et al, 1989; Harrison and Bers, 1989), even after compensating for the depressant effect of diabetes. In a similar preparation to the one used in our experiments, it has been recently observed (Lagidac-Gossmann and Feuvray, 1990) that administration of 10 mM caffeine produces an equivalent inotropic response in the presence or absence of diabetes. Although certainly not conclusive in this respect, this result does suggest that the responsiveness of diabetic tissues to agents known to alter myofilament Ca^{2+} sensitivity may be more or less intact. Consequently, a diabetes-related increase of myofilament Ca^{2+} sensitivity would tend to underestimate the quantitative reduction of contractility in the present experiments, and thus can not be responsible for the decrease of steady-state contraction or rapid cooling contracture amplitude.

Fractional release of Ca^{2+} from SR stores: The results shown in Figs. 45, 48 and 49 suggest that the fraction of total cytoplasmic Ca^{2+} for contraction released from SR stores may be diminished in diabetes. The accuracy of these calculations depends on the assumption that the reduction in Ca^{2+} -sensitivity of the myofilaments during the process of cooling in diabetic ventricle is not different from that of control muscles. On the basis of data obtained from skinned muscle fibre experiments, it has been suggested by Harrison and Bers (1989) that the envelope of rewarming spikes observed when rapid cooling contractures are terminated prematurely at various points in the time course of contracture appear to be due in part to a temperature-dependent decrease of myofilament Ca^{2+} sensitivity. Values calculated for the pCa required for half maximal tension in the rat ($K_{1/2}$ values predicted by the Hill Equation) were 5.32 at 36°C and 4.68 at 1°C , both of which are temperatures relevant to cooling contractures in the present experiments. An important feature of this relationship is that the reported $K_{1/2}$ values increase in a stepwise fashion from 1 - 36°C , although the slope of this rise is slightly greater in the lower part of the curve (1 - 8°C ; Harrison and Bers, 1989). This suggests that even if myofilament Ca^{2+} sensitivity were markedly reduced in diabetes, as long as the basal temperature-dependence of the myofilaments on cytosolic Ca^{2+} remained intact, then differences in calculated release fraction would represent a significant alteration in the source of Ca^{2+} for contraction in the presence of diabetes. The parallel and frequency-dependent dissociation of steady-state contraction amplitude from steady-state cooling contracture amplitude at either $[\text{Ca}^{2+}]_o$ in both control and diabetic muscles suggests that this may be the case. However, this data cannot be taken as an indication of normal myofilament temperature sensitivity, because such experiments necessitate time-gated measurements of the effects of premature rewarming which were not performed in this study.

If the temperature-dependence of the myofilaments to Ca^{2+} is enhanced or remains unaffected by diabetes, then reduction of the apparent "release fraction" makes possible the conclusion that other cellular processes are helping to maintain high levels of $[\text{Ca}^{2+}]_i$ during contraction. Likely candidates for such compensation include: (a) transsarcolemmal Ca^{2+} entry during the Ca^{2+}

current I_{Ca} , (b) ATP-dependent Ca^{2+} pumps located in the membranes of the SR and sarcolemma, and (c) the Na^+ - Ca^{2+} exchange process. Based on the observation that both sarcolemmal and SR Mg^{2+} -ATPase and Ca^{2+} - Mg^{2+} -ATPase activities are markedly depressed in diabetic animals (Penpargkul et al, 1981; Lopaschuck et al, 1983; Heyliger et al, 1987), it is unlikely that altered kinetics of Ca^{2+} uptake by the SR are responsible for this extra $[Ca^{2+}]_i$. Previous studies have shown that pharmacological inhibition of SR Ca^{2+} uptake is more likely to affect relaxation of the twitch, as opposed to peak contraction amplitude (Bouchard et al, 1989). Furthermore, the most profound changes in cytoplasmic Ca^{2+} due to reduced Ca^{2+} extrusion would likely occur too late to affect peak $[Ca^{2+}]_i$, which occurs ~40 ms after stimulation (duBell and Houser, 1989). Although membrane Ca^{2+} currents have not yet been investigated in the presence of diabetes, the reduction in the degree of membrane depolarization during the plateau of the diabetic action potential (Fein et al, 1983; Nordin et al, 1985) does provide some evidence in support of abnormal Ca^{2+} or K^+ conductances. A reduction of peak I_{Ca} would be consistent with reports of reduced cellular total Ca^{2+} content (Bergh et al, 1988; Tani and Neely, 1988; Horackova and Murphy, 1988) and steady-state rapid cooling contracture (this study), and may be related to the accompanying decrease of resting membrane potential (Fein et al, 1983; Nordin et al, 1985) and cellular cAMP levels (Ingebretsen et al, 1981); each of which has the capacity to significantly alter the availability and gating kinetics of membrane Ca^{2+} channels. Based on these considerations, it seems reasonable to suggest that cellular Ca^{2+} entry during depressed plateau voltages is either reduced or remains unaltered in diabetes. If the above assumptions are correct, this leaves the electrogenic Na^+ - Ca^{2+} exchange process as a possible means of maintaining or contributing to the rise of $[Ca^{2+}]_i$ during contraction. Recent work on voltage-clamped ventricular cells from a wide variety of species has indicated that force of contraction can be increased independent of changes in pooled SR Ca^{2+} stores, presumably due to quantitative changes in $[Na^+]_o$ -dependent cytosolic Ca^{2+} efflux (Brill et al, 1986; Boyett et al, 1987; duBell and Houser, 1989). This may be indirectly related to the reduced Na^+ - K^+ ATPase activity (Onji and Lui, 1980), elevated $[Na^+]_i$ (Regan et al, 1981), decreased resting membrane potential (Fein et al, 1983; Nordin et al, 1985), and prolonged action potential

duration (Fein et al, 1983; Nordin et al, 1985) reported in diabetic animals. Furthermore, the high degree of postrest potentiation in untreated rat ventricular muscle has been shown to be a composite function of beat-dependent recycling of Ca^{2+} through the SR (Orchard and Lakatta, 1985; Schouten et al, 1987) combined with a small net cellular Ca^{2+} gain during the course of rest through the activity of the Na^+ - Ca^{2+} exchange process (Shattock and Bers, 1989). Thus, the observation that the response of preparations obtained from control and diabetic animals were identical to stimulation patterns designed to increase the time spent by the muscle in a depolarized state (frequency-force relation), and gradual lengthening of the rest interval preceding the resumption of electrical stimulation or rapid cooling suggests that stimulation-dependent changes in the extent and direction of Na^+ - Ca^{2+} exchange may be relatively unperturbed at this stage of disease.

In summary, the positive correlation between the duration of diabetes and the marked reduction in twitch force and slowing of contraction was demonstrated in both 1.25 and 2.5 mM $[\text{Ca}^{2+}]_o$. The decreased force of contraction was associated with a proportional decrease of pooled SR Ca^{2+} content and release, assessed independently by rapid cooling contracture and postrest contraction. The observation that diabetes-related defects of SR Ca^{2+} handling could be reversed following an increase of $[\text{Ca}^{2+}]_o$ suggests that depressed contractions were the result of depleted SR Ca^{2+} stores, and not the consequence of chronic $[\text{Ca}^{2+}]_i$ -overloading. The ability of diabetic ventricular muscle to respond to changes in stimulus interval remained relatively intact, although changes in the fraction of cytoplasmic Ca^{2+} for contraction derived from SR stores suggest that compensatory mechanism(s) may evolve to oppose the negative inotropic effect of diabetes.

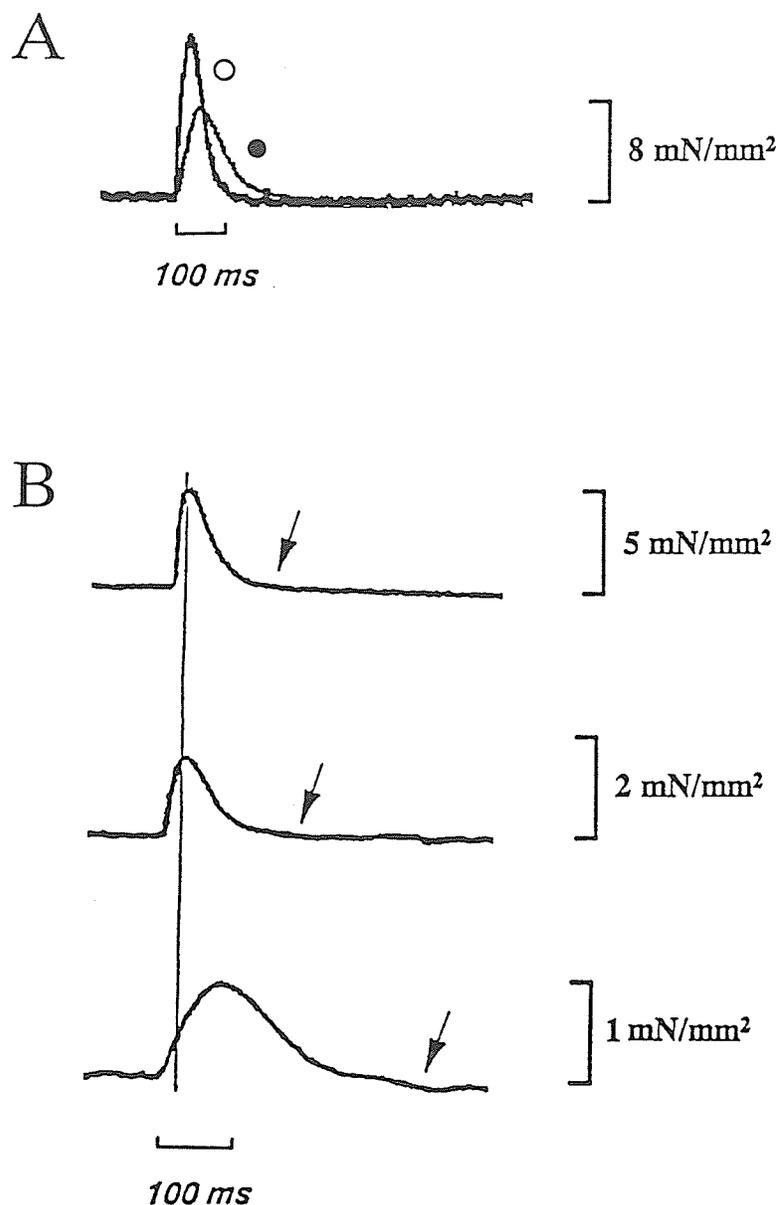


Figure 41. Influence of streptozotocin-induced diabetes on the peak and time course of isometric contraction. *A*) Superimposed recordings of contractions elicited in response to steady-state stimulation in 1.25 mM $[Ca^{2+}]_o$ in trabeculae isolated from eight week diabetic (●) and age-matched control (○) rats. *B*) Recordings of steady-state contraction at 0.2 Hz in muscles obtained from four week control (top), four week diabetic (middle), and eight week diabetic (bottom) rats. $[Ca^{2+}]_o$ was 1.25 mM. Vertical line indicates time to peak contraction in the control recording. Arrows illustrate the time required for complete relaxation for each contraction.

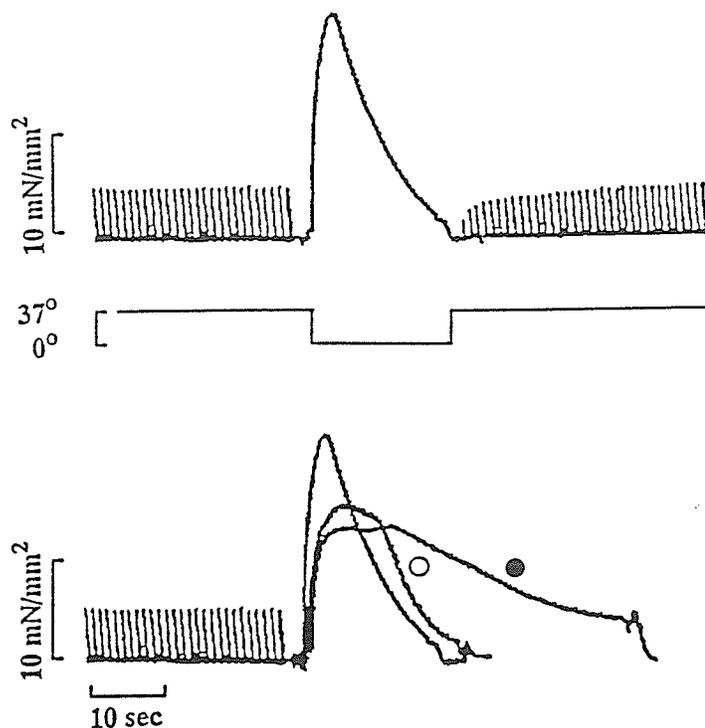


Figure 42. Steady-state rapid cooling contracture in the presence of 1.25 mM $[Ca^{2+}]_i$. A) Rapid cooling (< 1 sec) of a ventricular preparation isolated from a four week control rat heart produces a accompanying contracture which relaxes gradually toward the level of resting tension present before cooling. Following rewarming and the resumption of electrical stimulation, steady-state contraction is re-established over the next 60-90s. B) Superimposed cooling contractures obtained at 1.0 Hz stimulation from four week control (see top panel), and four (○) and eight week (●) diabetic muscles. Steady-state contractions have been scaled to facilitate comparison of RCC profiles.

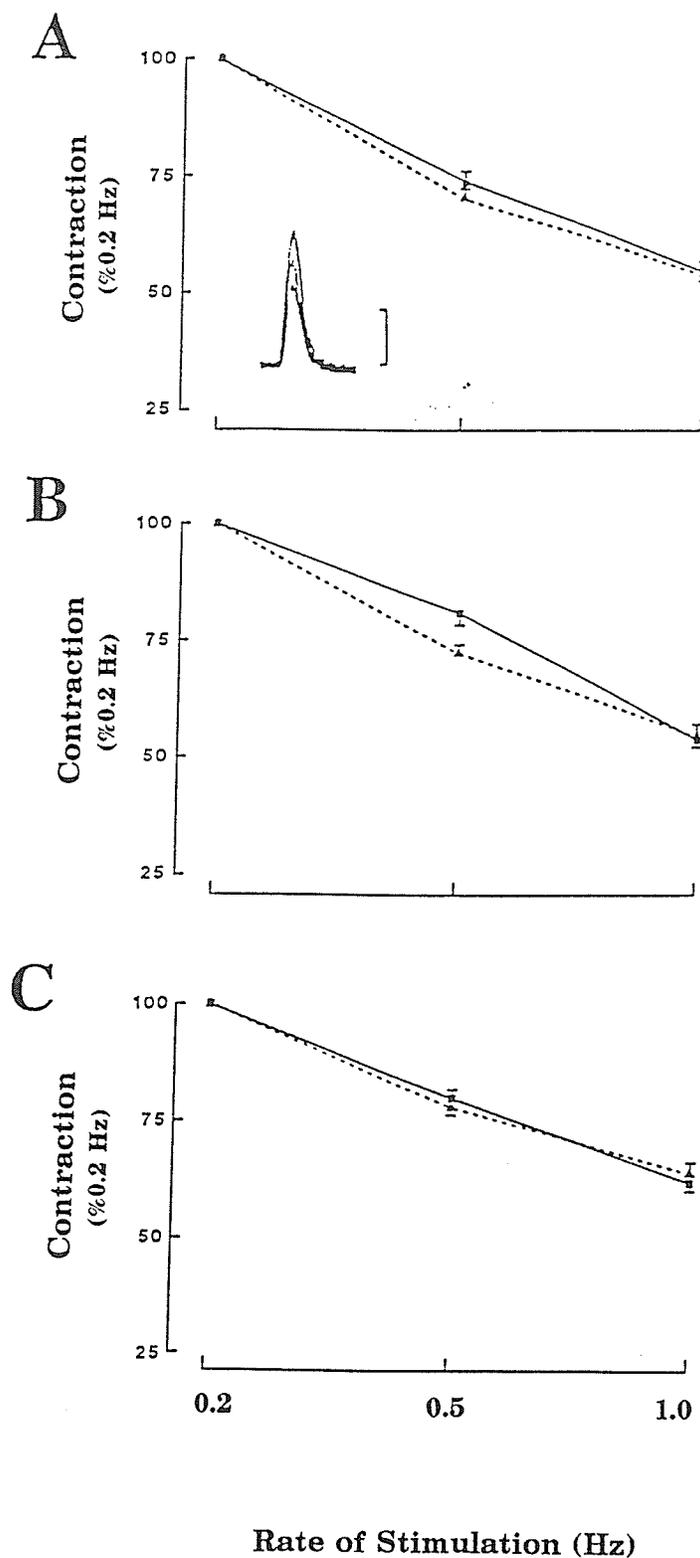


Figure 43. Influence of diabetes on the frequency-force relationship. The inset shows the effect of stimulus frequency on isometric contractions elicited from a trabecula obtained from a four week control rat bathed in 1.25 mM $[Ca^{2+}]_o$. Average frequency-responses are illustrated for muscles from four week control (top), four week diabetic (middle), and eight week diabetic (bottom) rats. Data are plotted for experiments conducted in 1.25 mM (dashed line) and 2.5 mM (solid line) $[Ca^{2+}]_o$.

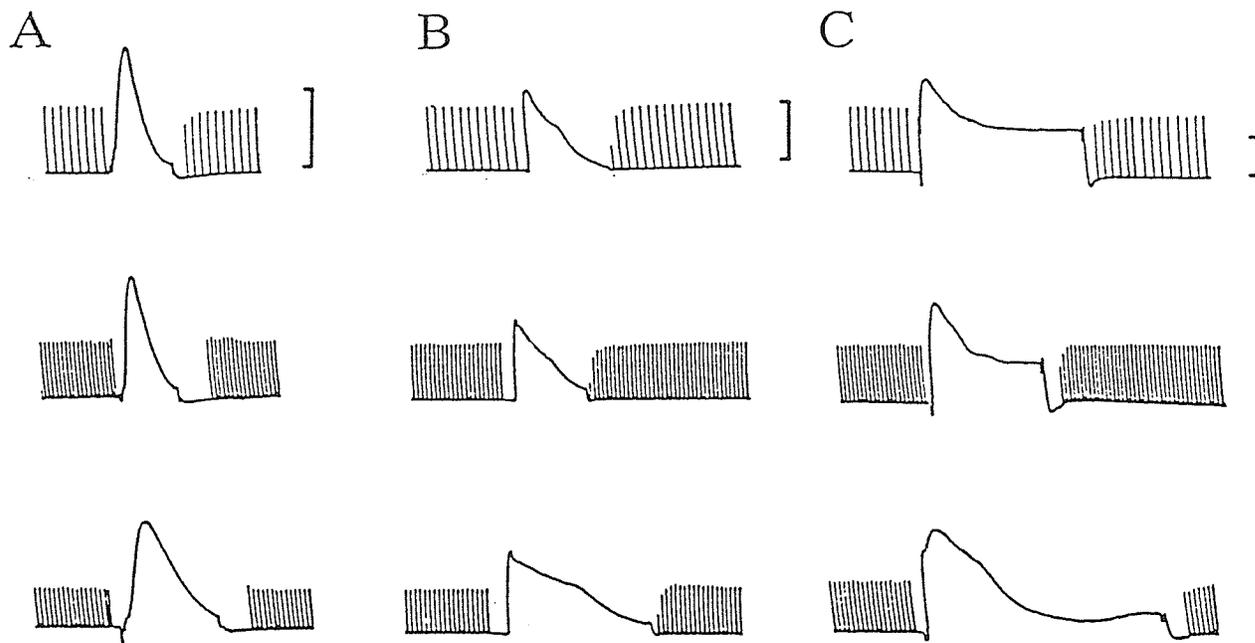


Figure 44. Effect of stimulus frequency on steady-state cooling contracture. Typical recordings obtained from four week control (*A*), four week diabetic (*B*), and eight week diabetic (*C*) rats. All muscles were bathed in 1.25 mM $[Ca^{2+}]_o$ and stimulated at 0.2 Hz (top), 0.5 Hz (middle), and 1.0 Hz (bottom). Vertical calibration bars denote force development of 15, 8, and 6.5 mN/mm² for panels *A-C*, respectively.

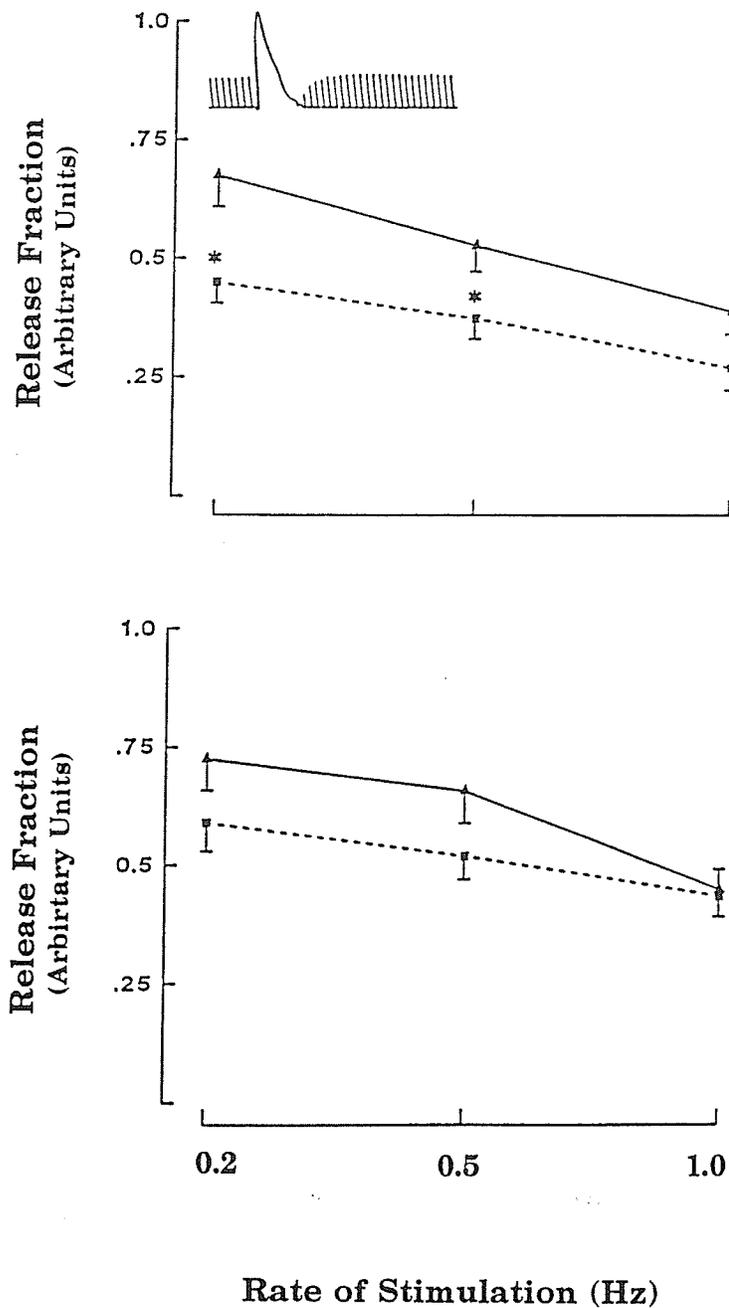


Figure 45. Calculated "release fraction" as a function of stimulus frequency. The stimulation protocol is shown in the inset. Values were calculated by obtaining the ratio of peak steady-state contraction to peak steady-state cooling contracture (SSC mN/mm²/SSRCC mN/mm²) at the 3 test frequencies. The responses of 6 eight week control (\blacktriangle) and 6 eight week diabetic (\blacksquare) muscles to cooling have been averaged. Data shown for experiments performed in 1.25 mM (top) and 2.5 mM (bottom) [Ca²⁺]_o. Note the partial restoring influence of increasing the rate of stimulation or [Ca²⁺]_o. Data are presented as the mean \pm S.E.M. of 6 experiments. * = $P < 0.05$.

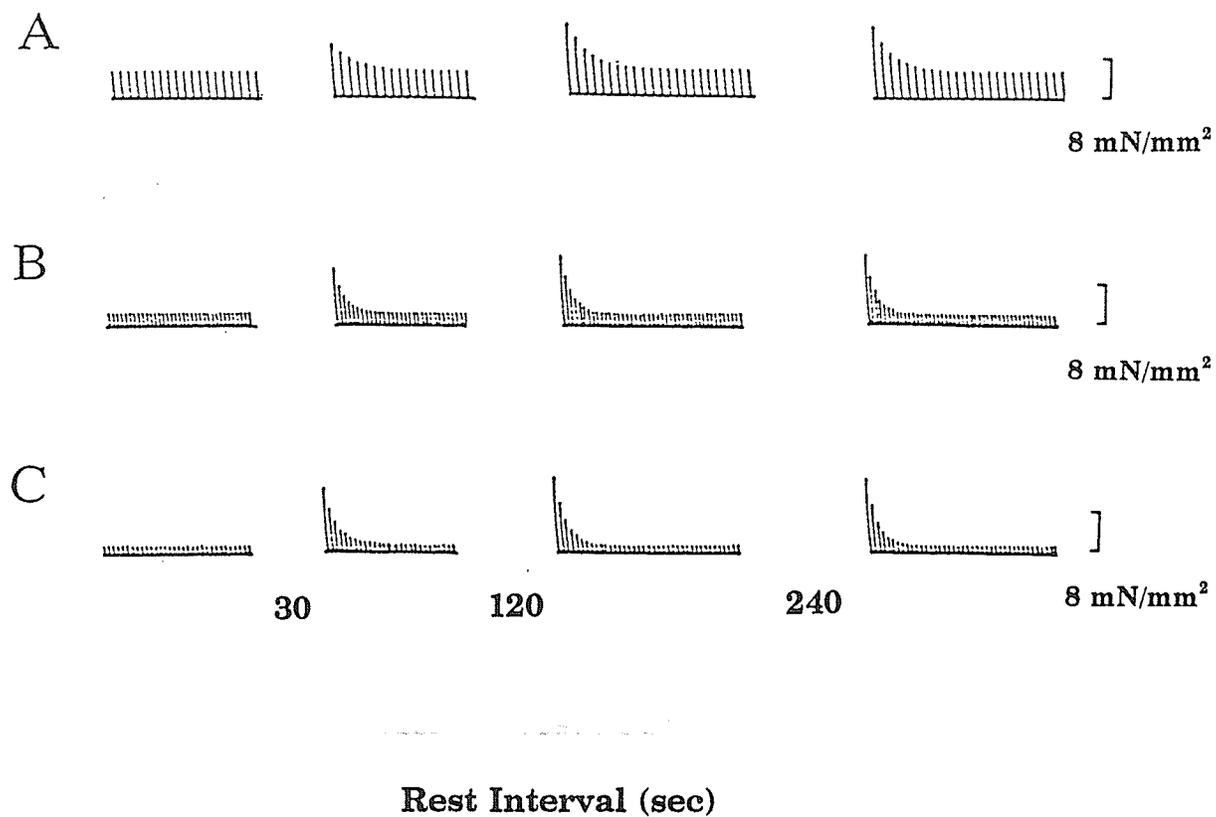


Figure 46. Postrest contractions recorded from a trabecula isolated from a four week control rat bathed in 1.25 mM $[Ca^{2+}]_o$. Rest intervals of 30-240s were interpolated between trains of steady-state stimulation at 0.2 (A), 0.5 (B), and 1.0 (C) Hz.

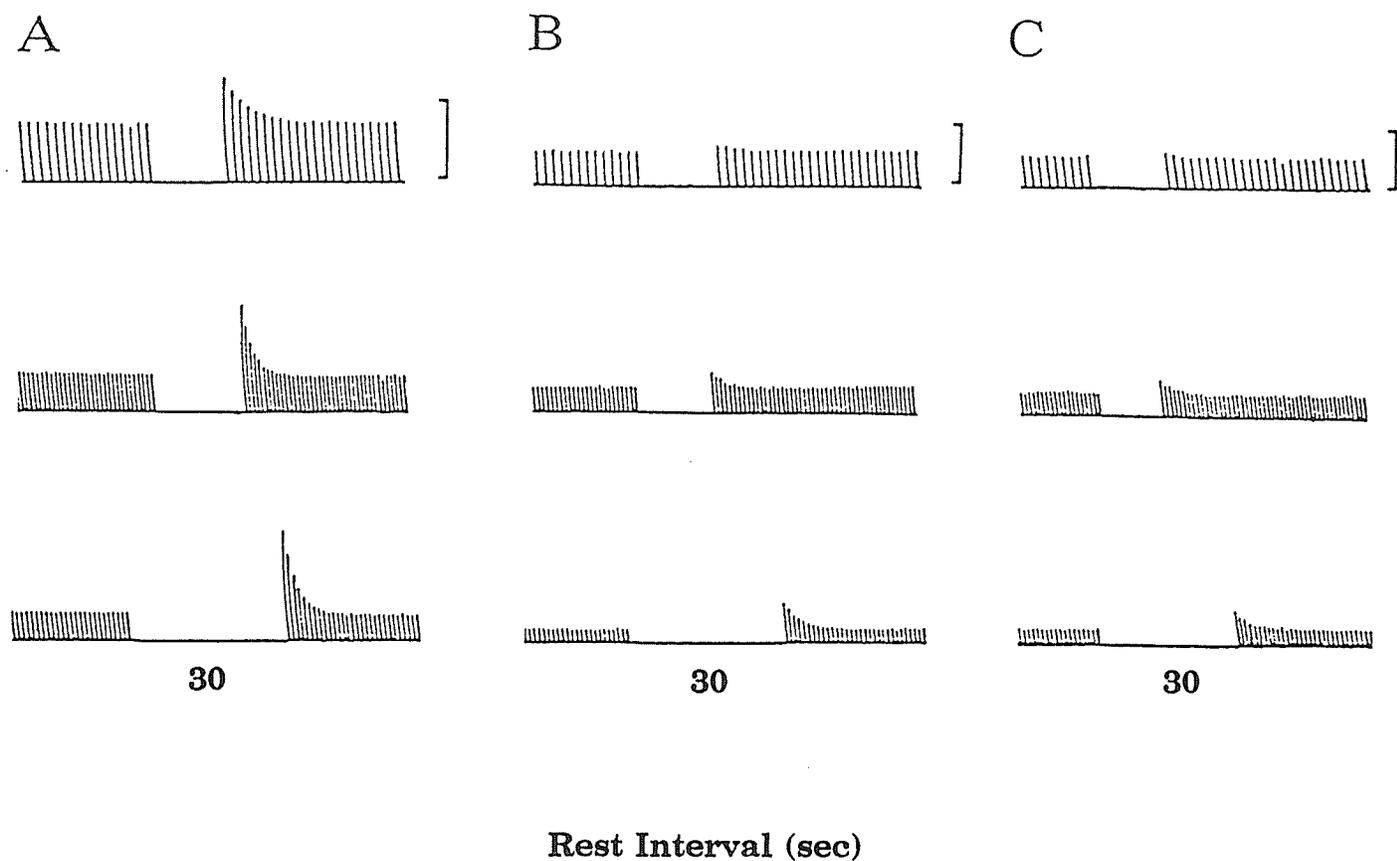


Figure 47. Influence of diabetes on postrest contraction. Representative recordings are illustrated for muscles obtained from four week control (*A*), four week diabetic (*B*), and eight week diabetic (*C*) rats in the presence of 1.25 mM $[Ca^{2+}]_o$. Steady-state stimulation frequency in these experiments was 0.2, 0.5 and 1.0 Hz and are arranged from top to bottom in each panel. Vertical calibration bars at the top of each panel represent force production of 20, 8, and 6.5 mN/mm² for panels *A-C*, respectively.

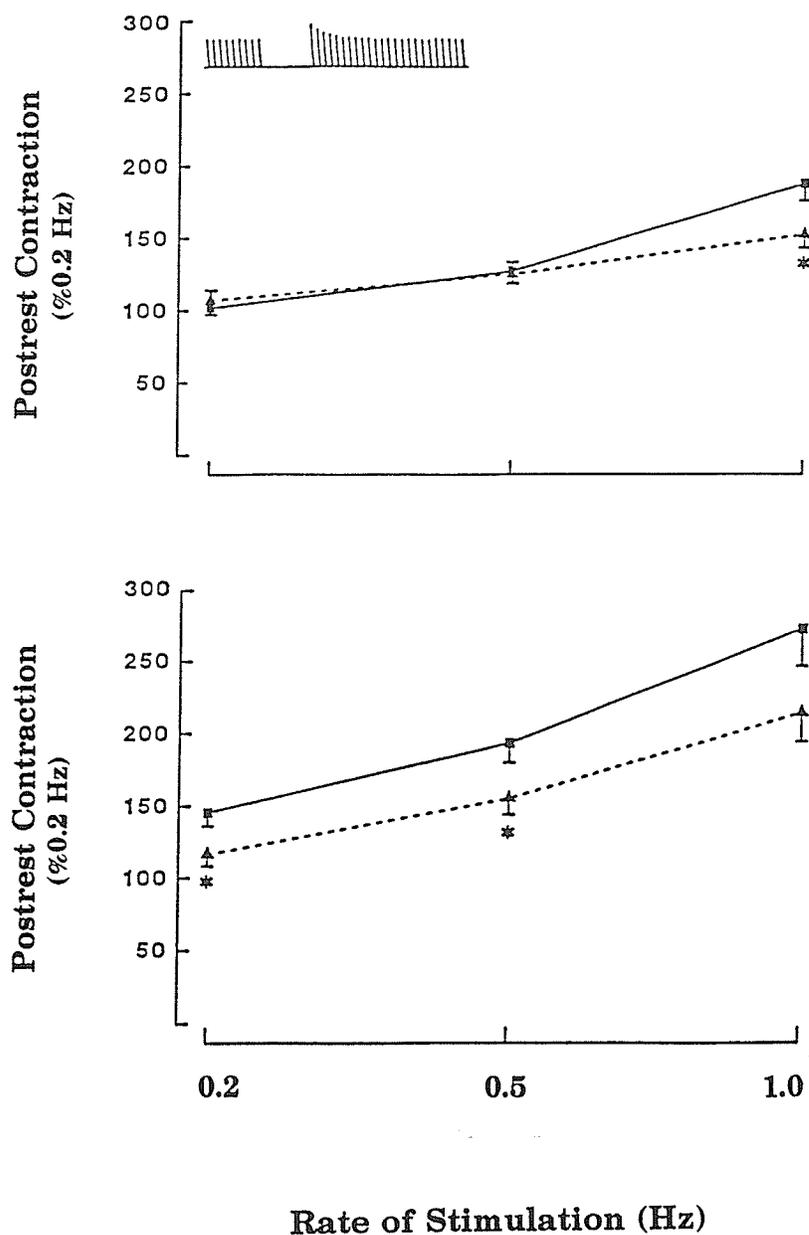


Figure 48. Effect of stimulus frequency on postrest potentiation in eight week control (■) and eight week diabetic (▲) rats. As shown in the inset, contractions were elicited following a 30s rest interval. The degree of postrest potentiation was estimated by calculating the difference between the steady-state and postrest contractions at each test frequency (PRC $\text{mN}/\text{mm}^2/\text{SSC}$ mN/mm^2) and comparing the values to those obtained at 0.2 Hz. Calculations were made from the pooled data given in Table 1. Each point represents the mean \pm S.E.M. of 10 experiments in each group. * = $P < 0.05$.

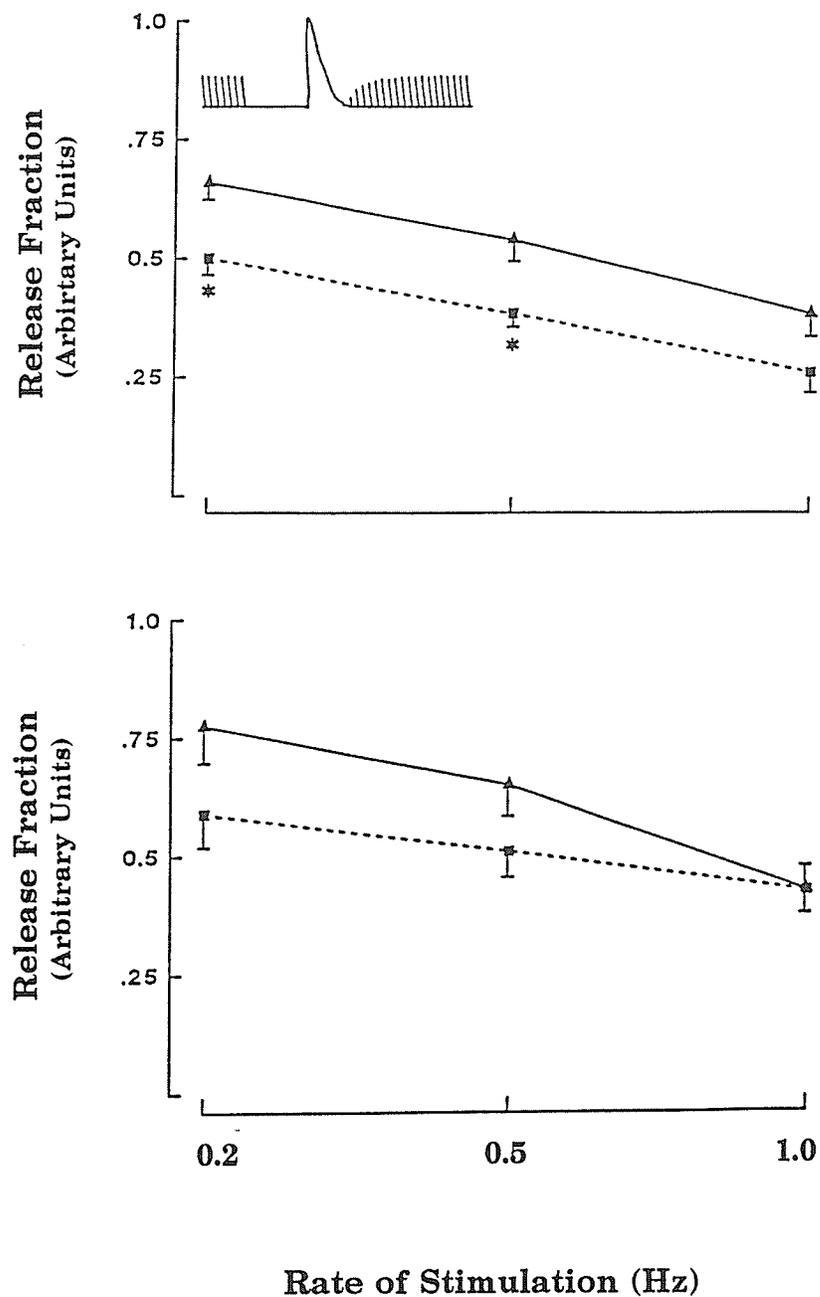


Figure 49. Effect of stimulus frequency on postrest RCC. The inset illustrates the stimulation protocol, in which RCCs were elicited after a 30s rest interval. A) Data averaged from 6 eight week control (▲) and 6 eight week diabetic (■) rats. $[Ca^{2+}]_o$ in these experiments was 1.25 mM. B) Data obtained from the same muscles after increasing $[Ca^{2+}]_o$ to 2.5 mM. Note that increasing the rate of stimulation in 1.25 mM $[Ca^{2+}]_o$ or elevating $[Ca^{2+}]_o$ to 2.5 mM partially restored depressed postrest RCC to the same degree as that observed earlier for postrest contraction (Fig.8) and steady-state RCC (Fig.5).

Table 6. Effect of streptozotocin-induced diabetes on the peak and time course of isometric contraction in rat ventricular muscle.

	[Ca] _o 1.25 mM				[Ca] _o 2.5 mM			
	4W CTL	4W DIAB	8W CTL	8W DIAB	4W CTL	4W DIAB	8W CTL	8W DIAB
DT (mN/mm ²)								
A. SSC								
0.2	4.6±0.5	2.9±0.3	6.4±0.77	3.0±0.29	11.3±1.1	5.7±0.6	15.6±1.9	5.2±0.6
<i>P</i>		<0.05		<0.05		<0.05		<0.01
0.5	3.1±0.37	2.0±0.2	5.0±0.6	2.2±0.2	8.5±0.93	4.8±0.5	12.9±1.5	4.3±0.47
<i>P</i>		<0.05		<0.05		<0.05		<0.01
1.0	2.3±0.3	1.5±0.16	3.6±0.43	1.7±0.15	6.0±0.6	3.3±0.45	8.2±0.98	3.3±0.3
<i>P</i>		<0.05		<0.05		<0.05		<0.05
B. PRC								
0.2	7.8±1.3	5.2±0.68	9.8±1.6	3.5±0.57	11.7±1.9	8.9±1.2	13.9±2.2	5.6±0.7
<i>P</i>		NS		<0.05		NS		<0.05
0.5	8.3±1.4	5.4±0.7	9.4±1.6	3.4±0.56	11.3±1.9	9.6±1.25	13.8±2.2	5.4±0.75
<i>P</i>		NS		<0.05		NS		<0.05
1.0	8.7±1.7	5.8±0.75	9.9±1.7	3.8±0.62	10.4±1.7	9.2±1.2	13.4±2.1	4.5±0.63
<i>P</i>		NS		<0.05		NS		<0.05
TPT (ms)	82.4±2.4	87.2±4.8	88.7±4.4	126.7±12	00±00	00±00	00±00	00±00
<i>P</i>		NS		<0.01		NP		NP
RT (ms)	140±13	195±7.9	140±6.7	227±22	00±00	00±00	00±00	00±00
<i>P</i>		<0.01		<0.01		NP		NP

DIAB= STZ-induced diabetic rats; CTL= Age-matched controls; DT= Developed tension; SSC= Steady-state contraction; PRC= Postrest contraction; TPT= Time to peak tension; RT= Time for complete relaxation; 0.2-1.0= Frequency of stimulation (Hz); NS= Not significant; NP= Analysis not performed; Values are presented as the mean ± S.E.M. of 10 experiments; *P* values refer to comparisons between diabetic and age-matched controls.

Table 7. Effect of streptozotocin-induced diabetes on the peak and time course of rapid cooling contracture in rat ventricular muscle.

		[Ca] _o 1.25 mM				[Ca] _o 2.5 mM			
		4W CTL	4W DIAB	8W CTL	8W DIAB	4W CTL	4W DIAB	8W CTL	8W DIAB
RCC (mN/mm ²)									
A. SS	0.2	16.9±2.0	8.6±0.9	16.7±1.8	7.3±0.6	16.8±2.5	12.6±1.6	21.5±2.3	8.8±0.7
	<i>P</i>		<0.05		<0.05		NS		<0.01
	0.5	15.7±2.1	7.8±0.86	16.1±1.6	7.0±0.63	16.0±2.4	12.8±1.2	19.6±2.1	8.2±0.5
<i>P</i>			<0.01		<0.05		NS		<0.01
	1.0	14.3±2.0	7.8±0.86	16.5±1.3	6.6±0.46	15.4±2.3	11.8±1.5	18.3±1.6	7.6±0.6
<i>P</i>			<0.01		<0.05		NS		<0.01
B. PR	0.2	16.7±2.0	9.2±0.83	16.5±1.5	7.4±0.51	17.1±2.0	11.4±0.85	20.3±1.8	8.6±0.6
	<i>P</i>		<0.05		<0.05		<0.05		<0.01
	0.5	15±1.75	8.1±0.6	16.7±1.5	7.0±0.5	15.7±1.9	12.5±0.93	19.5±1.7	8.2±0.55
<i>P</i>			<0.05	<0.05		NS		<0.01	
	1.0	13.4±1.6	9.3±0.7	17.3±1.4	6.5±0.45	15.6±1.8	12.3±1.0	19.0±1.7	7.6±0.52
<i>P</i>			<0.05		<0.01		NS		<0.01

DIAB= STZ-induced diabetic rats; CTL= Age-matched controls; SS= Steady-state RCC; PR= Postrest RCC; 0.2-1.0= Frequency of stimulation (Hz); NS= Not significant; Values are presented as the mean ± S.E.M. of 10 experiments; P values refer to comparisons between diabetic and age-matched controls.

GENERAL DISCUSSION

The aim of the experiments described above was to identify and measure the contribution of different cellular Ca^{2+} pools involved in raising the level of myoplasmic Ca^{2+} during contraction of mammalian cardiac muscle. In general, alterations in the rate and rhythm of stimulation resulted in inotropic changes which were consistent with accompanying alterations both of time-averaged Ca^{2+} entry into the cell per unit time expressed as Q_{Ca}/min , and shifts in the dominant mode of the sarcolemmal $\text{Na}^+-\text{Ca}^{2+}$ exchange process. The precise strength and time course of contraction during different patterns of electrical stimulation was demonstrated consistently to be proportional to the magnitude of Ca^{2+} release from intracellular stores, independent of frequency-dependent sarcolemmal Ca^{2+} movements mediated by $\text{Na}^+-\text{Ca}^{2+}$ exchange. Results obtained from experiments in which $[\text{Na}^+]_o$ was altered suggest that the exchange process can influence the strength of contraction both in concert with and independent of the amount of Ca^{2+} available for release from SR stores, and that this latter process may occur either by altering the trigger for release or by affecting the myofilaments in a more direct fashion. Changes in the amount of Ca^{2+} sequestered by the SR at different rates of stimulation were found also to depend on both time-averaged Ca^{2+} entry into the cell through dihydropyridine-sensitive Ca^{2+} channels, and the accompanying shift in the dominant mode of the exchange process. Finally, comparative data obtained from different species implies that the degree to which sarcolemmal Ca^{2+} entry via I_{Ca} and $\text{Na}^+-\text{Ca}^{2+}$ exchange (or inhibited efflux) influences contraction, via a frequency-dependent interaction with the SR, may depend significantly on the absolute size of the intracellular compartment involved, and hence, the relative buffering capacity for intracellular [free Ca^{2+}] within a given species.

A Working Hypothesis of EC-Coupling for Mammalian Ventricle

In order to facilitate a more complete understanding of the effect of altered stimulation frequency on contraction of isolated cardiac muscle we have constructed a schematic working hypothesis, or model. The model assumes that peak force during isometric contraction is proportional to the transient peak of intracellular [free Ca^{2+}] during contraction. A significant modification from the model described in the INTRODUCTION, is the inclusion of a third intracellular compartment from which Ca^{2+} can be derived for contraction. The location of this compartment inside the cell but separate from the SR is in general similar to the rapidly-exchanging "unsaturated fast pool" of Ca^{2+} described by Rich, Klassen and Langer (1988) or the "exchange compartment", $E_T(n)$ of Schouten, vanDeen, deTombe and Verveen (1987). However, its identification near the release site of the SR stems from experiments conducted with both the racemic form and purified enantiomers of the dihydropyridine Ca^{2+} channel agonist BAY K 8644, and confirmed in later work exploring the role of the $\text{Na}^+ - \text{Ca}^{2+}$ exchange process on the interval-dependence of contraction. Although our results do not allow a direct comparison with the 100 angstrom diffusion-restricted compartment discussed by Lederer, Niggli and Hadley (1990), they do raise the issue of how the frequency-dependence of contraction is coupled to accompanying sarcolemmal Ca^{2+} movements through voltage-gated channels and coupled transport mechanisms, and whether or not Ca^{2+} released in response to stimulation and the continual leak of Ca^{2+} from the SR are occurring from the same Ca^{2+} -channel complex within the terminal cisternae.

A schematic representation of the cellular mechanisms coupling depolarization of the membrane with contraction has been furnished in Fig. 50. Our data are consistent with the notion that the force of contraction is strongly dependent on the kinetics of Ca^{2+} movement between distinct functional compartments within an intracellular space. As discussed in the INTRODUCTION, the volume of Ca^{2+} taken up into the longitudinal tubules of the SR strongly influences the amount of Ca^{2+} available for release through its effect on the rate of Ca^{2+} transport

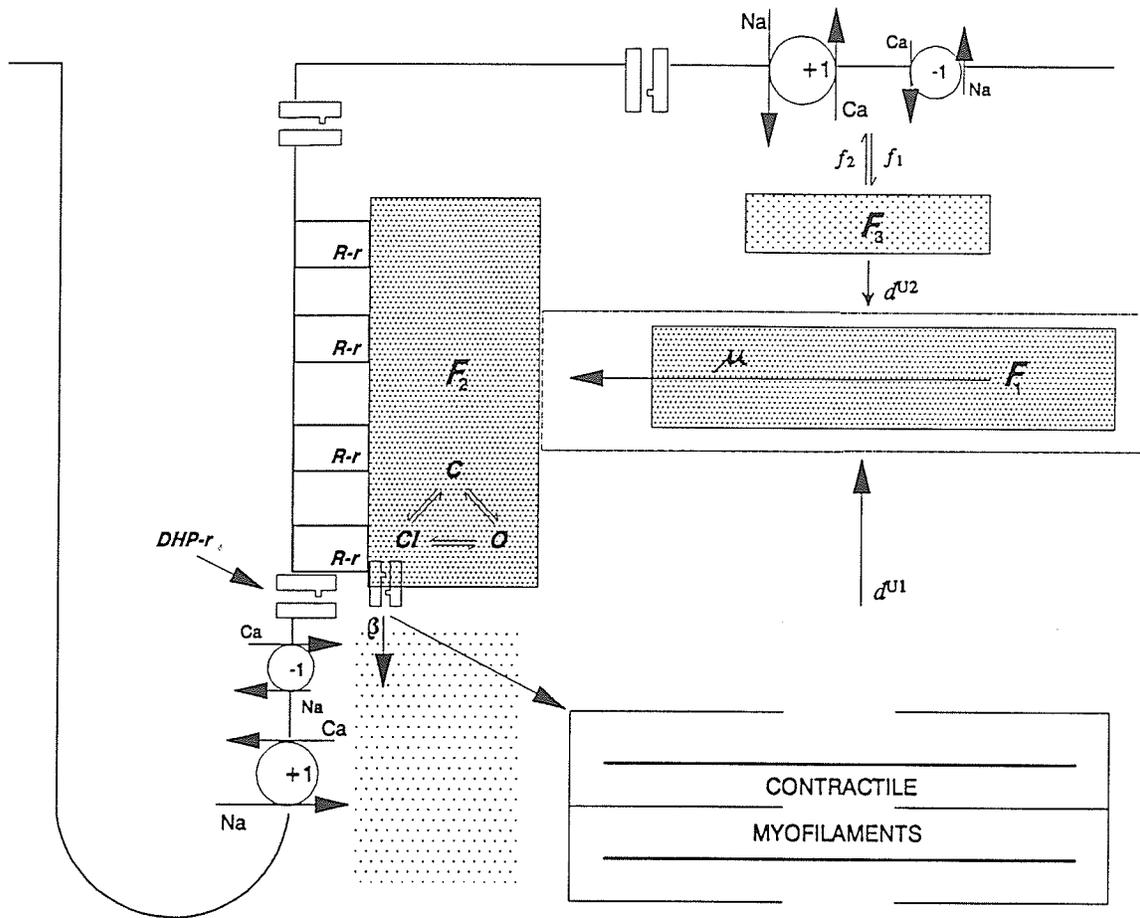


Figure 50. Schematic 3-compartment model of the cellular mechanisms mediating the rise of intracellular [free Ca^{2+}] during contraction of mammalian cardiac muscle. See following page for legend.

Figure 50. Schematic 3-compartment model of the cellular mechanisms mediating the rise of intracellular [free Ca^{2+}] during contraction of mammalian cardiac muscle. Distribution of total cell Ca^{2+} available for contraction within 3 functional intracellular compartments, F_1 , F_2 and F_3 . F_1 and F_2 represent the amount of Ca^{2+} present within the "uptake" and "release" compartments of the SR, whereas F_3 represents the volume of Ca^{2+} present within a variable-site "exchange" compartment. Assuming a constant state-conductance of the Ca^{2+} -release channel, the rate of flow of Ca^{2+} from F_1 to F_2 is defined by the term u and is directly proportional to the volume of Ca^{2+} within F_1 . The rate of Ca^{2+} leak from the release compartment F_2 is defined by the term B , and is directly proportional to the conducting-state of the Ca^{2+} -release channel (Open-conducting > Closed-inactivated > Closed). The volume of Ca^{2+} in the uptake compartment F_1 is a function of the instantaneous increase of cell Ca^{2+} during the flow of I_{Ca} , represented by the term d^{U1} , plus the volume contributed by the exchange compartment F_3 , defined by d^{U2} . Unlike the unidirectional Ca^{2+} movements between F_1 and F_2 , Ca^{2+} is proposed to move both into and out of F_3 during an individual cardiac cycle. Thus, the volume of Ca^{2+} present within this compartment at a given moment in time is equal to the product of the opposing tendency of Ca^{2+} to move into it (f_1) and Ca^{2+} exit from it (f_2). Junction of the SR with the sarcolemma occurs via the spanning protein/ryanodine receptor complex, here labelled $R-r$. Dihydropyridine-sensitive Ca^{2+} channels are labelled $DHP-r$. A small but significant fraction of these channels is physically linked with the release channel of the SR at the spanning protein/ryanodine receptor complex. Hence, the model predicts that the magnitude of Ca^{2+} release elicited by membrane depolarization is determined by the volume of Ca^{2+} present within F_2 prior to depolarization, which is itself a function of the rates of flow of Ca^{2+} into it (u), and the rate of Ca^{2+} loss from it (B) during the preceding diastolic interval.

during diastole. We have used the conceptual framework of Schouten et al (1987) and defined the rate constant for Ca^{2+} transport from the uptake compartment (F_1) to the release compartment (F_2) as u . The second main determinant of the volume of Ca^{2+} present within F_2 at a given time interval, t , is the rate of Ca^{2+} loss into the restricted volume of the t-tubule, which we have defined here as B . This time-dependent "leak" of Ca^{2+} from SR stores has been demonstrated in almost every mammalian species studied to date (Bers, 1983; 1989; Kurihara and Sakai, 1985; Bridge, 1986; Bose et al, 1988a; Bers et al, 1987; 1989; Hryshko et al, 1989a-c), with the single exception of rat, which appears to *gain* tissue Ca^{2+} during the diastolic interval (Kort and Lakatta, 1988b; Shattock and Bers, 1989). An important aspect of the leak process is that in the absence of Ca^{2+} -overload, the path taken by Ca^{2+} on its way from the release compartment to the extracellular space (Lakatta and Lappe, 1981; Stern et al, 1983; Bers, 1983; Hilgemann et al, 1984; Hilgemann, 1986a,b; Bers and MacLeod, 1986) does not include the myofilaments (Bose et al, 1988b; Hryshko et al, 1989b; Bouchard et al, 1989). Once in the intracellular space, this Ca^{2+} is either pumped back into the SR or extruded out of the cell by a combination of ATP-dependent Ca^{2+} pumps (not shown for clarity) or by the forward mode of the Na^+ - Ca^{2+} exchange process. Because of the large electrochemical gradient for Na^+ , \hat{u}_{Na} , the forward mode of the exchanger will be the dominant mode of operation in the equilibrium state (Mullins, 1979; 1981; Noble, 1986; Difrancesco and Noble, 1985; Hilgemann and Noble, 1987), as E_m will exceed E_{NaCa} by several tens of millivolts (assuming values for Na^+ and Ca^{2+} activities close to those discussed in SECTION 3). This is illustrated in the model as the larger scale depiction of the forward mode ($E_m > E_{\text{NaCa}}$), compared with the reverse mode ($E_{\text{NaCa}} > E_m$) of the exchanger. Taking into consideration the two opposing process of recirculation and leak, the volume of Ca^{2+} present at $F_2(t)$ is thus equal to the rates of flow of Ca^{2+} into it (u) and the rate of Ca^{2+} loss from it (B). Added to this relationship is the volume of Ca^{2+} present within the third compartment, F_3 . This fraction of Ca^{2+} is similar qualitatively to the "exchange compartment" of Schouten et al (1987) or the "fast pool" proposed by Rich et al (1988). A significant difference between this "rapidly" exchanging compartment and the previous two compartments described is that Ca^{2+} is transported both to and from it in a *bi-*

directional fashion, each with its own rate constant. Thus, the volume of Ca^{2+} present within the release compartment at a given time is strongly dependent on the dominant mode of the exchanger, due to the dependence of $dF_2(t)/dt$ on the term $(-u * F_1)$. The physical location of the compartment has been depicted as very near the inner surface of the sarcolemma, although it is highly probable that the precise nature and size of the compartment varies with distance from the t-tubule. We propose that it is this cellular space which Ca^{2+} enters during the process of leak, prior to its extrusion out of the cell. Conversely, Ca^{2+} enters into this compartment from the "other" direction through the reverse mode of the exchanger when $E_{\text{NaCa}} > E_m$ (Difrancesco and Noble, 1985; Hilgemann and Noble, 1987), or indirectly, during the flow of I_{Ca} . The amount of Ca^{2+} contained within this compartment is also a function of the *rate of loss* of Ca^{2+} from it, and hence any condition which retards Ca^{2+} extrusion, such as prolonged depolarization of the membrane (Brill et al, 1986; Boyett et al, 1987; Terrar and White, 1989) or reducing \hat{u}_{Na} , would increase the rate at which F_1 is replenished with Ca^{2+} from this compartment, termed f_1 . Removal of Ca^{2+} from F_3 , either by the forward mode of the exchange ($E_{\text{NaCa}} > E_m$) or ATP-dependent pumps, opposes the net gain of Ca^{2+} in F_1 , at the opposing rate constant, f_2 . As shown by Leblanc and Hume (1990), if the volume of Ca^{2+} present within F_3 changes fast enough ($< 1-5$ ms), the volume of Ca^{2+} within the release compartment will decrease by a certain fraction, due to Ca^{2+} -induced release into the myofilaments. Thus, due to the close physical location of the exchange compartment F_3 to both the uptake compartment (F_1) and the release compartment (F_2) at different points on the longitudinal axis of the cell, Ca^{2+} movement both to and from this compartment can influence the recirculating fraction of Ca^{2+} available for release from F_2 both in a positive (f_1) and a negative (f_2) fashion, depending to a large extent on the dominant mode of sarcolemmal Na^+ - Ca^{2+} exchange.

Leak vs Release of Ca^{2+} from the SR: The time-dependent passive leak of Ca^{2+} from the release compartment of intact cardiac tissues (Bridge, 1986; Hilgemann, 1986a; Bers et al, 1987; 1989; Bouchard et al, 1989; Hryshko et al, 1989c) can be dramatically accelerated by application of compounds which bind to the Ca^{2+} -release channel complex and convert this channel into an

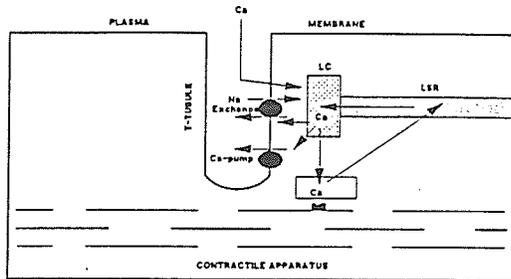
open conducting (Rousseau et al, 1989; Bull et al, 1989) or subconducting state (Rousseau and Meissner, 1987; Ashley and Williams, 1990). The racemic form of the dihydropyridine Ca^{2+} channel agonist BAY K 8644 or the purified (-) enantiomer is unique in this regard because, in addition to its known effects on sarcolemmal Ca^{2+} conductance (Hess et al, 1984; Brown et al, 1984; Thomas et al, 1984), it also markedly accelerates the loss of Ca^{2+} from the SR (Bose et al, 1987; 1988b; Hryshko et al, 1989a,b; Bouchard et al, 1989; Saha et al, 1989; Saxon and Gainullin, 1990). As discussed above, once this Ca^{2+} crosses the membrane of the SR into the myoplasm, it is either extruded out of the cell or back into the SR. An important implication of the influence of BAY K 8644 on SR function is that the channels reputed to control Ca^{2+} release in response to membrane depolarization may be the same pores through which Ca^{2+} "leaks" from the SR during diastole. If so, then these channels may exist for the large majority of diastole in a closed-inactivated (or open-subconducting) state (*C*) with rapid state-transition kinetic fluctuations, which may help explain why increasing the rate of diastolic Ca^{2+} recirculation (μ) reduces the rate constant for recovery of extrasystolic contractions, or why application of ryanodine (Wier and Yue, 1986; Bose et al, 1988a) or racemic BAY K 8644 (Hryshko, 1987) results in a situation where the strength of extrasystolic contraction exceeds that of preceding steady-state contraction. This possibility has been discussed by Cohen and Lederer (1988) who have also suggested that a small but significant fraction of dihydropyridine and ryanodine binding sites are connected by spanning proteins at the junctional face of the t-tubule. Thus, state-transitions of those dihydropyridine-sensitive channels attached to spanning proteins would affect the gating properties of the SR Ca^{2+} -release channel, and hence the volume of Ca^{2+} available for release upon stimulation. Our data are consistent with this interpretation, as well as with that of the restricted diffusion-limited space, from which the macromolecular constituents of the ryanodine-receptor/junctional-SR complex exert their respective influences on cell function. An interesting aspect of the data obtained in low- Na^+ solutions is that the leak of Ca^{2+} from the release compartment, F_2 , can augment the volume of Ca^{2+} present within the uptake compartment (F_1) under conditions where the reverse mode of the Na^+ - Ca^{2+} is inhibited, and that this occurs in the absence of significant changes of resting tension.

Differences in the restitution of postrest contraction and post-extrasystolic potentiation in 140 and 70 mM $[\text{Na}^+]_o$ suggest that reducing \hat{u}_{Na} does not inhibit the rate of Ca^{2+} leak (B), but through its effects on F_3 and f_1 increases the volume of Ca^{2+} taken up into the uptake compartment. The observation that significant diastolic myofilament oscillations can be demonstrated only when the cell is overloaded with Ca^{2+} (Fabiato, 1972; 1975; Lakatta and Lappe, 1981; Stern et al, 1983; Ishide et al, 1984; Allen et al, 1985; Bose et al, 1988b) is compatible with the concept that marked shifts in the concentration of free Ca^{2+} in this diffusion-restricted space may take place without significant activation of the myofilaments. Alternatively, the observation that application of either ryanodine, caffeine or BAY K 8644 significantly prolongs contraction (to differing degrees) seems to suggest that the path taken by Ca^{2+} during the process of leak may be different than that taken during depolarization-induced release.

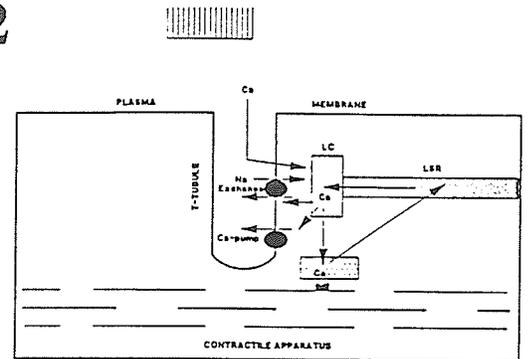
Interval-dependence of contraction

Altered rhythm of stimulation: Figure 51 illustrates the effects of imposing a variable period of rest on Ca^{2+} movements within the SR of canine ventricular muscle. For clarity, only the uptake and release compartments F_1 and F_2 are shown. In panel A1, the primary routes of Ca^{2+} entry and exit from the cell are depicted along with the relative concentration of Ca^{2+} present within each compartment, the latter of which is indicated by the shading density of the individual pools. Resumption of stimulation, shown in panel A2, results in the release of Ca^{2+} from the release compartment into the myofilaments. During rhythmic stimulation, the cycle of contraction and relaxation continues until a steady-state is established with respect to the fraction of Ca^{2+} available for release from F_2 , that present within the uptake compartment, F_1 , and their respective transport and leak rate constants, u and B . The precise value for the fraction of total Ca^{2+} released from F_2 during the process of Ca^{2+} -induced release of Ca^{2+} is unknown, with values estimated from mathematical simulations of intracellular Ca^{2+} movements ranging from 0.75 (Capogrossi et al, 1988; Stern et al, 1988) to 1.0 (Schouten et al, 1987; Jorgenson et al, 1988; see also Bers et al,

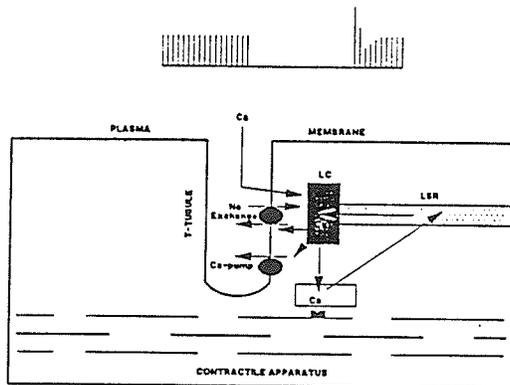
A1



A2



B1



B2

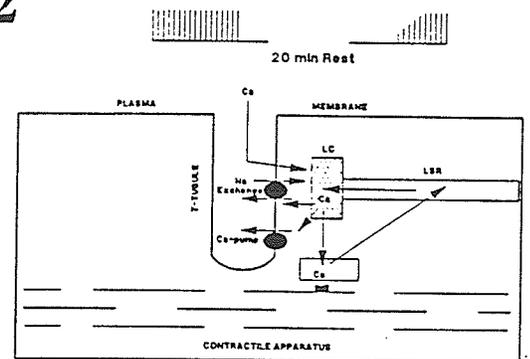


Figure 51. Schematic representation of the effect of alterations in the rhythm of stimulation on intracellular Ca^{2+} movements. The relative volume of Ca^{2+} present within functional uptake (F_1) and release (F_2) compartments is represented by the shading density during the different stimulus conditions. See text for details.

1989). Panel B1 illustrates the influence of artificially prolonging the diastolic interval on the recirculating fraction of Ca^{2+} . In the absence of Ca^{2+} entry into the cell during the period of mechanical quiescence, there is a net loss of cell Ca^{2+} . This occurs at a rate determined by B , which can be measured experimentally as the slope of the time-dependent decay of postrest rapid cooling contracture amplitude (Bridge, 1986; Bers et al, 1987; 1989; Hryshko et al, 1989c; Bouchard et al, 1989) or as a time-dependent repletion of extracellular [total Ca^{2+}] (Hilgemann et al, 1984; Hilgemann, 1986a,b; Bers and MacLeod, 1986). However, due to the large volume of F_1 compared with that of F_2 in canine ventricle, the recirculating fraction of Ca^{2+} available for release in response to stimulation increases up until the point at which Ca^{2+} accumulation within the release compartment is exceeded by the time-dependent leak out of this compartment (see panel B1). For canine ventricular muscle stimulated at 0.5 Hz in 2.5 mM $[\text{Ca}^{2+}]_o$, the peak of mechanical restitution is 120-sec. In skinned canine cardiac Purkinje fibres bathed in solutions maintained at 22°C, Ca^{2+} has been demonstrated to reaccumulate in the release compartment in a biphasic fashion: the first, rapid, phase is complete by 750 ms, and a slower, more gradual, phase completed by ~16-sec (Fabiato, 1985b). Determination of the equivalent restitution of the Ca^{2+} -induced release of Ca^{2+} process indicated that the refractory period for channel reactivation was ~750 ms, after which the amplitude of intracellular Ca^{2+} transients elicited in this fashion restituted quickly, becoming parallel in their time course with caffeine-induced transients after 3.5-sec. Due to the Q_{10} of ~4 for the time-dependence of channel reactivation (Fabiato, 1985b), it seems reasonable to suggest that both short-term (200-1000 ms) and long-term restitution (1-120 sec) curves for intact preparations maintained at 37°C are a reflection of the kinetics of Ca^{2+} transport into and out of the SR. Hence, after 20 minutes of rest (B2), the gradient for Ca^{2+} translocation from F_1 to F_2 is negligible, and postrest contraction is markedly depressed.

The best example of the ability of Ca^{2+} *translocation* within the SR to support contraction comes from the negative staircase in rat ventricular muscle. Here the relatively small changes of pooled SR Ca^{2+} content do not account for the marked reduction of twitch force as the rate of stimulation is progressively enhanced in the 0.2-2.0 Hz range. Close inspection of the differences in

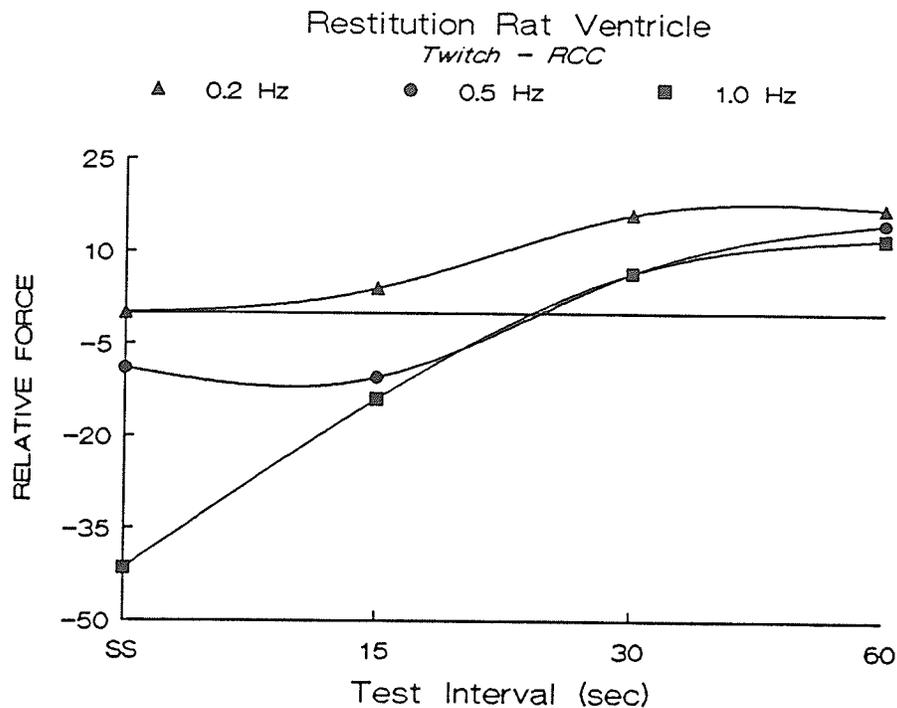


Figure 52. Effect of stimulation frequency on the recirculating fraction of Ca^{2+} available for release in rat ventricular muscle. Each point was obtained by dividing contractions expressed as a function of steady-state contraction at 0.2 Hz by the amplitude of rapid cooling contractures expressed as a function of steady-state cooling contracture amplitude at 0.2 Hz. Points less than unity reflect depressed contractions due to limited repriming of the release compartment with Ca^{2+} (greater change of contraction than accompanying cooling contracture), whereas those points greater than unity reflect increases of contractile force due primarily to time-dependent replenishment of total SR Ca^{2+} content. Data was obtained in 140 mM $[\text{Na}^+]_o$ /1.25 mM $[\text{Ca}^{2+}]_o$ (n=3).

the restitution curves for contraction and rapid cooling contracture amplitude shown in Fig. 52 indicates that the recirculating fraction of Ca^{2+} available for release is reduced in a frequency-dependent fashion. This process is completely overcome by allowing sufficient rest for repriming of the release compartment with Ca^{2+} , which occurs as a composite function in rat ventricle of both Ca^{2+} recirculation (Ragnarsdottir et al, 1982; Schouten et al, 1987; Capogrossi et al, 1988) and net cellular gain of Ca^{2+} via the reverse mode of Na^+ - Ca^{2+} exchange (Kort and Lakatta, 1988b; Shattock and Bers, 1989). The frequency-dependent reduction in the recirculating fraction of Ca^{2+} is likely related both to the extensive physical development of the sarcotubular network in the rat compared to other species (Sommer and Johnson, 1979; 1981; Sommer and Jennings, 1986) and the possibility that stimulation in rat ventricular muscle may be associated with a net cellular loss of Ca^{2+} into the extracellular space via the forward mode of sarcolemmal Na^+ - Ca^{2+} exchange (Shattock and Bers, 1989).

Altered frequency of stimulation: In the working hypothesis discussed above, the recirculating volume of Ca^{2+} available for release from ryanodine-sensitive stores was suggested to be a function of a number of cellular processes which influence the kinetic parameters of Ca^{2+} movements both into and out of the SR ($F_1 + F_2$), as well as those mediating Ca^{2+} movements across the sarcolemma ($I_{\text{Ca}} + \text{Na}^+$ - Ca^{2+} exchange). At the present time there is little information available regarding the relative role of rate-dependent changes in membrane Ca^{2+} conductance to these contractile phenomena. Even without distinguishing between "L-type" and "T-type" Ca^{2+} current, in response to an increase in the rate of rhythmic stimulation peak I_{Ca} per action potential may be either reduced, in conjunction with depressed plateau voltages (Reuter, 1973; Simurda et al, 1976; Mitchell et al, 1985; Mitra and Morad, 1986), increased (Noble and Shimoni, 1981; Mitra and Morad, 1986; Lee, 1987; 1990; Schouten and Morad, 1989), or remain unaltered (Mitchell et al, 1985; London and Kreuger, 1986; Schouten and Morad, 1989). To explain the rate-dependent increase of SR Ca^{2+} availability (Gibbons and Fozzard, 1971; Kurihara and Sakai, 1985; Bouchard and Bose, 1989) or release observed in similar situations (Fabiato, 1985c), it has been necessary to

propose that net cellular gain of Ca^{2+} is due in part to an increase of the integral of time-averaged Ca^{2+} influx (Morad and Goldman, 1973; Allen et al, 1976; Bridge, 1986; Hilgemann and Noble, 1987; Lewartowski and Pytkowski, 1988). We have demonstrated that a significant fraction of this increase of SR Ca^{2+} availability and release (30-35%) is due to sarcolemmal Na^+ - Ca^{2+} exchange. The link between the increase of intracellular [free Ca^{2+}] and uptake by the SR is provided by the strong relationship between myoplasmic Ca^{2+} levels and ATP-dependent Ca^{2+} pump activity (Tada and Katz, 1983) and velocity (Froelich et al, 1978). We have obtained ample proof in support of the proposal (Orchard and Lakatta, 1985; Schouten et al, 1987) that the rate of Ca^{2+} recirculation within pooled SR compartments is directly proportional to the volume of Ca^{2+} within the uptake compartment. This was shown in experiments conducted on isolated canine ventricular muscle bathed in 140 mM $[\text{Na}^+]_o$ as a frequency-dependent increase in both the peak and rate of mechanical restitution, as well as peak post-extrasystolic potentiation and recovery times required for the preceding extrasystolic contractions. The equivalent response observed after lowering $[\text{Na}^+]_o$ to 70 mM indicates that the influence of pooled SR Ca^{2+} content on the mechanical restitution curve is not limited to steady-state conditions. Thus, even in the absence of frequency-dependent alterations of peak steady-state cooling contracture, the restitution curve is shifted up and to the left by the increase of Ca^{2+} uptake by the SR during the extended diastolic interval.

The effect of increasing or decreasing the volume of Ca^{2+} within pooled SR compartments on the recirculating fraction of Ca^{2+} (and hence the force of contraction) can be quite profound, and may become apparent in subsequent beats within the millisecond range (e.g., Lipsius et al, 1982). For example, we found that experimental manipulations which increase SR Ca^{2+} content in rat, canine and guinea-pig ventricular muscle uniformly reduced the time constant for recovery of extrasystolic contractions at coupling intervals between 150-700 ms. The simplest model of unidirectional Ca^{2+} transport predicts that any condition which favours net cellular Ca^{2+} gain would result in an increase in the volume of Ca^{2+} available for release upon stimulation. Hence an increase of $[\text{Ca}^{2+}]_o$ or reduction of $[\text{Na}^+]_o$ increases Ca^{2+} uptake and release by the SR during steady-state stimulation, and decreases the time constant for the exponential recovery of

extrasystolic contraction. Data obtained from the present work suggests that differences in the frequency-dependence of steady-state contraction, peak post-extrasystolic potentiation and postrest potentiation, and rapid cooling contracture amplitude are all consistent with an increase of Ca^{2+} uptake and release by the SR, tending toward saturation in $2.5\text{mM } [\text{Ca}^{2+}]_o/70\text{mM } [\text{Na}^+]_o$ solution (e.g., frequency-dependence of post-extrasystolic potentiation is abolished, with a rank order of $1.25\text{mM } [\text{Ca}^{2+}]_o/140\text{mM } [\text{Na}^+]_o > 2.5\text{mM } [\text{Ca}^{2+}]_o/140\text{mM } [\text{Na}^+]_o \gg 2.5\text{mM } [\text{Ca}^{2+}]_o/70\text{mM } [\text{Na}^+]_o$). This relationship breaks down, however, when extrapolated to perturbations in the strength of contraction resulting from changes in the rate of stimulation. There is no obvious explanation for why the relationship between contractile strength and the time constant for recovery of the extrasystolic contraction becomes quantitatively uncoupled subsequent to altering the rate of stimulation. In the absence of strong support for inactivation of the Ca^{2+} -release channel (and assuming the recovery pattern of extrasystolic contractions is an adequate measure of the recirculating fraction of Ca^{2+}), this difference may be related to the relatively unknown effects of altering the ionic composition on the frequency-dependence of action potential configuration and the underlying currents.

An interesting observation made in SECTION 3 was that an increase in the apparent recirculating fraction of Ca^{2+} can significantly reduce the time constant for recovery for extrasystolic contraction while having no effect on the 150 ms refractory period for premature excitation of the preparation. One possible interpretation of this result is that the release channel of the SR has a minimum of three functional states seen from the *trans*-, or "inside", of the release compartment: (a) a open fully-conducting state, (b) a closed refractory state, and (c) a closed-inactivated (or partially-conducting) state. In this simplified analysis, the open channel configuration would conform to the state elicited by a rapid rise of intracellular [free Ca^{2+}] following depolarization of the membrane. Subsequent to the release of Ca^{2+} , the channel would occupy a closed-refractory state, during which time no further depolarization could elicit a Ca^{2+} release. This period corresponded in our experiments to 150 ms, during which time no clearly observable extrasystolic contraction could be elicited in the absence of drugs known to bind to

dihydropyridine-sensitive Ca^{2+} channels or the junctional-SR/ryanodine receptor complex, despite the production of action potentials displaying both potentiated and prolonged plateau phases (Hryshko, 1987). Recent experiments by Nabauer and Morad (1990) have suggested that an instantaneous release of approximately 25-50 μM intracellular [free Ca^{2+}] by flash-photolysis of DM-nitrophen activates a significant contraction in rat or guinea-pig cells, even if the light pulse was applied immediately after or during a depolarization-induced contraction. Although this result seems to provide evidence against Ca^{2+} -inactivation of Ca^{2+} -induced release of Ca^{2+} from the SR, the applicability of this result to the control of *in vivo* Ca^{2+} release is hampered by the high affinity of DM-nitrophen not only for Ca^{2+} (K_d of 3×10^{-9} M), but also for Mg^{2+} (cf. Fabiato, 1989). Competitive binding of Mg^{2+} to DM-nitrophen would be expected to significantly lower the resting intracellular [free Mg^{2+}], as well as result in a large release of Mg^{2+} during flash photolysis. Thus, although the 150 ms "refractory period" in our experiments may have been related to the restitution of the action potential, its disappearance in the presence of BAY K 8644 or ryanodine (despite differences in the inotropic state of the preparation) suggests that this 150-200 ms period may correspond to the 750-800 ms refractory period for Ca^{2+} -induced release of Ca^{2+} demonstrated in skinned canine cardiac Purkinje fibres at 22°C (Fabiato, 1985b).

Data provided in Figs. 32-36 are consistent with the proposal that once the Ca^{2+} -release channel passes through its apparent refractory state (however brief), that it enters a closed-inactivated state. We have tentatively labelled this a closed-inactivated, as opposed to an open-subconducting, state because our pharmacological data suggests that repriming of the release compartment may not be the limiting factor in determining the strength of extrasystolic contractions elicited in the 100-200 ms range. Wier and Yue (1986) have suggested that the mono-exponential kinetics of recovery for extrasystolic contraction may reflect either reaccumulation of Ca^{2+} within the release compartment, or state-transition of SR Ca^{2+} -release channels as a first-order kinetic process. Our data suggest that both of these processes may be operant in intact tissues, and while an increase in the amount of Ca^{2+} within pooled SR stores can lead to an increase in both the rate and volume of recirculating Ca^{2+} available for release, the second

process can not be altered in the absence of pharmacological intervention. Although no data is presently available regarding the effects of BAY K 8644 on the conductance and gating kinetics of the ~ 90 pS Ca^{2+} -release channel (Meissner, 1986; Smith et al, 1985; Rousseau et al, 1987; Inui et al, 1987; Lai et al, 1987; Ashley and Williams, 1990), past work from this lab has indicated that $1 \mu\text{M}$ BAY K 8644 (Hryshko, 1987; Bouchard, Chau and Bose, unpublished) or 10 nM ryanodine (Bose et al, 1988a; Bouchard, Chau and Bose, unpublished) both abolish the period of time when extrasystolic contractions are smaller than preceding steady-state contractions. This result may be interpreted to suggest that enough Ca^{2+} may be present within the release compartment (F_2) to initiate significantly larger contractions than are observed in the absence of such agents. If so, this conclusion would be significantly tempered by the observation that both BAY K 8644 and ryanodine markedly accelerate B , the rate at which Ca^{2+} is lost from the SR. The consequence of increasing B in intact tissues is that the recirculating fraction of Ca^{2+} at very short coupling intervals would be increased, due to the increased rate of repriming of the release pool. Complex gating properties of the SR Ca^{2+} -release channel have been reported previously (Fabiato, 1983; 1985a-c; Meissner; 1986; Lai et al, 1987; Bull et al, 1989; Ashley and Williams, 1990) and different kinetic models have been constructed to fit the increasingly complex gating kinetics of these and other channels present within the membrane system of the junctional-SR (Wang et al, 1989; Coronado et al, 1989; Escande et al, 1989; Bull et al, 1989; Ashley and Williams, 1990). In so far as validating the present, rather simple, hypothesis is concerned, an important theoretical limitation of the protocol used in these experiments is that it does not permit an estimate of how long a functional closed-inactivated state may last, as the shortest test interval in which cooling contractures were elicited following termination of rhythmic stimulation was 15 sec. Future experiments are needed to resolve this and other limitations discussed in the above analysis.

In summary, the experiments described within this Thesis have illustrated the importance of Ca^{2+} release from intracellular stores in mediating the rise of intracellular [free Ca^{2+}] during contraction. In addition, contractile force was demonstrated to be related to the amount of Ca^{2+} located within a second intracellular "compartment", which was found to influence contraction both in concert with and independent of the total volume of Ca^{2+} present within pooled functional stores residing within the SR. Alteration in the rate and rhythm of stimulation was associated with changes in the force of contraction which could be explained by a combination of the accompanying differences in SR Ca^{2+} uptake and release, and shifts in the dominant mode of operation of the sarcolemmal Na^+ - Ca^{2+} exchange process. Cross-species experiments suggested that the degree to which contractile force depends on the kinetics of Ca^{2+} transport depends strongly on the relative volume of the sarcotubular network within a given species. A 3-compartment model of intracellular Ca^{2+} transport for mammalian ventricular muscle was developed based on data from different mammalian species obtained both before and after altering the ionic composition of the bathing media or in the presence or absence of drugs known to affect various stages of the EC-Coupling cascade. This model was used successfully to attribute contractile changes in a previously established model of cardiomyopathy to defective Ca^{2+} handling by the SR. Although some insight has been gained into the mechanisms controlling the interval-force relation in mammalian ventricular muscle, more experiments are necessary to elucidate (a) the precise role of sarcolemmal Ca^{2+} entry through various voltage-gated Ca^{2+} channels in mediating the frequency-dependence of contraction, (b) the dependence of contractile force on the amount of Ca^{2+} located within the poorly-defined exchange compartment, and how sarcolemmal Na^+ - Ca^{2+} exchange influences contraction independent of the amount of Ca^{2+} available for release from the SR, and (c) the physical relationship between the "diffusion-restricted" compartment of the junctional-SR/t-tubule and the contractile apparatus, as it appears that it is this relatively small volume of the cell from which Ca^{2+} appears to be both released from in response to membrane depolarization and leaks out into during the diastolic interval.

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