An Investigation of the Effects of the Calcium Channel Agonist, BAY k 8644, on Post-Rest Potentiation in Canine Ventricular Muscle

A Thesis Presented to the University Of Manitoba

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

by

Larry Hryshko

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BY

LARRY HRYSHKO

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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Dedicated

to

my family

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ABSTRACT

The effects of the calcium channel agonist, BAY k 8644, were examined on the interval-force relationship in canine ventricular muscle. Rest, post-extrasystolic, and frequency potentiation were examined. These potentiation phenomena are prevented by agents which impair the sarcoplasmic reticulum such as caffeine and ryanodine. Conversely, potentiation is not affected by inhibition of extracellular calcium entry. BAY k 8644 was found to alter the interval-force relationship in a manner which suggested that the ability of the sarcoplasmic reticulum to augment its output was impaired.

This hypothesis was tested using an experimental paradigm which allows distinction between intracellular and extracellular contributions of activator cation to individual contractions. In this model, strontium replaces the majority of calcium in the bathing solution. Biphasic contractions are produced with the initial peak indicative of intracellular calcium release whereas the second peak represents extracellular strontium entry. When interval alterations were imposed on biphasically contracting muscles, potentiation was found to be mediated by an enhanced intracellular calcium release from the sarcoplasmic reticulum. Potentiation was not observed in the presence of BAY k 8644 as augmentation of intracellular release was prevented by this agent.

Electrophysiological studies in untreated muscles suggested that extracellular calcium entry was decreased during potentiated beats as the action potential plateau was depressed. In the presence of BAY k 8644, an apparent increase in extracellular calcium entry was observed for corresponding beats but potentiation did not occur. To investigate the possibility that BAY k 8644 altered the interval-force relationship by producing calcium overload, mechanical and electrical experiments were repeated with different extracellular calcium concentrations. Elevated extracellular calcium partially reversed the inhibitory effects of BAY k 8644 which negated the calcium overload hypothesis. Changes in electrical and mechanical events produced by BAY k 8644 could also be partially reversed by reducing the extracellular sodium concentration or adding ouabagenin.

The size of the intracellular calcium pool was estimated by rapidly cooling the muscle. This technique allows relative comparisons of the sarcoplasmic reticular calcium pool during interval alterations. Untreated muscles exhibited a continual decrease in the size of the SR pool when subjected

to increasing durations of rest. The rate of diastolic efflux was increased in the presence of BAY k 8644. This effect was also partially reversed by elevating extracellular calcium or reducing extracellular sodium.

These results indicate that BAY k 8644 alters the interval-force relationship by impairing sarcoplasmic reticular function. Ryanodine and caffeine were also examined to see if these agents produced similar effects. While both BAY k 8644 and ryanodine accelerate the diastolic efflux of intracellular calcium, they do not share similar electrical effects. Caffeine and BAY k 8644 appear to exert their effects by entirely different mechanisms.

PREAMBLE

This thesis is primarily concerned with an investigation of the effects of a new positive inotropic agent, BAY k 8644, on rest potentiation in canine ventricular muscle. This drug represents a novel approach to increasing cardiac inotropy and provides a useful means of studying mechanisms which control this. My specific focus has been to examine the way in which BAY k 8644 can alter the inotropic memory of canine ventricular muscle. Rest potentiation was the most frequently used paradigm although initial studies included both frequency and post-extrasystolic potentiation. Other pharmacological and inotropic interventions were also examined to gain a better understanding of factors which can alter the interval-force relationship in canine ventricle.

INTRODUCTION

INOTROPIC MEMORY IN CARDIAC MUSCLE

The strength of any given cardiac contraction is dependent on the previous stimulation history of the muscle (Koch-Weser and Blinks, 1963). This is referred to as **inotropic memory**. For example, if an isolated muscle is stimulated at a constant rate, tension development will also be of constant magnitude. However, if a premature beat or extrasystole is imposed on this regular train of stimulation, then both the extrasytole and post-extrasystolic beat yield different tensions than beats from the regular train. Obviously, an isolated electrically stimulated muscle is unaware of the timing of the next stimulus. Therefore, inotropic memory must reside in the restorative processes which enable the regular twitches to be of constant magnitude. At the time of each regularly stimulated beat, the recovery processes are at such levels that the twitch is indistinguishable from the preceding one. Since perturbations in stimulus timing can be in any direction, the result is either a prolongation or reduction in the time allowed for these recovery processes to occur. Thus, the interval-force relationship depends upon the effects of different interval alterations on the recovery processes which influence contractile strength. Examples of different perturbations are considered in the following sections. Throughout this thesis, results from isolated muscle preparations are discussed unless stated otherwise.

FREQUENCY ALTERATIONS

Changing the stimulation frequency results in profound changes in both short and long-term tension development in mammalian cardiac muscle. These changes are referred to as **staircase phenomena** with both positive and negative force staircases being observed (Koch-Weser and Blinks, 1963). The direction of force change depends on numerous factors including initial and new stimulation frequency, metabolic condition of the muscle, and species examined. In a number of mammalian and amphibian species, increasing the frequency of stimulation leads to an increase in developed tension once a new steady state is reached. However, if very high frequencies are examined, tension development is

found to decline. Mammalian cardiac muscle generally shows short-term tension changes which are directionally different from long-term or steady state changes. That is, on going from a lower frequency to a higher one, tension of the beats at the new higher frequency will decrease initially before increasing towards a larger steady state value. Thus both positive and negative staircases are observed for a single frequency intervention. This behavior is also observed when the frequency alteration occurs in the opposite direction. Changing stimulation frequency to a lower value results initially in potentiation before tension reaches a smaller steady state level.

Unfortunately, the above generalizations do not apply to results from all studies. While it is not my intention to provide a complete description of all deviations and anomalies concerning the interval-force relationship, several of these require mention as they provide insight into the mechanism of the more generalized behavior when mechanisms are considered. For this reason, some well known anomalies are discussed. Rat ventricular muscle is unique in that it shows a negative staircase with increasing stimulation frequency although more recent reports suggest this only occurs in thick muscle preparations (0.2-1.2 mm) (Schouten and ter Keurs, 1986). Mouse heart also exhibits a negative staircase relationship with increasing frequency (Stemmer and Akera, 1986). Amphibian cardiac muscle such as frog is also unique in that it only exhibits a monodirectional change with altered frequency. Increases in frequency lead to increased tension without the initial negative staircase observed in most mammalian tissues. The significance of these peculiarities is discussed when mechanisms of the interval-force relationship are considered.

POST-EXTRASYSTOLIC POTENTIATION

Post-extrasystolic potentiation (PESP) describes the ability of a single premature beat to augment the tension developed by a subsequent normally timed beat. Provided the muscle is not refractory during the premature stimulus, post-extrasystolic potentiation is increased with increasing prematurity of the extrasystole. Conversely, force development of the extrasystole is inversely related to its prematurity. This behavior is not observed in frog heart muscle where the extrasystole can actually be

larger than the regular twitches (Morad and Orkand, 1971).

Paired-pacing stimulation can be considered as a variation of PESP. In this paradigm, an extrasystole is coupled to every beat of the regular train. The coupled beats are small (depending on their prematurity), and the beats of the regular train are markedly potentiated. This approach to increasing cardiac inotropy was even considered for clinical use although it has not proved successful.

REST POTENTIATION

Rest potentiation (RP) refers to the augmentation of contraction when a train of regular stimulation is interrupted for a variable interval and then stimulation is resumed. There is wide disparity in the responses of different species to this protocol. For example, rest potentiation is observed in cat (Koch-Weser and Blinks, 1963), rat (Bers,1985), rabbit atria (Hilgemann, 1986), guinea pig and dog. Conversely, post-rest tension is depressed in rabbit and frog ventricle (Bers, 1985). Even within species, the rest response varies depending on which region of the heart is examined. The post-rest response is also dependent on the duration of rest and the stimulation frequency preceding the rest. If very long rest intervals are examined, the post-rest contraction eventually becomes independent of rest interval. This condition is referred to as the **"rested state contraction"** (Koch-Weser and Blinks, 1963).

EXCITATION-CONTRACTION COUPLING

Excitation-contraction (EC) coupling refers to the processes which couple sarcolemmal depolarization to the contractile event. Any useful theory of EC coupling must provide an explanation for the interval-strength relationship as well as numerous other experimental findings. There are several excellent reviews of EC coupling which will not be reiterated (Morad and Goldman, 1973; Wohlfart and Noble, 1982; Chapman, 1983; Winegrad, 1979). This thesis will focus on a composite model of EC coupling and its utility in explaining the obtained experimental results. Current information from newer experiments are integrated into the model where appropriate. The focus is not to provide an overview of

EC coupling but to use available information for interpretation of results.

THE MODEL

The following is a description of the EC coupling model used to assess our experimental results. Conversely, we have used our experimental results to test the model. This model is based on a compilation of our own experimental findings, results from other laboratories, and other EC coupling models. In this last regard, it is very similar to models proposed by Wood, Heppner, and Wiedmann (1969) and subsequent modifications by Wohlfart and Noble (1982) and Bers (1985). Only the shallowest attempt at providing a historical perspective is made since the volume of recent literature in this area precludes the inclusion of older data.

As previously stated, any model of EC coupling must be capable of explaining observed experimental results. Prior to discussing these results, the model is presented (Figure 1). The model assumes that calcium is required for contraction. This concept, originating from the discovery by Ringer in 1883, has progressed to dogma. The sliding-filament theory of contraction is assumed throughout and more recent discoveries postulating a contractile role for myosin are not considered (Pollack, 1986). Reluctantly, the link between energy metabolism and EC coupling is largely ignored as it exceeds the breadth of this thesis. By precluding many exciting and useful bodies of knowledge, the author hopes to avoid presenting a "milky" thesis. Much information must be assumed as dogma otherwise qualifications and reservations will assail the reader. While "Is this true?" could be asked following every sentence, this approach will not be taken. On with "fact".

Both intracellular and extracellular sources are involved in providing the calcium transient responsible for contraction. Evidence for the role of extracellular calcium (Ca_o) is essentially irrefutable because contraction does not occur in the absence of Ca_o. The strength of contraction increases or decreases in the same direction as changes in calcium concentration in the extracellular medium over a wide range. This is different from that observed in skeletal muscle where contraction can continue unaltered for long periods of time with very low concentrations of extracellular calcium. The small size of myocardial cells (2-10 um in diameter) and the absolute dependence on Ca_o has made it attractive to

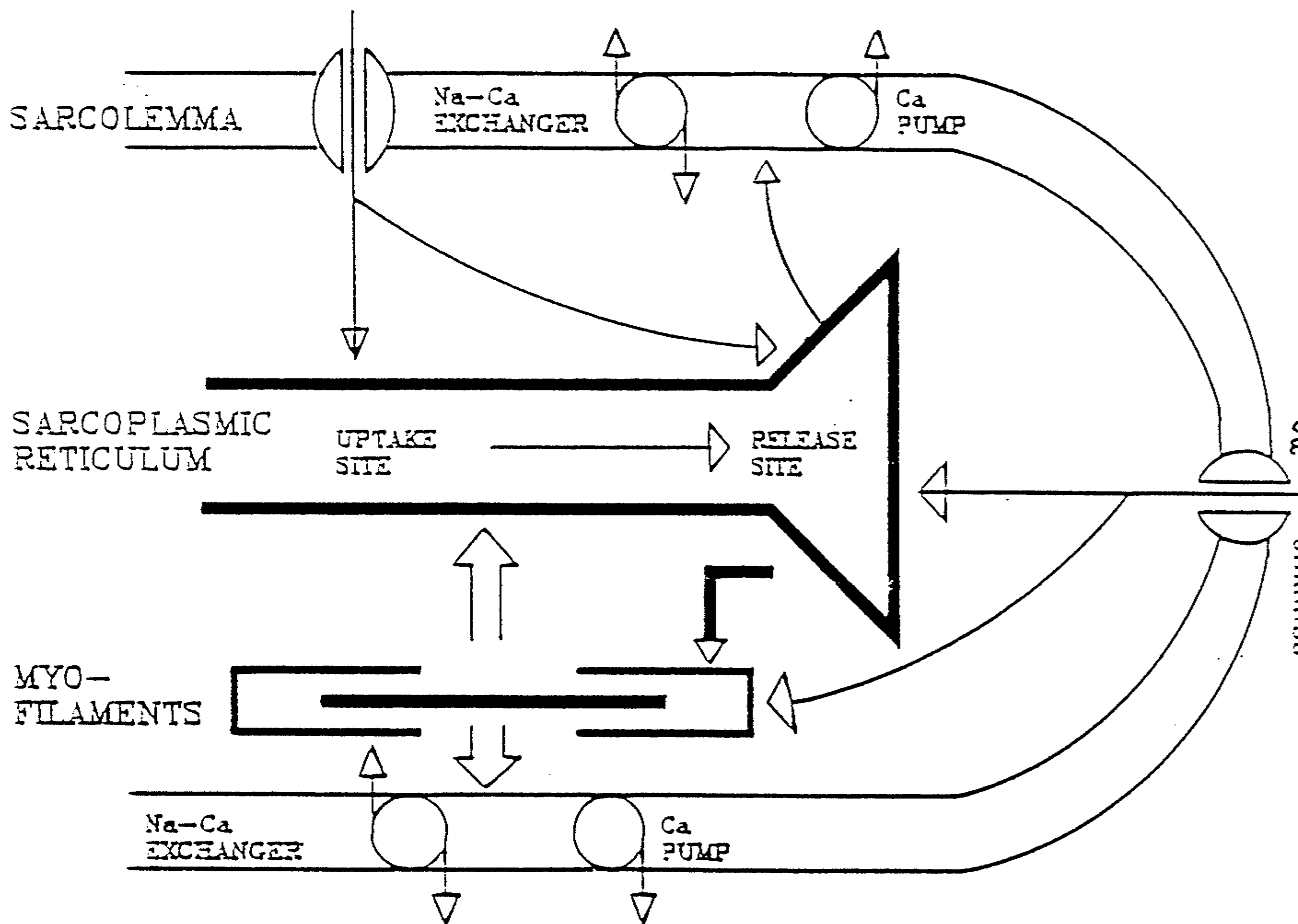


DIAGRAM 1: Model cell illustrating the numerous pathways involved in the regulation of intracellular calcium.

speculate that the contractile proteins are directly activated by extracellular calcium entry. Indeed, this appears to be the case for amphibian heart muscle where tension is exquisitely sensitive to calcium entry during the cardiac action potential. Several current theories of excitation-contraction coupling place major emphasis on the role of Ca_o entry in regulating and supporting the majority of contraction. Included are models by Langer (1972) and Mensing (1981) which suggest extracellular calcium entry is the primary determinant of contractile force development.

In summary, evidence in favor of a dominant role for extracellular calcium entry include:

1. The absolute dependence of contraction on the presence of extracellular calcium.

2. The small size of myocardial cells (relative to skeletal muscle) which should allow for rapid diffusion of Ca_o into and out of the cell.

3. The ability of inorganic cations such as lanthanum and cobalt to uncouple excitation and contraction. These cations displace extracellular calcium from the sarcolemma.

Evidence for the role of intracellular calcium (Ca_i) in contributing to the elevation of cytosolic calcium during contraction comes from different sources. In voltage clamp studies, Ca influx during a beat could be augmented without altering contractile strength of that particular beat. However, contractile force of the subsequent beats were augmented despite normal entry of calcium from the extracellular space. This led these workers (Wood et al, 1969) to postulate that the additional extracellular calcium entry produced by this intervention was not available for direct myofilament activation but was used to provide additional calcium in the intracellular store(s). This additional calcium influx augmented contraction of the next several beats. In experiments where a reverse approach was taken, i.e. calcium entry was decreased during a particular beat, an opposite effect was observed but the same explanation applied (Wood et al, 1969). The beat with the depressed calcium entry was unaffected but subsequent beats had impaired tension development despite normal calcium entry. Again it appeared that this intervention produced this effect by decreasing the size of the intracellular calcium pool. Other critical evidence comes from Fabiato (1975, 1983) who has shown that small amounts of Ca are capable of inducing calcium release from the sarcoplasmic reticulum in skinned cardiac fibres. The sarcolemma is

removed by microdissection and the skinned fibre is then placed in a low calcium containing medium. If small amounts of calcium (less than that required for tension development) are microinjected near this preparation, a larger calcium release is produced which generates tension. This tension production is not directly due to the microinjected calcium acting on the contractile apparatus as tension development is suppressed if the concentration of microinjected calcium is increased. This observation shows that there is an optimal trigger for calcium release and higher levels (which would increase tension if they were directly involved in producing contraction) suppress tension development by causing a supraoptimal trigger for calcium induced calcium release. In most species, it is believed that extracellular calcium entering the cell could not reach the myofilaments without first inducing a calcium release from the SR (Fabiato, 1983).

At present, considerable evidence supports critical roles for both intracellular and extracellular calcium in transiently elevating the intracellular free calcium responsible for contraction. Quantitative arguments revolving around the importance of these are not the subject of this thesis as both are deemed important by this author and their importance varies with so many factors that the focus of the controversy is easily lost. Therefore, experimental findings are now summarized with the intent of providing a coherent account of myocardial cell function as well as an explanation for the interval-force relationship. Properties of each source of calcium are considered in detail with the intent of determining where various 'memory' processes reside.

EXTRACELLULAR CALCIUM ENTRY

At present, two possible routes of extracellular calcium entry receive the majority of attention. These are:

1. Calcium entry through slow calcium channels.
2. Calcium entry by sodium-calcium exchange

Prior to a discussion of these, a rudimentary review of the cardiac action potential is provided.

The cardiac action potential differs from that of skeletal muscle and nerve in that its time course is much longer. It ranges from 100-500 msec compared to a few milliseconds in the others. Furthermore,

its duration and configuration are variable. The reasons for these differences are evident when one considers the different roles played by these tissue types. Nerves transmit frequency coded signals and thus must be capable of rapidly varying signal frequency. Skeletal muscle function ranges from precise and rapid movements (blinking) to sustained and arduous movements (standing). To achieve this, skeletal muscle requires both twitch and tetanic capabilities. Physiologically, this response is permitted by having muscles respond by twitches but also being capable of developing sustained force through summation of twitches. A rapid action potential with a short refractory period is ideally suited for these needs. An entirely different requirement exists in cardiac muscle. The function of the heart is to periodically pump blood and this is best achieved by a phasic contraction followed by a interval allowing for refilling of the ventricle. As such, capabilities for rapid or tetanic contractions are inappropriate and in fact, undesirable. The protracted cardiac action potential serves to eliminate this possibility as the electrical refractory period and contraction are virtually coterminous.

The ventricular action potential can be separated into four distinct phases (diagram 2). Phase 0 or rapid depolarization is mediated by entry of sodium ions through fast sodium channels. Phase 1 represents a rapid repolarization which is mediated by an increase in potassium conductance with a concomitant inactivation of the fast sodium channels. This phase is generally more pronounced in Purkinje fibres and is occasionally absent in the ventricle. Phase 2 is indicative of an inwardly directed calcium conductance balanced by an efflux of potassium. It is this phase which imparts the prolonged refractory period to cardiac muscle. As well, the calcium which enters during this phase is intimately related to contraction. During phase 3, a rapid repolarization to diastolic potential occurs due to an increase in potassium conductance and decreased calcium conductance. Phase 4 represents the maintenance of resting potential in ventricular muscle as there is generally no spontaneous depolarization in this tissue.

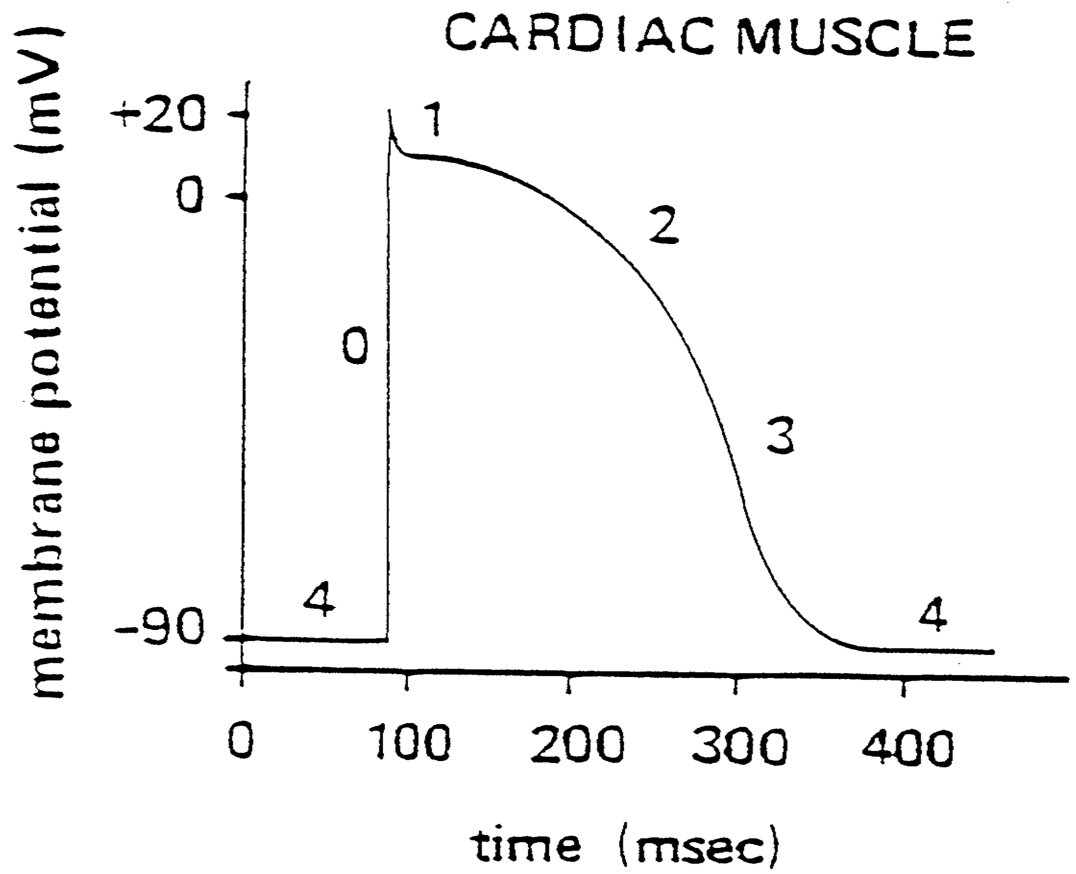


DIAGRAM 2: Typical cardiac action potential. (From Katz, 1977)

THE ROLE OF EXTRACELLULAR CALCIUM ENTRY IN FORCE DEVELOPMENT

This section provides a review of studies which have examined the properties of extracellular calcium entry and the role of this in force production in cardiac muscle. Several different approaches have been used in this regard including:

1. radioisotope flux measurements
2. action potential measurements
3. voltage-clamp studies
4. patch-clamp studies
5. extracellular calcium-selective electrodes
6. extracellular calcium-sensitive dye measurements

Many studies have employed pharmacological or ionic interventions and have examined the effect of different stimulation protocols on measured parameters. This discussion will focus on the latter five techniques as these have time resolutions compatible with rapid interval alterations. Unfortunately, different experimental protocols, and species or tissue examined create a diversity of results and this conundrum is not easily sorted through. Therefore, only salient features from each type of study are discussed. These results still provide a detailed framework from which mechanisms can be ascertained.

ACTION POTENTIAL AND VOLTAGE CLAMP STUDIES

Several excellent reviews (or large papers) have dealt with the electrical events in cardiac tissue and the effects of stimulation protocols, drugs, and ions on these (Boyett and Jewell, 1980; Carmeliet, 1977; Wood et al, 1969; McDonald, 1982; Reuter, 1979). I will exclude information on the rapid depolarization phase of the cardiac action potential as it is of less relevance to this thesis. Information was selected to relate to the results which will be presented later. The focus is on the plateau phase of the cardiac action potential and inferences which can be made about calcium fluxes.

Unlike the all-or-none action potential observed in skeletal muscle and nerve, the cardiac action potential is extremely sensitive to alterations in frequency. In general, the duration of the cardiac action potential is found to shorten with increasing frequency. The necessity for this behavior is obvious when one considers the long duration of the cardiac action potential and its relation to contraction. For example, if we assume an action potential duration of 250 msec at a rate of 60 beats\minute, then the heart is in electrical systole for 25% of the time. If this rate is suddenly increased to 180 beats\minute, the heart would remain in electrical systole for 75% of the time. If changes in both electrical and mechanical duration did not occur, the diastolic intervals would decrease to such short periods that any effect of augmented rate would be offset by inadequate filling time. Fortunately, both durations are markedly decreased in response to increasing frequency and thus diastolic filling time is adequate for augmented cardiac output. The mechanisms for these properties are now considered.

The plateau phase of the cardiac action potential (phase 2) is characterized by a very slow rate of potential change prior to terminal repolarization. This indicates that net currents are either very small or a fine balance exists between inwardly and outwardly directed currents (which could be of large or small magnitude). Again, numerous differences exist between species and different regions of the heart. Presumably, this reflects the existence or prevalence of the ionic currents responsible for the plateau configuration. Several ionic currents are known to be involved during this phase. This includes the slow inward calcium current (I_{si}) which now appears to be comprised of at least two and possibly three separate currents. These are fast ($I_{Ca,f}$) and slow ($I_{Ca,s}$) currents through distinct calcium channels and a current generated by the sodium-calcium exchanger ($I_{si,2}$ or I_{NaCa}) (Hume,1986). A transient inward current (I_{TI}) has also been reported (Colquhoun et al, 1981) which appears to be activated by intracellular calcium. This current has characteristics similar to I_{NaCa} and it remains uncertain whether or not these two currents are distinct (Noble, 1986). Several outwardly directed potassium currents have also been observed during this phase of the cardiac action potential. The time independent, inwardly rectifying, background potassium current (I_{K1}) is present in most tissue types excluding primary or subsidiary pacemaker tissue (Giles et al, 1986). Two potassium currents (I_{X1} and I_{x2}) involved in terminal repolarization during the later portion of the plateau were observed in Purkinje fibres. As the

reversal potential for these currents was positive to E_K, they were called I_{x1} and I_{x2}. More recent studies have shown time and voltage dependent outward currents which appear to be carried exclusively by potassium. These have been termed I_K, I_A, and I_{TO} and their occurrence and prevalence differs in different cardiac tissues (Noble, 1986; Hume et al, 1986). In Purkinje fibres, a slow TTX sensitive sodium current (window current) also exists during the plateau phase and may be responsible for the greater plateau duration observed in this tissue (Coraboeuf et al, 1979).

Prior to discussing experimental findings concerning these currents, it is important to compare the different types of information obtained from voltage clamp and action potential studies. Action potential measurements yield composite information on all the simultaneous and interactive ionic currents which contribute to the cardiac action potential. Configuration changes can be analysed in terms of the known ionic currents responsible for the various phases but the information is largely inferential and speculative. Conversely, the majority of voltage clamp studies are designed to isolate a specific current unmodified by simultaneous (and often competing) currents. This approach permits a very detailed analysis of a particular current but under extremely artificial conditions. Ionic interactions are not easily obtained, but if so, the analysis again becomes largely inferential. The most successful approaches to overcoming this Catch-22 situation combine voltage-clamp and action potential measurements to provide a useful foundation for inference. The primary focus will now be to examine the slow inward current, its influence on contractility, and its properties.

The classic paper of Wood, Heppner, and Weidmann (1969) will now be considered in detail as it has greatly influenced the direction of EC coupling studies. This study employed a single sucrose gap voltage clamp technique and calf or sheep ventricular tissue was examined. The initial approach was to examine the effect of depolarizing currents which produced contracture in these tissues. Following this intervention, and after returning to repolarized conditions, a residual potentiation of the next several beats was observed. The potentiation occurred despite little alteration in the configuration of the accompanying action potentials. Interestingly, the decay of potentiation was markedly protracted if the tissue was not restimulated (average $t_{1\backslash 2}$ 95 sec.) whereas about 8 sec. (at 1\sec) were required in the stimulated tissue. The degree of potentiation achieved by this intervention was also far greater than that

produced by paired pulse stimulation. The second experimental protocol involved the application of depolarizing or hyperpolarizing currents during the plateau phase of the action potential. Thus the plateau could either be prolonged or shortened, or increased or decreased in amplitude. An increase in plateau height or duration resulted in potentiation of successive beats whereas decreases in plateau height or duration impaired contraction of the subsequent beats. These interventions produced positive or negative residual effects with similar decay dependencies as the contracture experiments. From these studies, the following conclusions were made:

1. It is the presystolic level of intracellular calcium bound to various storage sites which determines tension development of a given beat.
2. The amount of intracellular calcium available for subsequent beats is determined by;
   i. the intersystolic intervals of the preceding beats in an inverse manner.
   ii. the magnitude and duration of the action potential plateau in a direct manner.

The primary importance of this study was that it conclusively demonstrated that the inotropic state of cardiac muscle was largely predetermined by preceding events. While this information was known previously in terms of force development (Koch-Weser and Blinks, 1963), this paper revealed that the slow inward current influenced the inotropic memory by altering intracellular calcium levels and not calcium entry during the altered beats.

The above study can be considered a landmark in the history of EC coupling. However, newer studies reveal a more complicated picture. Specifically, the action potential shows major changes in configuration in response to various interval related contractility changes. Prior to a discussion of these, the familiar caveat should be re-emphasized. There are considerable differences in action potential configuration changes between species and even within species depending on region of myocardium studied and laboratory reporting the results. This makes generalizations difficult, but these will still be attempted with the knowledge that conflicting information also exists. The majority of the following discussion will pertain to results from ventricular muscle. Prior to a consideration of possible mechanisms, a description of changes is given.

In response to an increase in frequency, ventricular muscle from the majority of species exhibits a

decrease in action potential duration (Boyett and Jewell, 1980). This decrease is manifested by a reduction in the duration of the plateau phase of the action potential, with changes in the rate of terminal repolarization being less frequently observed. A depression of plateau magnitude is often seen at high rates of stimulation. When low rates of stimulation are examined, increases in plateau duration are observed in many species. Thus, from numerous studies it appears that tension development is inversely related to the time-voltage area of the cardiac action potential. Another commonly observed phenomenon is that action potential changes of the initial beats at a new frequency will show an opposite change to that observed prior to stabilization. This behavior has been reported in dog ventricular tissue (Miller et al, 1971) and may or may not be seen in other preparations (Boyett and Jewell, 1980).

Extrapolating this finding to previous hypotheses from the work of Wood, Heppner, and Weidmann (1969) where the availability of intracellular calcium stores was found to be rate dependent, it appears that the positive inotropic effect of increasing frequency is at least partly mediated by enhanced intracellular calcium utilization. The absolute magnitude of extracellular calcium entry during frequency alterations cannot be surmised from action potential measurements and it is not known whether increased electrical systolic time compensates for the abbreviated plateau. Other approaches to answering this question are discussed in later sections.

The configuration of the action potential is affected by other interval alterations, as well. When an extrasystole is imposed on a steady train, the action potential of the extrasystole is variable. Even in studies examining the same tissue type, different responses have been observed. For example, extrasystoles in dog ventricular muscle can show a depression of plateau height and duration (Miller et al,1971) or "supernormal" extrasystoles can be observed where plateau height and duration are increased above the control responses (Greenspan et al, 1967; Miller et al, 1971; my studies). Despite this variability, tension is decreased for early extrasystoles. Post-extrasystolic tension is increased in accordance with the prematurity of the extrasystole but the accompanying action potential usually shows depressed plateau height and duration (dog ventricular muscle; Edmands et al, 1966). If plateau height and duration are used as indicators of extracellular calcium entry, it appears that post-extrasystolic potentiation is mediated by intracellular calcium stores whereas the greater extrasystolic calcium entry

serves only to fill these stores for utilization by subsequent beats, as tension development is poor for this beat.

After resumption of stimulation following a rest period, tension development can be depressed or augmented depending on species and duration of rest. Greenspan et al (1967) reported a lengthening of action potential duration with an accompanying depression of plateau phase (also observed in my studies). Similar behavior has been reported in cat ventricle (Boyett and Jewell, 1978 and 1980). Other species such as rabbit show a depression of the action potential following rest periods (Boyett and Jewell, 1980). Again, it appears that in species which demonstrate positive inotropic responses to rest (eg. dog ventricle), the calcium entry is decreased during this beat. This information provides additional support for the hypothesis that intracellular calcium can have a major role in tension development. The unique aspect of rest potentiation however, is the fact that calcium is not being actively introduced into the cells during the rest period. This is not the case for extrasystoles, paired pulse experiments, or voltage clamp experiments where calcium entry is promoted by different means. Thus another mechanism must exist which alters the availability of intracellular calcium independent of loading.

Prior to a consideration of possible mechanisms responsible for changes in the action potential configuration, basic features of the elementary currents will be reviewed. The focus will be on voltage-clamp studies of the slow inward calcium current but potassium fluxes will also receive some attention. The original voltage-clamp studies implicating calcium as the charge carrier during the plateau phase were conducted by Reuter in 1967. Since then, a great deal has been learned including methodological problems with the technique (Reuter, 1979; McDonald, 1982). The following equation has successfully been used to describe I_{si}:

$$I_{si} = g_{si} \cdot d(V,t) \cdot f(V,t) \cdot (V_m - V_{si})$$

where d and f are voltage and time dependent activation and inactivation variables respectively. I_{si} is activated in cardiac cells at intracellular potentials more positive than -50 mV and activation is believed to follow a monoexponential time course with time constants ranging between 5-30 msec reported (McDonald, 1982). Far less agreement exists regarding inactivation parameters especially when patch-clamp studies are considered. Voltage clamp studies have revealed a hundred fold difference in selectivity

of the divalent cation channel for calcium over potassium or sodium. Selectivity for divalent cations from indirect measurements suggests Ba > Sr > Ca >> Mg.

The inactivation of I_{si} has received considerable attention given its importance in contributing either directly or indirectly to tension development. The question as to whether inactivation is calcium or voltage dependent is a longstanding and unresolved issue. This controversy will be discussed further when patch-clamp and whole cell voltage-clamp studies are reviewed. There is general concurrence that the time constant of inactivation (t_f) increases at intracellular potentials more positive than -20 mV and inactivation proceeds exponentially, but wide disparity exists concerning estimates of t_f (10-200 msec) and its voltage dependence (McDonald, 1982). Recovery from inactivation has been reported to have similar time constants as inactivation itself.

Several distinct potassium currents are present in cardiac cells. Whether intracellular calcium is involved in regulating the conductance of any or all of these is an area of active research. In a series of papers, Isenberg (1977a,b,c) examined the influence of iontophoretically applied calcium or EGTA on calcium and potassium fluxes. An increase in intracellular calcium was shown to increase I_{k1} and I_{k2} whereas opposite results were obtained following the intracellular injection of EGTA. Less concurrence exists in other studies addressing this same question but using different methods to augment intracellular calcium (reviewed by McDonald, 1982). Changes in I_{k1}, I_{k2}, and I_X were not observed when extracellular calcium was increased (DiFrancesco and McNaughton, 1979).

VOLTAGE AND PATCH CLAMP EXPERIMENTS IN SINGLE CELLS

The recent advent of voltage and patch-clamping of single myocytes has had tremendous impact on our understanding of the elementary currents which produce the cardiac action potential. These techniques afford distinct advantages over conventional tissue voltage-clamp experiments but also introduce new concerns and problems. The primary advantages of single cell voltage-clamping are uniformity in voltage control throughout the cell (ie spatial and temporal control) and ease and uniformity of iontophoresis and\or internal dialysis (Smith et al, 1985). The former advantage alleviates a problem which has plagued voltage clampers historically. In essence, voltage clamp experiments attempt to tame the ionic currents responsible for the action potential and render the cell(s) at an isopotential directed by the experimenter. This has been achieved by employing negative feedback circuitry which supplies current to an electrode so that the desired membrane potential sensed by a second electrode is maintained. However, since the two electrodes are in different regions of the tissue, it has not always (in fact, seldom) been possible to assume that current injection in one region affects membrane potential uniformly in all regions. The use of single cells has alleviated many of the problems associated with tissue preparations. The second advantage (ie. ease of internal dialysis) is important since it assists in the isolation of overlapping currents which was less easily achieved in larger preparations. It also permits control of the intracellular and extracellular environments and thus the effects of different ionic and pharmacological interventions can be studied with reasonable surety of intracellular homogeneity. Thus, we are now able to control both the intracellular and extracellular environments of the cell, obtain good spatial and temporal control of membrane potential, and examine the influence of this on cellular currents. Modifications of the technique permit voltage clamping with a single electrode by rapidly switching current injection and sensing functions of the same microelectrode. This technology alleviates the numerous difficulties associated with introducing two electrodes into a single cell.

The patch-clamp technique permits resolution of the current pulses flowing through individual ionic channels. Currents- of a few picoamperes are readily visualized and the kinetics of the transitional

states as well as the amplitudes of unitary conductances are easily obtained. Thus, the requirement to infer kinetic and permeation properties of ionic channels from whole cell or tissue preparations is no longer required as the molecular processes underlying these macroscopic currents can be directly visualized and measured (Smith et al, 1985).

The patch clamp technique isolates a small region of membrane (usually a few square micrometers) within the pipette tip. This 'patch' can be extracted from the cell or left in place for whole cell patch-clamp recording. This approach offers several advantages over other voltage clamp techniques. Since currents are only measured across the membrane in the pipette tip, there is excellent spatial control and series resistance problems (associated mainly with voltage clamping of whole tissue) are avoided. With patch excision, the ionic milieu on both sides of the patch can be readily altered and osmolarity of the solutions is no longer important. Unfortunately, it is not yet possible to measure movement of calcium through single channels from cardiac tissue after patch excision, as rundown of the channels is very rapid in this preparation.

As discussed later, there is considerable disparity in results from voltage-clamp studies in multicellular preparations and those from single cell experiments. This may well be due to the numerous 'advantages' of single cell techniques. These advantages possibly create very artificial or nonphysiological environments for the cells. The isolation techniques used to produce myocytes invariably employ enzymatic dispersion. To what extent these procedures alter the glycocalyx of the cell is not certain. As demonstrated by Langer and workers (1979, 1983), altering the extracellular matrix of cardiac cells has pronounced effects of calcium binding properties and thus tension. Also, multicellular preparations have restricted extracellular spaces where ionic depletions and accumulations are likely to occur. This physiological property is unlikely to exist for isolated myocytes. Thus, many of the quantitative differences between isolated cells and multicellular preparations may be due to the creation of artificial conditions for myocytes. Regardless, a wealth of information has been obtained from these approaches even though the technique is still in its infancy.

The marriage of patch-clamp and single cell voltage-clamp experiments has provided tremendous insight into the mechanisms of calcium channel function and its modulation by various interventions. In

guinea pig ventricular cells, the permeability sequence for divalent cations was Ca^{2+} > Sr^{2+} > Ba^{2+} with Mg^{2+} showing no permeation. Monovalent cation permeation exhibited the following sequence, Li^+ > Na^+ > K^+ > Cs^+ but this was much less than that observed with divalent cations. This result was based on measurements of the unitary current amplitude and reversal potential (E_{rev}) in cell attached patch-clamp experiments and by E_{rev} measurements from whole cell voltage clamping. However, when single channel conductances were measured, the following sequence (and values) were obtained; Na^+ (85 pS) > Li^+ (45 pS) > Ba^{2+} (20 pS) > Ca^{2+} (9 pS). Thus ionic fluxes are lower for cations with greater permeability. Low concentrations of calcium (less than 2uM) were shown to reduce whole cell currents produced by sodium to half. From these and previous results, the following mechanisms of calcium channel permeation were proposed (Hess et al, 1986).

1. Ions pass in single file through the pore and interact with multiple binding sites.

2. Selectivity is primarily determined by ion affinity to these binding sites and not by exclusion by a selectivity filter.

3. Occupancy by a single calcium ion can block the high conductance of monovalent cations.

4. Double occupancy is required for rapid permeation of calcium ions. This only occurs at millimolar concentrations of $[Ca]_o$ where electrostatic repulsion or other ionic interactions can be overcome.

5. If double occupancy occurs, the ionic interactions accelerate calcium exit from the pore.

The blocking actions of inorganic cations on unitary calcium channel currents were examined in guinea pig ventricular cells (Lansman et al, 1986). In this study, 50 mM barium was used as the charge carrier and long lasting unitary currents were promoted by the addition of the calcium channel agonist, Bay K 8644 (5 uM). At micromolar concentrations, cadmium, cobalt, manganese, and lanthanum were found to convert long lasting channel openings into rapidly flickering pulses suggestive of rapid transitions between blocking and unblocking events. This behavior was also observed with magnesium but at millimolar concentrations. Calcium also appeared to block the barium conductance but by a different mechanism. Rather than convert the openings into rapid flickering events, calcium decreased the amplitude of the unitary currents. This evidence supports the contention that double occupancy of the

pore (as would be expected with 50 mM Ba) will accelerate calcium efflux. This effect requires millimolar calcium concentrations whereas univalent cation fluxes are blocked by micromolar concentrations of calcium. Other important observations from this study were regarding voltage and concentration dependence. For instance, rate of block was found to be voltage independent but dependent on the concentration of blocking agent. The rate of unblock however was accelerated by hyperpolarization or increases in concentration of the charge carrier.

The results from these patch-clamp studies (Hess et al, 1986; Lansman et al,1986) have had a major influence on concepts regarding both ion permeation and blockade during the plateau phase of the cardiac action potential. Several theories have been proposed to account for the blocking action of certain divalent cations including:

1. a screening of negative surface charges which would decrease the local calcium concentration around the pore (Muller and Finkelstein, 1974).
2. direct competition with permeant cations within the pore itself (Hagiwara et al, 1974).
3. effects on a regulatory site outside the channel (Kostyuk et al, 1983).

The results from studies by Lansman, Hèss, and Tsien (1986) have provided strong evidence which would suggest that all these blocking cations are themselves capable of transit through the pore. Their blocking effect merely results from extremely slow transport through the pore. In fact, these cations appear to lodge within the pore producing the flickering of current pulses. However, increasing charge carrier concentration or hyperpolarization accelerates the exit of the blocker through the pore. This observation is difficult to reconcile if local charge screening or external regulatory site hypotheses are considered. They have thus characterized several cations on the basis of blocking and permeation qualities, where these qualities form a spectrum or progression rather than an absolute category. Thus, all cations examined were both permeators and blockers but to different degrees. (see diagram 2 from Lansman et al, 1986).

POTENT BLOCK	RAPID PERMEATION: NO	RAPID PERMEATION: YES
YES	Cd La ultrastrong binding	Ba Sr Ca Mn strong binding
NO	Mg slow dehydration	Li Na K Cs weak binding

An important question which has been addressed in several voltage clamp studies concerns the mechanism of inactivation of calcium channels. Certain evidence exists which favors calcium induced inactivation while other evidence supports voltage dependent inactivation. In favor of calcium dependent inactivation are the following observations:

1. Increasing the extracellular calcium concentration accelerates the rate of inactivation (Kohlhardt et al, 1975)

2. The rate of inactivation is decreased when extracellular calcium is replaced with barium (Marban and Tsien, 1981) or strontium (Vereecke and Carmeliet, 1971).

3. Double pulse experiments revealed a U-shaped inactivation relationship (Marban and Tsien, 1981). In these experiments, current of a test pulse is plotted against the voltage step of a previous conditioning pulse. As the voltage of the conditioning pulse is elicited closer to the reversal potential for calcium (where calcium entry would decline), an increase in current of the test pulse would be expected if inactivation was calcium induced. This was the effect observed. However, other evidence argues against calcium induced inactivation of calcium current. For example, Isenberg (1977) has shown that the intracellular injection of calcium lead to an increase in the slow inward current. An opposite effect was observed when EGTA was injected suggesting that calcium enhances its own entry. Reuter and Scholz (1977) have reported an increase in I_{si}

caused by B-adrenergic agents yet inactivation kinetics were not altered. This is difficult to reconcile with calcium induced channel inactivation but recent evidence (Bean et al, 1984) suggests that the beta-adrenergic effect is mediated by channel recruitment as postulated by Reuter (1979). In a recent study combining voltage clamp studies of Purkinje fibres and isolated myocytes, Lee et al (1985) demonstrated that calcium channel inactivation depended on both intracellular calcium and membrane potential. Evidence for calcium dependent inactivation came from experiments showing decreased inactivation when extracellular calcium was replaced with either strontium or barium, and an enhanced rate when extracellular calcium was elevated. Also, double pulse experiments revealed that maximum inactivation of the test pulse coincided with maximum inward current generated by the conditioning pulse. However, voltage dependence was also demonstrated as in the absence of extracellular calcium, outwardly directed cesium currents (through the calcium channels) were found to inactivate. This occurred when barium replaced calcium or in the complete absence of permeant divalent cations which excludes the possibility of divalent mediated inactivation. Also the rate of inactivation increased with increasing depolarization. Therefore, it appears that both mechanisms are involved in regulating the influx of calcium through slow channels. These authors have suggested that the calcium induced inactivation might serve a negative feedback role during individual contractions while the membrane control of inactivation prevents excessive calcium entry once intracellular calcium levels are lowered (which occurs prior to termination of the action potential). Another possibility is that intracellular calcium regulates the voltage dependence of channel gating. Such possibilities are currently being examined.

The role of intracellular calcium in the regulation of potassium fluxes has received a great deal of attention as considerable precedence for this behavior is found from studies in other tissues (Meech, 1978; Inoue et al, 1986; Mitra and Morad, 1985). Despite this, little conclusive evidence exists to suggest this regulation is present in mammalian myocardium with the possible exception of Purkinje fibres. In this tissue both I_{k1} and I_{k2} were reportedly increased following pressure injection of calcium into sheep Purkinje fibres whereas intracellular EGTA injection led to a decrease in these currents (Isenberg, 1977).

However, elevation of extracellular calcium was found to have no effect on I_{k1}, I_{k2} or I_x in another study. Studies in ventricular and atrial tissue have also produced conflicting results (McDonald, 1982 and references therein). The patch clamp technique may soon provide answers to this question.

Several excellent studies have combined voltage clamp techniques with indices of intracellular calcium release. These include studies measuring contraction with and without pharmacological alteration of the SR (Mitchell et al, 1985) or aequorin luminescence measurements (Wier and Isenberg, 1982). These results will be considered after the next section.

EXTRACELLULAR CALCIUM FLUX MEASUREMENTS

Two different approaches have been used to measure extracellular calcium fluxes during contractions of myocardial tissue. Hilgemann has used optical measurements of absorbance changes in an extracellular calcium-sensitive dye. Bers has employed extracellular calcium-selective microelectrodes. Both of these techniques measure changes in the concentration of extracellular calcium which would result from at least five factors:

1. Calcium entry through slow calcium channels.
2. Calcium entry through sodium-calcium exchange.
3. Calcium efflux by sodium-calcium exchange.
4. Calcium efflux through outwardly directed calcium pumps.
5. Calcium binding and diffusion from the extracellular matrix.

Therefore, a given replenishment or depletion signal reflects the sum of these processes. The utility of these approaches resides in the fact that they compliment studies which measure calcium current directly and thus allow inference on calcium flux from other pathways. As well, they both permit at least a crude estimate of extracellular calcium entry. These studies have also provided insight into the contributions of intracellular and extracellular calcium during various stimulation protocols.

Using double barreled extracellular calcium-selective microelectrodes, Bers (1983) demonstrated transient depletions of calcium associated with individual contractions. The magnitude of the depletions

were found to correlate with increases in tension development produced by elevating extracellular calcium. The depletion signal was abolished by cobalt and enhanced by the reduction of extracellular sodium or isoproterenol. Following a rest interval however, the magnitude of the depletion was decreased. After the initial beat, tension then increased in parallel to the depletion signal. Using this technique to estimate calcium influx per beat, estimates were within the range to allow for direct activation of the myofilaments by transmembrane influx of calcium.

In subsequent studies, this technique was used to assess the importance of extracellular calcium entry for tension development in a number of species. The study examined postrest tension development and the effect of calcium influx inhibitors and agents inhibiting the SR on tension and postrest recovery patterns. In all tissues studied (rabbit atria and ventricle, rat ventricle) except frog ventricle, the initial beat following a rest interval was highly dependent on SR calcium release following which calcium influx increased. Postrest tension development in mammalian ventricle was impaired by caffeine and ryanodine, agents known to impair SR function. However, postrest tension was insensitive to cobalt or lanthanum which impair extracellular calcium entry again supporting the notion that tension development is primarily dependent on the SR during the initial beat. Based on these experiments and tension sensitivity to SR inhibitors, the following order of SR dependence for the different tissues was proposed; adult rat ventricle > rabbit atrium > rabbit ventricle > frog ventricle. A more extensive list was referred to in the discussion where the importance of calcium influx to contraction was ranked as follows (A, atria; V, ventricle):

frog V = toad V > fetal V (human, cat and rabbit) > rabbit V > guinea pig V > cat V > rabbit A > dog V > ferret V > rat V > calf Purkinje fibre

This result is in reasonable accord with the threshold for calcium-induced calcium release phenomenon reported by Fabiato and Fabiato (1978).

In rabbit ventricular muscle, Bers and McLeod (1986) used extracellular calcium-selective microelectrodes to measure cumulative extracellular calcium depletions following a number of

interventions. In control preparations, a net calcium depletion was observed following resumption of stimulation from rest and this calcium returned to the extracellular space once stimulation was discontinued. The magnitude of this depletion was enhanced after the addition of the calcium channel agonist, Bay k 8644 (1 uM), or with increases in stimulation frequency. This depletion could be prevented by lanthanum and cobalt as expected from their ability to impair extracellular calcium entry. Both decreases in extracellular sodium (70 mM) and the addition of acetylstrophanthidin (1-3 uM) decreased extracellular calcium depletions. The explanation favored by these authors was that these interventions lead to an accumulation of calcium in the SR which would limit the capacity for further uptake during stimulation. In control muscles, calcium is continually lost from the SR during rest and thus the SR has the ability to sequester extracellular calcium during stimulation which yields the depletion signal. Two classically studied agents which impair SR function, ryanodine and caffeine, were also examined. Caffeine was shown to prevent the depletion signal presumably due to inhibition of SR uptake of calcium and thus abolition of an intracellular sink into which extracellular calcium can be stored. In contrast to this, ryanodine markedly increased the magnitude of the depletion signal and also accelerated the diastolic efflux of calcium once stimulation was terminated. This effect was accounted for by proposing that ryanodine accelerates the SR uptake during repetitive stimulation and accelerates SR calcium efflux during diastole. The authors were not able to determine if the enhanced uptake was due to enhanced diastolic loss (and therefore a larger sink into which calcium could subsequently be loaded into). Previous evidence has shown ryanodine to accelerate SR calcium uptake (Jones et al, 1979) which might also be sufficient to account for this observation. Furthermore, evidence was not available to suggest that this calcium efflux occurred during regular stimulation.

The optical techniques employed by Hilgemann et al (1983) may offer some advantage over calcium-selective microelectrode studies although the results obtained are generally similar. This technique measures extracellular calcium concentration changes by introducing an extracellular calcium-sensitive dye into the bathing medium. A thin, translucent portion of guinea pig right atrium is then illuminated and multi-wavelength absorption levels (or transmittance) are monitored during various interventions. The use of multiple wavelength spectroscopy permits accurate resolution of changes due to

motion artifact and that due to changes in extracellular calcium concentration. Isosbestic wavelength monitoring provided a reasonable control for motion artifact while properties of the dye allow calcium changes to be monitored. This was achieved by measuring absorption at two different wavelengths which showed directionally opposite changes in response to calcium concentration changes. The primary advantage of this approach over calcium-selective microelectrodes is that the measurements reflect calcium changes in the dye accessible space. Therefore, calcium changes include those occurring in restricted areas as well, whereas the microelectrode technique only measures calcium changes in a very limited area. This may result in "well" formation and artificial results (Hilgemann et al, 1983). The similarity of results from these two different techniques argues against this latter reservation.

Hilgemann et al (1983) have reported a depletion of extracellular calcium following resumption of stimulation after rest periods. The magnitude of this depletion was enhanced by isoproterenol and replenishment occurred after discontinuation of stimulation with an apparent $t_{1\backslash 2}$ of 25-60 seconds. Ryanodine was found to greatly enhance the amount of depletion as well as accelerate the replenishment during subsequent diastolic periods similar to results reported by Bers and McLeod (1986). Caffeine and nifedipine were also shown to suppress the magnitude of the depletion signal.

More recent studies by Hilgemann (1986a,b) have centered on extracellular calcium fluxes during single excitations in rabbit atrium. Action potential configuration changes and tension were also integrated into these studies. Rabbit atrium was selected for this study as the action potential undergoes considerable change in configuration in response to rest periods. Following periods of quiescence, the plateau phase is greatly suppressed but an additional late phase (or slow phase of repolarization) is observed. A significant result from these studies with regard to the interval-force relationship was the observation that during potentiated beats following a brief burst of stimulation at 4 Hz, there was a net accumulation of extracellular calcium. During the burst, the tissue accumulated 5-15 uM calcium but the potentiated beats following the burst resulted in an increase in extracellular calcium of 4-8 uM for a single contraction. That is, total calcium fluxes resulted in a net increase in extracellular calcium presumably due to efflux from intracellular stores. This provides strong evidence to support the notion that extracellular calcium entry is not responsible for post-stimulatory potentiation. This potentiation declines over a few

beats and this correlates with a negative staircase of the late phase of the action potential. When the extracellular sodium concentration was reduced and the same protocol was repeated, the extracellular calcium depletion during the burst was diminished as was calcium accumulation during subsequent beats. The loss of contractility (ie negative staircase) was diminished for post-rest contractions although these were not potentiated. Suppression of the slow repolarization of the action potential was also observed. Ryanodine was found to facilitate calcium entry during rapid stimulation as well as promote efflux in subsequent diastolic periods. If intervals were then examined which would normally have produced post-stimulatory potentiation (> 5 sec), both potentiation and extracellular calcium accumulation were not observed. Tension development then follows the positive staircase of the early action potential. Both isoproterenol and 4-aminopyridine (which enhances the action potential plateau by decreasing repolarizing currents) could diminish the rate of decay of post-stimulatory potentiation. When this occurred, calcium accumulations were observed for several potentiated beats. It was concluded that extracellular calcium accumulations and depletions represent the depletions and fillings of an intracellular calcium store. Potentiation typically results in extrusion of the additional calcium across the sarcolemma and thus a net calcium accumulation is observed.

In another study which focused on extracellular calcium depletions during individual contractions, Hilgemann (1986b) reported that the depletion began very early after excitation (within 2-4 msec). During regular stimulation, the depletion continued into the early in rising portion of tension development. Replenishment of extracellular calcium was then observed. This occurred at the midpoint of contraction or earlier and continued for variable durations during the contraction. With decreases in frequency, the magnitude of the depletion signal was diminished. Paired pulse stimulation revealed augmented depletion for the coupled beats and augmented extracellular accumulation for the potentiated beats. This evidence supports the contention that the coupled beats serve to augment the size of the intracellular calcium pool. The observation that enhanced calcium entry during these coupled beats is ineffectual in tension development negates a major role for extracellular calcium entry in tension development in this author's opinion. This result is very similar to observations by Wood, Heppner, and Weidmann (1969) who found extracellular calcium entry to be an important determinant of contractile

strength in subsequent beats but not an individual preceding beat. Potentiation of post-stimulatory contractions were unique in that depletions were not observed during these potentiated beats. Rather, an extracellular calcium accumulation was observed throughout again negating a critical role for extracellular calcium entry.

The studies of Hilgemann and coworkers (1983, 1986a,b) and Bers and coworkers (1983, 1985, 1986) are significant in that the measured quantity yielded net calcium flux. This allows inference about the role of sodium-calcium exchange and outwardly directed calcium pumps during various types of contractions. As will be discussed later, sarcolemmal calcium pumps do not appear to have a primary role in relaxation. Sodium-calcium exchange has been proposed to be the primary mediator of calcium efflux (Barry et al, 1986). If we accept this for the sake of argument, then the results from the studies of Bers, Hilgemann, and coworkers suggest that during contractions and especially during potentiated beats there is an efflux of calcium presumably due to sodium calcium exchange. At least for potentiated beats (where voltage clamp studies show diminished but definitely measurable calcium current) sodium-calcium exchange is not contributing calcium for contraction but in fact removing it. This conclusion will be reinforced in subsequent sections.

Other studies have examined the role of the slow inward calcium current on contractions with the emphasis on the influence of this current on intracellular calcium release. Wier and Isenberg (1982) have examined this in voltage-clamped canine Purkinje fibres using the bioluminescent protein, aequorin. This preparation exhibits a luminescence signal comprised of two peaks which the authors refer to as L_1 and L_2. L_1 has several properties which suggest that it may arise from calcium entry through the slow inward channel. Under conditions which only generate the early peak, L_1, tension development is very small. The peak of the slow inward current and the peak of L_1 share a similar voltage dependency and occur at roughly the same time. They also share a similar threshold potential.

In the presence of L_2, tension development is much greater than when L_1 is observed alone. Both L_1 and L_2 were abolished by D600, a calcium channel antagonist. Several characteristics of L_2 suggest that it arises from calcium release from an intracellular store. First, L_2 reprimes much slower than L_1. This was determined by measuring L_1 and L_2 during paired pulse stimulation. L_1 was only slightly

depressed during the closely coupled beat whereas L_2 could be abolished during this beat. If repetitive clamp steps of 50 msec duration were given, only L_1 was observed and its magnitude was similar to that observed for 500 msec clamp steps where L_2 was also observed. When 50 and 500 msec clamps were alternated, L_2 was found to be greater for the 50 msec clamps. This observation suggests that calcium accumulates intracellularly during prolonged clamp steps (500 msec) whereas shorter steps (50 msec) do not allow adequate filling and thus L_2 is not observed. This study provides considerable evidence which suggests that intracellular calcium is critical for force development and the function of the slow inward current is to provide calcium to this pool. Strikingly similar conclusions were obtained by Wood, Heppner, and Weidmann (1969).

Using a different approach, Mitchell et al (1985) have examined the influence of the slow inward current on contraction as well as the influence of intracellular calcium release on this current. Rat ventricular cells were voltage-clamped to achieve this. As well, action potentials were measured in some preparations and the apparatus was capable of altering resting potential by constant current application. While rat ventricular muscle is unique in that it exhibits a negative force-frequency relationship, the conclusions of this study seem applicable to other species. The voltage clamp duration was varied between 10 and 100 msec. If the stimulation frequency was maintained at 0.3 Hz, the amplitude and time course of contraction and the slow inward current were similar at either duration of the clamp step. However, if the stimulation frequency was increased to 3 Hz, both the slow inward current and the evoked contraction were found to decrease for 100 msec clamp steps. Aftercontractions also appeared as well as transient inward currents. This was not observed for the 10 msec depolarizing steps. Contractions, and the amplitude of the slow inward current were maintained even if frequency was increased to 4.2 Hz. At the longer depolarizing steps, the decline in calcium current at high frequency could be largely prevented by the intracellular application of EGTA. This intervention also abolished contractions and transient inward currents. Ryanodine also abolished the appearance of aftercontractions and prevented the large decrease in calcium current observed in control muscle. These data indicate that a significant proportion of calcium-induced calcium channel inactivation occurs due to the intracellular release of calcium, presumably from the sarcoplasmic reticulum. This effect appears to be more important than build-up of

intracellular calcium at the mouth of the pore as inactivation was not observed for brief depolarizations which did not impair peak inward current. Only when long depolarizations (which would fill SR stores and cause subsequent release) were examined was inhibition observed. This conclusion was substantiated by double pulse experiments which examined slow inward current recovery following alternating short (10 msec) and long (100 msec) depolarizing pulses. The test (or coupled) pulse was 100 msec in both cases and was introduced at varying intervals to the conditioning pulses. For these experiments, an exponential recovery of inward current was observed when long conditioning pulses were examined. For the short conditioning pulses, an additional component of inactivation or incomplete recovery was observed. This additional component mirrored the time course of contraction generated by the conditioning depolarization. The authors reasoned that this inactivation resulted from calcium release from the SR. Further, this conclusion was supported by the abolition of extra-inactivation when ryanodine or intracellular EGTA were applied to the cells. Thus it appears that intracellular calcium release is very important in the modulation of the slow inward current. As well, the generation of aftercontractions with or due to associated electrical events is caused by intracellular calcium release.

INTRACELLULAR CALCIUM RELEASE

The notion that intracellular calcium stores play a prominent role in excitation-contraction coupling is based on several lines of evidence. Several of the preceding studies measuring some form of calcium influx has suggested this. However, more direct approaches provide compelling evidence for the role of intracellular calcium stores in modulating contraction. These include:

1. Studies on the properties of isolated sarcoplasmic reticulum.
2. Studies examining skinned cardiac fibres.
3. Pharmacological studies using agents with well documented effects on the sarcoplasmic reticulum. This approach generally is included in other types of studies.
4. Electron-probe microanalysis studies of calcium movements during various phases of the cardiac excitation-contraction cycle.

5. Rapid cooling contracture studies.

6. Scattered light intensity fluctuation measurements.

7. Electron microscopy studies.

Despite considerable evidence in favor of electrical and mechanical modulation of contraction by intracellular calcium, its importance is not universally appreciated. However, even theories which place the emphasis on trans-sarcolemmal modulation of contraction (eg. Langer, 1974) necessarily include some role for the SR during certain types of contractions.

STRUCTURE AND FUNCTION OF CARDIAC SARCOPLASMIC RETICULUM

The sarcoplasmic reticulum (SR) of cardiac muscle is a network of membranous tubules which invest the myofibrils in an irregular manner. It can be categorized into distinct regions based on appearance and cellular location. This includes free or network SR and junctional SR which is further divided into extended junctional SR, lamellar junctional SR, and corbular SR. For description of these various categories, the interested reader is referred to Sommer and Johnson (1979). The extent and degree of development of SR is much less in cardiac muscle than its well studied counterpart, skeletal muscle. It is also extremely variable amongst different species and thus all-encompassing theories are not likely to ever exist as different species probably possess different mechanisms of excitation-contraction coupling. It is very well established that skeletal muscle utilizes SR calcium to support contraction and autoradiographic studies (Winegrad, 1965, 1968, 1970) have revealed that calcium is released from the terminal cisternae of the SR which form triads at the T-tubules. While it is dangerous to extrapolate this observation to cardiac muscle, X-ray microanalysis studies have revealed a similar result in guinea pig ventricular muscle (Wendt-Gallitelli, 1985). In this study, contractions of delayed onset (eg. "rested state" contractions) were associated with a loss of SR calcium whereas contractions with normal time to peak tensions showed calcium accumulations in the SR. Despite the fact that cardiac muscle does not show the degree of SR specialization observed in skeletal muscle, there are regions where smaller subsarcolemmal

cisternae are in close apposition to both the plasma membrane and to the larger diameter cardiac muscle T-tubules (Sommer and Johnson, 1983). Radioimmunographic and immunocolloidal labelling studies have shown that specialization occurs at the protein level as well. Calsequestrin, a calcium binding protein is localized in the terminal cisternae while calcium-ATPases are primarily found in the network SR (Jorgensen et al, 1985). In fact, the presence of calsequestrin and ryanodine sensitive calcium channels are now be used as criteria to identify junctional SR (Brandt et al, 1985). Thus a similar situation may exist in both cardiac and skeletal muscle but this possibility must still be considered speculative.

Experiments on isolated SR have shown that it is capable of rapidly accumulating calcium in the presence of ATP (Weber et al, 1966). More recent studies using oxalate (Chamberlain et al, 1983; Feher and Lipford, 1985) and phosphate (Feher and Lipford, 1985) facilitated calcium accumulation have demonstrated rates sufficient to account for muscle relaxation. One unique feature of cardiac muscle SR is that the calcium pumps are modulated by phospholamban, a protein which is phosphorylated by both calmodulin dependent and cAMP dependent protein kinases. This is not observed in skeletal muscle. Phosphorylation results in greater than doubling of the calcium uptake rates (Tada and Katz, 1982) which provides a reasonable explanation for the accelerated relaxation observed when muscle are treated with beta-agonists.

Cardiac muscle SR releases its accumulated calcium in response to a number of stimuli. Weber and Herz (1968) have demonstrated calcium release after the administration of caffeine. Ryanodine has been shown to cause calcium release at low concentrations (Meissner, 1986) whereas higher concentrations prevent calcium release (Meissner, 1986; Chamberlain et al, 1984). Perhaps the most relevant stimulus for calcium release, and one with a putative role in EC coupling, is calcium itself. Fabiato and Fabiato (1975) have demonstrated that small amounts of calcium are capable of inducing a much larger release of calcium from the SR. This hypothesis forms the cornerstone of the majority of theories regarding EC coupling today.

CALCIUM-INDUCED CALCIUM RELEASE FROM THE SARCOPLASMIC RETICULUM

The original proposition for calcium-induced calcium release from the SR was put forward by Endo as a possible mechanism for EC coupling in skeletal muscle. While he rejected this theory later due to the 'unphysiological' conditions necessary for its demonstration (Fabiato, 1983), interest has resurfaced as calcium channels have been reported in large density in the skeletal muscle T-tubule. Also, as techniques improve for rapidly applying calcium, the conditions for demonstration of calcium-induced calcium release become more acceptable (Fabiato, 1983). In 1975, Fabiato and Fabiato proposed calcium-induced calcium release from the SR of cardiac muscle as the potential link between excitation (and calcium entry) and contraction (due largely to calcium release from the SR). This notion has been expanded upon and characterized over the years and numerous species have been examined. The basic tenets of this theory are as follows. The calcium-induced calcium release hypothesis states that the transsarcolemmal calcium influx, irrespective of magnitude, does not directly activate the myofilaments but induces a release of calcium from the sarcoplasmic reticulum which itself activates the myofilaments. In all mammalian and avian species examined by Fabiato, including those with poorly developed SR, no simulated influx of calcium of any magnitude could directly trigger a contraction without first producing a calcium release from the SR. This statement is supported by observations in mechanically skinned fibres which were examined prior to and after the destruction of the SR by detergents. When the SR was rendered nonfunctional, much greater concentrations of calcium were required for tension development whereas in the presence of a functioning SR, lower concentrations of microinjected calcium (including those which did not produce tension with disrupted SR) were found to produce tension. Two potential candidates for calcium release other than the SR have also been excluded. Using inhibitors of mitochondrial calcium metabolism have not altered results and thus mitochondria were excluded. Sealed over T-tubules which may have been involved were also excluded as tissues which lack a T-tubule system also exhibit a calcium-induced calcium release.

The trigger for calcium release from the SR is the rate of change of free calcium outside the SR and not merely a particular concentration (Fabiato and Fabiato, 1975). If calcium is applied slowly, it results in filling of the SR and not a calcium release. This property may hamper studies in skeletal muscle whose much greater diameter prevents rapid changes in free calcium. In cardiac muscle, it seems likely

that the trigger for calcium release is the rapid component of calcium entry whereas the remainder of the calcium current merely serves to load the SR for subsequent contractions. Fabiato believes that calcium channels exist within the SR that are gated by free calcium in the submicromolar and micromolar range. The possibility that gating is mediated by the same proteins which control uptake has been discarded (Fabiato, 1983).

Another tenet of the theory is that free calcium outside the SR also serves a negative feedback role which terminates the calcium release process. Calcium release is inhibited by high calcium concentrations outside the SR. Thus once release occurs, the local build-up of calcium outside the SR terminates further release. This proposal has been verified using two different approaches. First, under identical loading conditions and triggering time courses, there is an optimal level for the calcium concentration used for the trigger. Greater or lesser concentrations result in less tension development. This observation is extremely important. It eliminates the possibility that the calcium injection used for triggering causes or enhances the contractions. If this were so, then supraoptimal concentrations would be expected to enhance contractions. It also demonstrates the negative feedback relation in which free calcium outside the SR terminates further release. Secondly, Fabiato (1983) has shown that during an optimally triggered calcium release, an additional injection of calcium at supraoptimal levels during the ascending phase of the calcium transient, will curtail the release and tension is decreased for that same contraction.

With regard to the force-interval relationship, the following characteristics may be deemed important. Calcium-induced calcium release from the SR is not an all-or-none process. Graded calcium release is determined by numerous factors including:

1. Rate of change of free calcium outside the SR.
2. Concentration of calcium used to trigger release.
3. Level of calcium preloading of the SR.
4. Interval from preceding calcium release.

This last factor refers to the observation that calcium requires time to reaccumulate in the SR before another calcium-induced calcium release can occur or that time is required for recovery of the calcium

release channels. This provides a reasonable mechanism for the observation that extrasystoles generate less and less tension as their coupling interval is decreased. It is also possible that the trigger for calcium release during extrasystoles is less efficient. In conclusion, the results of Fabiato suggest that transsarcolemmal calcium influx modulates contraction by altering the calcium release from the SR. This is achieved by triggering the calcium release and by loading the SR with calcium for subsequent beats. However, it is the SR calcium release which determines the strength of any given contraction.

RAPID COOLING CONTRACTURES IN CARDIAC MUSCLE

Although studies by Fabiato provide a framework from which changes in contractility can be examined, they do not provide answers to several question regarding the interval-force relationship. For example, why does post-rest potentiation or post-rest depression occur? What mechanism underlies the 'rested state contraction? These phenomena suggest more dynamic mechanisms of SR function than merely uptake and release. While it remains possible that transsarcolemmal calcium triggering controls the interval-force relationship, the majority of evidence discussed above does not support this idea. The technique of rapid cooling contracture measurements provides a different approach to investigate SR function.

Rapid cooling contracture (RCC) refers to the tension produced by muscles in response to a rapid lowering of temperature. This behavior was first observed (Sakai, 1965) in skeletal muscle which had been treated with subthreshold caffeine concentrations (caffeine itself induces contracture in skeletal muscle at higher doses)(Sakai, 1986). In contrast, cardiac muscle exhibits RCC in the absence of caffeine. This phenomena has recently been well characterized by Kurihara and Sakai (1985) in the guinea pig ventricle and by Bridge (1986) in rabbit ventricular muscle. Earlier studies by Fujino and Fujino (1980) will not be discussed as caffeine was employed in these. As well, studies in amphibian ventricle are omitted as they exceed the breadth of this thesis (Sakai and Kurihara, 1974; Chapman, 1973). The following studies which will be considered are the more recent ones.

The study by Kurihara and Sakai (1985) was intended to develop and characterize a technique to independently assess intracellular calcium pools without an accompanying membrane depolarization. Cooling ventricular muscle from $37^{o}C$ to below $18^{o}C$ resulted in contracture provided the muscle was stimulated prior to contracture. Contracture tension was shown to depend on cooling temperature and pre-cooling stimulation frequency and duration. Maximum tension development was observed when cooling was to $5^{o}C$ after which a plateau in tension height was reached. The majority of experiments were conducted in this plateau region. RCC tension development increased with increasing frequency and

duration of stimulation although this later variable also exhibited a plateau as tension approached a steady state level. These RCC were accompanied by a small depolarization but this was subthreshold for contraction. Thus, RCC appeared to depend solely on intracellular calcium stores. Further support for this was provided by experiments in the presence of 4 mM cobalt. As previously discussed, this cation blocks calcium channels at micromolar concentrations (Lansman et al, 1986). This intervention abolished tension development during electrical stimulation but failed to prevent RCC tension development. In fact, RCC was larger than control after rapid stimulation. RCC tension development was also increased by elevating extracellular calcium or lowering extracellular sodium. Even in the absence of stimulation, RCC could be produced after long period of quiescence in the presence of low sodium (less than 69 mM). While it is not difficult to envision increased extracellular calcium increasing the intracellular pool size, the effect of reduced sodium is quite interesting. This observation demonstrates the reversibility of Na-Ca exchange at low extracellular sodium concentration as net calcium influx must be occurring during this period. Quiescent preparations were also found to develop RCC tension if the preparation was depolarized prior to cooling. RCC tension increased with increasing degrees of depolarization produced by the addition of potassium. In summary, these results show that rapid cooling contractures arise from calcium release from intracellular stores. These calcium stores require loading prior to RCC production. This can be achieved by calcium entry due to the slow inward current or by Na-Ca exchange. Low extracellular sodium or membrane depolarization by KCl addition appear to load the stores by Na-Ca exchange operating in a calcium entry-sodium extrusion mode. Depolarization appears to favor the calcium entry mode of the sodium-calcium exchanger due to both intracellular sodium loading and alteration of membrane potential. Though the voltage dependence of the sodium-calcium exchanger remains unclear (Noble, 1986), some evidence suggests that calcium entry is favored during depolarization (Hume, 1986). While the authors (Kurihara and Sakai, 1985) could not unequivocally identify the intracellular calcium store as the sarcoplasmic reticulum, this was implied throughout and appeared to be the favored hypothesis.

The work of Bridge (1986) extends the conclusions of Kurihara and Sakai (1985) and provides additional information regarding diastolic calcium fluxes. Bridge also observed RCC in response to rapid

cooling following stimulation. When these two studies are compared, the magnitude of the RCC was larger in guinea pig ventricle at a stimulation frequency of 0.5 Hz (ie about 30-50% of twitch tension in rabbit, greater than twitch tension in guinea pig). This observation is expected knowing the difference in SR development (and interval-force responses, sensitivity to calcium-induced calcium release) reported for the two species. The decay of RCC during increasing periods of rest following stimulation was examined. A continuous decay in RCC magnitude was observed as the rest interval increased. This compares favorably with reports from Bers (1985) where post-rest tension development was shown to decline continuously with increasing intervals of quiescence. Thus the rest decay in rabbit ventricular muscle may well result from continuous diastolic depletion of intracellular calcium. The decay of post-rest RCC was curtailed and to some extent reversed when extracellular sodium concentration was reduced implying a role for sodium-calcium exchange in removal of intracellular calcium during diastole. If caffeine was added during the rest interval, the rate of RCC decay was accelerated. Most likely, this occurs due to the sensitization of calcium release produced by this agent, as reported by Fabiato (1983) and Blayney et al (1978). However, if caffeine was present for longer periods including the stimulation period, both post-rest RCC and the RCC generated immediately after stimulation were abolished. This result supports the contention that the SR is the intracellular site responsible for RCC. Bridge (1986) also measured intracellular calcium content before and after a 20 minute rest interval using atomic absorption spectroscopy. The measured decay in RCC was consistent with the results obtained from calcium elemental analysis. Thus RCC was concluded to provide a useful index for estimating calcium availability from the sarcoplasmic reticulum. The decay in tension development following rest correlates well with the decline in intracellular calcium in the rabbit ventricle. The diastolic loss of calcium was suggested to result from the passive efflux of calcium from the SR which subsequently is exported from the cell by Na-Ca exchange or outwardly directed Ca-ATPases in the sarcolemma.

SCATTERED LIGHT INTENSITY FLUCTUATION MEASUREMENTS

The measurement of scattered light intensity fluctuations (SLIF) has been used extensively by Lakatta and coworkers (Lappe and Lakatta, 1980; Lakatta and Lappe, 1981; Stern et al, 1983; Kort and Lakatta, 1984) in the last seven years. The utility of this technique is that it permits assessment of diastolic myofilament oscillations supposedly due to spontaneous, asynchronous calcium release from the SR. These microscopic oscillations are generally undetectable in force tracings as they are both asynchronous and minute in magnitude. Under certain conditions of enhanced inotropy or toxicity however, the augmentation of these oscillations is manifest as an elevation of resting tension or decreased diastolic compliance. Thus, SLIF measurements permit assessment of the asynchronous diastolic calcium oscillations prior to overt manifestations of toxicity as well as when these occur.

In this approach, coherent light is passed through a thin segment of muscle which causes scattering of the incident beam and a 'speckle' pattern is monitored. This scattering is due to the random and relatively unordered array of myofilaments due to stacking of cells within a whole tissue (as opposed to the ordered structure within single cells which yields a diffraction pattern indicative of myofilament spacing). Alterations in the speckle pattern (or diffraction spacings of single myocytes) occur due to myofilament motion. The observation of intensity fluctuations in quiescent cardiac muscle led Lakatta and others to propose that mechanical oscillations occur in cardiac tissue even during rest. This result is in contrast to similar measurements in skeletal muscle where increases in the frequency of intensity fluctuations are only observed during activation and are unmeasurable in quiescent muscle. From many approaches, it has been inferred that the mechanical oscillations (which produce the change in frequency of intensity fluctuations) are due to asynchronous calcium release from the sarcoplasmic reticulum. The parameter measured was $f_{1/2}$ which is the half-decay time of the autocorrelation function which measured the frequency of intensity fluctuations (see Lappe and Lakatta, 1980). Resting force measured at high gain was found to correlate with $f_{1/2}$ for a variety of inotropic interventions. Elevations of extracellular calcium produced parallel increases in both parameters. In the presence of a fixed

extracellular calcium concentration, ouabain also increased both $f_{1/2}$ and resting tension. Skinned preparations did not exhibit measurable $f_{1/2}$ in the absence of calcium but this reappeared when calcium was added to the perfusate. This last result negates the necessity for an intact sarcolemmal barrier in the production of SLIF.

Support for the proposal that SLIF is generated by spontaneous sarcoplasmic reticulum calcium release was provided by a study in which the actions of caffeine and ryanodine were compared in rat papillary muscle (Sutko et al, 1986). Both of these agents were found to decrease $f_{1/2}$ in accord with their known inhibitory effects on SR. However, caffeine was also shown to increase $f_{1/2}$ initially prior to decreasing this parameter. Similar effects of caffeine have been reported by Fabiato (1985) with regards to the effect of caffeine on calcium-induced calcium release. Caffeine was shown to initially produce myofilament activation and enhance the sensitivity of calcium-induced calcium release prior to abolishing it. On the basis of several other experiments, the authors concluded that ryanodine and caffeine do not share similar mechanisms of action. However, the decrease and abolition of SLIF by SR inhibiting agents provides strong support for the role of the SR in SLIF production.

The validity of the results from Lakatta and coworkers has been recently challenged by Ishide et al (1984) and Bose et al (1986). The focus of the study by Ishide et al (1984) was to determine whether the diastolic oscillations observed was an inherent property of quiescent cardiac muscle or merely a result of cellular damage as previously reported by Krueger and Pollack (1975). In contrast to results reported by Lakatta, spontaneous sarcomere motion was not found to be homogeneous throughout the muscle. It appeared primarily at the cut ends of the muscle and pronounced reduction in SLIF occurred towards the central undamaged regions. Lappe and Lakatta (1980) reported less than 10% variation in SLIF over the entire muscle. Moreover, Ishide et al (1984) demonstrated that calcium paradox augmented SLIF measurements. This paradigm is known to produce cellular damage. The conclusion favored by these authors was that SLIF measurements were useful for the detection of damaged regions of myocardium but were not truly indicative of a physiological process.

This controversy is by no means resolved although recent studies using different approaches have provided insight into this issue. In a study using calcium-overloaded ferret papillary muscle, Allen et al

(1985) have described a mechanism by which calcium overload or spontaneous calcium oscillations could impair tension development. This study simultaneously measured tension development and aequorin light emission for a variety of inotropic conditions. Over a range of concentrations, raising extracellular calcium resulted in an increase in the aequorin light signal and in developed tension. However, further increases in extracellular calcium (or in the presence of increasing concentrations of strophanthidin) lead to a decrease in developed tension despite similar or larger aequorin signals. This behavior of cardiac muscle is commonly referred to as **calcium overload.** The observation that tension declines during calcium overload despite the lack of a similar decline in the aequorin signal yields important clues to the mechanism given the following information. During calcium overload, the amplitude of the systolic light signal exhibited large variability. It was also observed that diastolic light signals and occasional after-contractions occurred during calcium overload. The magnitude of the after-contractions did not correlate well with the size of the corresponding aequorin emission. The most informative clue to the mechanism of negative inotropy during calcium overload was the fact that the size of the systolic aequorin light signal depended on whether diastolic calcium fluctuations were observed prior to the systole. If a diastolic release occurred close to the systolic period, the systolic light signal was decreased. Conversely, if the diastolic period was free of aequorin emissions or these occurred at least 1 second before systole, then a larger systolic light signal was observed. These results led the authors to propose that calcium-overload reduces tension by causing regional inhomogeneity of the SR calcium contributions. That is, certain regions of the SR which released calcium during diastole are incapable of discharging their normal amounts of calcium during the ensuing systole. Since these regions or cells would only generate modest amounts of tension, they act as a series compliance with the remainder of the tissue and thus reduce the force generated as a whole. Accepting this, it becomes apparent that little physiological benefit is derived from spontaneous SR calcium oscillations. Moreover, in order to demonstrate spontaneous oscillations, it is necessary to enhance the inotropic state of the muscle to very high levels or even beyond optimal inotropic conditions. While this argument does not refute the evidence of Lakatta's group with regards to SLIF, it argues against its physiological relevance.

Using an improved indicator for intracellular calcium (Fura-2), Wier et al (1987) have shown

that three different population of cells can be found in a suspension of rat ventricular cells. These have been categorized according to cellular appearance and behavior. These are:

1. Mechanically quiescent cells which show low and stable intracellular calcium levels which are constant throughout the cytoplasm. Average [Ca^{2+}] = 134 nM

2. Cells which contract spontaneously. These cells have uniformly higher intracellular calcium levels and also show periodical propagating waves of calcium elevation which disrupts the homogeneity. Average [Ca^{2+}] = 270 nM

3. Hypercontracted cells with the greatest amount of intracellular calcium. Average [Ca^{2+}] = 955 nM

This categorization could also be considered as a hierarchy of cellular viability. The authors have used these results to caution workers using cell suspensions as the interpretation of results are not likely to be representative of a homogeneous population of cells. This population variance might also extend to multicellular preparations. Fabiato (1985) has also extended a cautionary note regarding the difference between cells exhibiting spontaneous mechanical oscillations and those quiescent during diastole. He states that these oscillations are due to calcium overload and not due to the calcium-induced calcium release process responsible for normal contractions. Although both activities are impaired by ryanodine application and thus may share the same channel for calcium release, they respond quite differently with regard to calcium injection in skinned preparations (Fabiato, 1985). For example, the slow application of relatively high calcium concentrations would enhance cyclic aftercontractions whereas would impair calcium-induced calcium release. Thus considerably more evidence in support of a physiological role for spontaneous oscillatory calcium release will be required before this behavior can be woven into EC coupling models.

A possible indirect demonstration of the existence of spontaneous calcium oscillations is the fact that cardiac muscle continuously loses calcium during diastole. Over sufficient diastolic intervals, this leads to the rested state contraction which depends primarily on extracellular calcium entry. One can envision that if spontaneous calcium release occurred during diastole, the calcium released into the myoplasm would partition between reuptake into the SR and export from the cell. Over time, this would

lead to a net decrease in SR calcium stores. Thus, spontaneous oscillations could account for the postulated leak of SR calcium which is incorporated into several EC coupling theories. However, two considerations decrease the likelihood of this possibility. First, diastolic calcium oscillations are promoted by calcium overload conditions. If these were responsible for diastolic calcium efflux, the process should become self-limiting as the overload was reduced and the SR should still retain sufficient calcium for normal twitch development. Secondly, the rest response is an artificially created condition and it is difficult to envision a similar physiological process. While calcium homeostasis is a critical cellular concern, the underlying systems are designed for contracting hearts. The results from rest cannot simply be transposed onto this physiological priority. The deleterious effects of spontaneous oscillations on tension development would appear to exclude any apparent benefits. Thus the locus of the putative leak site remains elusive. Studies of the behavior of cardiac muscle after a period of rest may yet reveal this.

MECHANISMS OF DIASTOLIC CALCIUM EXTRUSION

As previously discussed, cardiac cells lose intracellular calcium during diastole. This was shown by the rapid cooling contracture technique (RCC) where RCC were found to continually decline with increasing rest. Bridge (1986) has also shown that elemental calcium levels, as determined by atomic absorption spectroscopy, are decreased during long rest intervals. Prior to detailed considerations of mechanisms, Koch-Weser and Blinks (1963) described the rested state contraction as one which occurred after a long rest when the ill-defined negative inotropic effect of activation no longer influenced tension development. Pharmacological studies have revealed that the rested state contraction relies primarily on extracellular calcium entry with little or no contribution from the SR (Lewartowski et al, 1978; Reiter et al, 1984). Thus, a mechanism surely exists to achieve this. EC coupling theories by Mensing and Hilgemann (1981) and earlier EC coupling models describe some leak process or route of calcium efflux to explain the rested state contraction. Yet despite a consensus regarding its existence, its nature and locus have remained unclear.

As shown by Bridge (1986) and Kurihara and Sakai (1985), alterations in the extracellular

sodium concentration have pronounced effects on the diastolic efflux of calcium. These results imply a role for sodium-calcium exchange in the process. In a study by Sutko et al (1986), the role of sodium-calcium exchange in postrest inotropy was examined in the rabbit ventricle. Interventions which impaired calcium efflux via sodium-calcium exchange were found to augment postrest tension to a greater degree than they increased steady state tension. These included Na,K-ATPase inhibition by acetylstrophanthidin or reduced extracellular potassium, lowered extracellular sodium (with the $[Ca^{2+}]_o$ / $[Na^+]_o$ maintained constant), or elevation of extracellular calcium. Conversely, the enhanced inotropy produced by isoproterenol, norepinephrine, and histamine had similar effects on both steady state and postrest contractions. Thus, the effects on postrest tension observed after interventions which retarded calcium efflux were not simply due to the enhanced inotropic state of the muscle. At a time when ryanodine (1 uM) produced only modest changes in steady state force development, there were pronounced decreases in postrest tension. Rabbit ventricular muscle does not show positive inotropic responses to rest (Sutko et al, 1986; Bers, 1985). However, in the presence of acetylstrophanthidin, the usual negative response to rest is converted into rest potentiation. This potentiation is also impaired by ryanodine but is not affected by cobalt, a calcium channel blocker (Sutko et al, 1986). The important point from this observation is that even rabbit ventricle, which has a very poorly developed SR (Bers, 1985; Fabiato, 1978), is capable of an SR mediated potentiation. The fact that it normally does not demonstrate this behavior implies that sodium-calcium exchange mediated diastolic calcium efflux is very effective or that extremely high loading of the SR is required to demonstrate rest potentiation. However, the machinery or mechanisms to produce rest potentiation still exist in this tissue.

The relative magnitudes of calcium efflux via sodium-calcium exchange and outwardly directed Ca-ATPases were compared in embryonic chick ventricular cell monolayers by Barry et al, (1986). This study examined shortening, radioactive calcium fluxes, and intracellular calcium levels by atomic absorption spectroscopy. Contractures were produced in the monolayers by rapid exposure to 20 mM caffeine. In the absence of extracellular calcium and sodium, this intervention resulted in a large contracture which relaxed with a $t_{1/2}$ of 9 seconds. If the same protocol was used in the presence of 140 mM extracellular sodium, less than half the tension development was observed and relaxation occurred

more than 10 times faster. This dramatically illustrates the effect of sodium-calcium exchange on intracellular calcium efflux. Using a variety of interventions, the authors estimated that calcium export via sodium-calcium exchange has at least a fivefold greater capacity than Ca-ATPase mediated efflux. They also postulate that sodium-calcium exchange, in addition to calcium reuptake by the sarcoplasmic reticulum, represent the physiological means of cellular calcium removal. The calcium pump is believed to provide little to calcium removal during beat-to-beat relaxation.

EFFECTS OF THE CALCIUM CHANNEL AGONIST, BAY k 8644

A variety of calcium channels are known to exist in different tissues (Reuter, 1985; Nilius et al, 1985; Nowycky et al, 1985; Bean 1985). Undoubtedly, these serve the numerous functions and requirements of intracellular calcium signalling. The recent advent of the patch-clamp technique has been instrumental in the identification and characterization of these. In ventricular muscle, two different types of channels have been reported (Nilius et al, 1985). These differ in conductances, voltage dependencies, and sensitivity to pharmacological interventions. The newly identified channel (T-type) has a smaller measured conductance, and unlike the other commonly studied channel (L-type, Hess et al 1986), does not show differences in conductance when calcium or barium are used as charge carriers. This channel type is also insensitive to dihydropyridine calcium channel blockers and is less affected by the inorganic cation, cadmium. The following discussion deals with the formerly identified L-type channel.

The most potent and specific calcium channel blockers are dihydropyridine compounds of which over 7000 have been synthesized. This group includes compounds used clinically for a variety of cardiovascular disorders including hypertension, congestive heart failure, certain arrhythmias and angina. The effects of these compounds on calcium channel function have been well described (see reviews by Janis and Triggle, 1983, 1984). Cardiac action potentials exhibit a decreased plateau height and duration indicative of a decrease in calcium entry via the slow inward current (Fleckenstein, 1983; Nayler and Grinwald, 1981). Calcium current measured in tissue and single cells also reveal a decrease (Hess et al, 1984). This effect is not mediated by decreasing the unitary conductance of individual channels. Hess et

al (1984) have characterized three modes of calcium channel gating. These are:

Mode 1: brief openings of short duration

Mode 2: long lasting openings with only brief closings

Mode 0: no openings due to channel unavailability

The dihydropyridine calcium channel blockers favor the probability of mode 0. Thus, the decrease in macroscopic current is due to unavailability of some population of calcium channels. In 1983, a novel dihydropyridine was reported to have opposite actions, suggesting an enhancement of calcium entry (Schramm et al, 1983). This compound was a derivative of nifedipine which has negative inotropic effects and relaxes smooth muscle. The new compound, BAY k 8644, was found to enhance cardiac contractility as assessed by measurements of left ventricular pressure in isolated perfused guinea pig heart and the anesthetized dog. It was also found to increase peripheral resistance in the dog as well as enhance contractions in isolated rabbit aortic strips. All of these effects were competitively inhibited or reversed by nifedipine administration. Since this initial report, many studies have appeared confirming these effects or examining the effects of augmented calcium influx in a variety of preparations.

Electrophysiological studies from a number of laboratories have demonstrated augmented calcium influx in the presence of BAY k 8644. Action potential duration was shown to increase in bovine ventricular muscle and Purkinje fibres (Thomas et al, 1985), an effect which was reversed by nisoldipine. Using strontium as the charge carrier, current through calcium channels was increased and the time and voltage dependency was altered in Purkinje fibres. The largest increases were observed at weak depolarizations and a hyperpolarizing shift was observed in the current-voltage relationship. Tension development in isolated guinea pig atria was also examined in this study. Bay k 8644 increased tension development in a frequency dependent manner. The maximal inotropic effect was observed at a stimulation frequency of 0.5 Hz whereas no potentiation was observed at 0.003 Hz. This latter frequency results in the rested state contraction in guinea pig atria (which exhibits potentiation unlike ventricular tissue). Unlike isoproterenol, Bay k 8644 did not alter the time course of contraction. Thus, the effect was not due to cAMP production. Isoproterenol still produced a positive inotropic response in the presence of maximally effective concentrations of Bay k 8644, in support of the last statement.

Voltage and patch-clamp techniques have been used extensively to investigate the mechanism of action of BAY k 8644. In a study which examined guinea pig and neonatal rat ventricular cells, the following observations were made:

1. Action potential duration was increased in a concentration dependent manner.

2. Whole cell calcium currents were increased. With calcium as the charge carrier, only peak inward current was increased without a hyperpolarizing shift in the current-voltage relationship. This effect was blocked by cobalt.

3. From patch clamp studies using calcium as the charge carrier, the probability of channel reopening after opening and closing was found to increase. No evidence of greatly prolonged openings was observed. However, if barium was used as the charge carrier, a marked prolongation of channel open time was observed.

Another newly developed calcium channel agonist (CGP 28 392) was examined in this study and similar though weaker effects were observed.

In contrast to the above report, several other studies have demonstrated a hyperpolarizing shift in the current-voltage relationship. This has been reported by Thomas et al (1985), Sanguinetti et al (1986), Hess et al (1984), Tsien et al (1986) to name a few. This has been observed regardless of the charge carrier used. The reason for this discrepancy is unknown but the possibility that it results from loss of voltage control (due to the increase in magnitude of the current) has been ruled out (Sanguinetti et al, 1986). Hess et al (1984) propose that Bay k 8644 acts by favoring long lasting openings with only brief closings of calcium channels (Mode 2 gating behavior). The most pronounced effects of Bay K 8644 occur at relatively weak depolarizations and less enhancement is observed at higher voltages. The reversal potential is not altered by this agent.

Studies have revealed that BAY k 8644 can act both as a calcium channel agonist and antagonist depending on experimental conditions. The balance between agonist and antagonist effects was determined by the membrane holding potential in voltage-clamped calf Purkinje fibres (Sanguinetti and Kass, 1984; Sanguinetti et al, 1985). If the holding potential was maintained below -55 mV, the agonist effect was favored whereas holding potentials more positive than -40 mV yielded antagonist effects. These

results have led to the categorization of BAY k 8644 as a partial calcium channel agonist. Other experimental approaches have led to a similar description. While conflicting reports exist regarding the frequency dependence of dihydropyridine calcium channel blockers (Chappell et al, 1985), several studies have shown that the effects of agonist compounds are frequency or use dependent. BAY k 8644 had no effect on "rested state" contractions in guinea pig atria and a bell-shaped frequency dependence of the inotropic effect was observed as higher frequencies were examined (Thomas et al, 1985). The maximum inotropic effect was observed at a stimulation frequency of 0.5 Hz in this study. Similar results were obtained by Kennedy and Seifen (1985). The calcium channel agonist, CGP 28 392, has also been shown to have a decreased inotropic effect at higher frequencies (Kamp et al,1985). These reports have also described BAY k 8644 as a partial calcium channel agonist.

The enantiomers of BAY k 8644 have been shown to have distinctly different modulatory actions on the calcium channel (Thomas et al, 1985; Bellemann and Franckowiak, 1985; Marinov and Saxon, 1985; Franckowiak et al, 1985). These studies have demonstrated that one stereoisomer promotes calcium entry whereas the other behaves as a calcium channel antagonist. This has also been found for a related dihydropyridine compound (202-791) (Hof et al, 1985). The demonstration of stereoselectivity at the dihydropyridine binding site is an exciting discovery. However, it also complicates the interpretation of other studies which have mostly employed the racemic mixture. Specifically, the demonstration of a partial agonist effect for BAY k 8644 may well be due to the presence of these two stereoisomers. It does not seem unreasonable to postulate that the affinities of the two distinct species may be different and possess different voltage dependencies. If chirality is sufficient to impart different modulatory effects, the above possibility is no less likely. Thus, the classification of BAY k 8644 as a partial calcium channel agonist may be incorrect, or at least premature.

My studies have focused on the effects of BAY k 8644 on diastolic calcium movements in canine ventricular muscle. Specifically, the mechanism by which BAY k 8644 inhibits rest potentiation was examined. A premise of this investigation has been that in order to inhibit rest potentiation, BAY k 8644 must affect the sarcoplasmic reticulum either directly or indirectly. There are several ways that this could be achieved including:

1. impairing the trigger for calcium release from a normally functioning SR.
2. directly impairing the calcium release process from the SR.
3. decreasing the size of the releasable pool of calcium from the SR.
4. production of cellular toxicity by calcium overloading.
5. enhancing calcium uptake by the SR during post-rest beats.

Several less obvious mechanisms may also be responsible.

In the few studies which have examined the effects of BAY k 8644 on sarcoplasmic reticular function, there appears to be little or no effect. This observation is perhaps expected as the entire class of calcium channel blockers only affect SR function at extremely high concentrations (Colvin et al, 1982). In guinea pig atria, rested state contractions were not affected by BAY k 8644 (Thomas et al, 1985). The "rested state" beat of this preparation shows potentiation and is impaired by agents which affect the SR. These authors eluded to the fact that EC coupling differs in atria and ventricle but results from ventricular muscle were not reported. Another group (Beyer et al, 1986) examined postrest contractions in guinea-pig papillary muscle where an enhancement of tension was found. As this contraction appears to rely exclusively on extracellular calcium entry (Beresewicz and Reuter, 1977), Beyer et al (1986) concluded that BAY k 8644 increased the "rested state" contraction by promoting extracellular calcium entry. This was supported by the observation that the postrest contraction showed an further increase in time to peak tension when BAY k 8644 was present. In a study using skinned atrial fibres, BAY k 8644 did not affect the calcium sensitivity of the contractile apparatus nor did it cause calcium release from intracellular stores (Thomas et al, 1985). The calcium release channels in the SR of skeletal muscle were also not affected by BAY k 8644 (Zorzato et al, 1985). Thus it appears that the primary effect of BAY k 8644 seen in the published studies is to enhance calcium entry through modulation of the sarcolemmal calcium channel.

MATERIALS AND METHODS

GENERAL:

All experiments examined right ventricular trabeculae from canine heart. Dogs of either sex (3-10 kg) were anesthetized with intravenous pentobarbital (30 mg\kg). Hearts were excised through a left lateral thoracotomy and the coronary vasculature was retrogradely flushed via the aorta with ice-cold Krebs-Henseleit (KH) buffer solution. The heart was then placed in ice-cold KH oxygenated with 95% O_2 - 5% CO_2 and the right ventricular wall was dissected from the remainder of the heart. This tissue was then pinned to a dissecting chamber containing the same solution. Thin, free running trabeculae were then tied using 6-0 silk thread and dissected from the wall. Right ventricular trabeculae were chosen as these are generally more abundant than in the left ventricle. The thinnest trabeculae were always used although size was extremely variable. Usually tissue about 1 mm in diameter could be found but larger samples were occasionally used for exploratory experiments. These larger tissue were less desirable as tension tended to decline over time presumably due to insufficient oxygenation of the interior of the trabeculae.

TENSION MEASUREMENTS

Ends of isolated trabeculae were attached to silk loops and were transferred to a vertical muscle bath. One end of the muscle was attached to the base of an acrylic stimulating device. Tissue was placed between two platinum electrodes for field stimulation. Punctate electrodes were also present on the acrylic rod but these were less frequently used. The other end of the muscle was attached by silk thread to a Grass FT-O3C force transducer mounted on a rack and pinion arrangement which permitted length alteration. The transducer was connected to a Grass model 5D polygraph recorder. Four muscles could be examined simultaneously as four baths were identically arranged. Muscles were stimulated at 0.5 Hz via the field electrodes attached to a Grass model SD44 stimulator or a Pulsar 6b stimulator (Frederick Haer Instruments). Pulses were of 4 msec duration at an amplitude between 50-100 % above threshold. The stimulators were driven from a stimulus sequencer (Boyechko and Bose, 1984). During the 45-60 minute equilibration period, muscles were progressively stretched until maximal tension development

occurred. Muscles were stimulated at 0.5 Hz during the equilibration period and between test intervals. The muscle baths contained KH buffer maintained at 37°C and continuously oxygenated with 95% O_2 - 5% CO_2 unless stated otherwise.

BIPHASIC CONTRACTION EXPERIMENTS

Biphasic contraction experiments were conducted in the same apparatus as above using the method of King and Bose (1983). In this study, strontium replaces calcium in the KH buffer solution. Over time, this leads to the development of contractions with greatly prolonged time to peak tensions and slow rates of tension development. At this point, if small amounts of calcium (0.05-0.1 mM) are re-introduced to the bathing solution, contractions showing two phases are produced. The first phase of contraction or P1 has characteristics similar to the contractions in normal KH buffer. The second phase (P2) retains the characteristics of contractions in the buffer where strontium replaced calcium (King and Bose, 1983). Once this preparation has stabilized, interval and pharmacological interventions can be examined. Previous studies have shown that the initial peak (P1) is sensitive to agents which affect SR function (eg. caffeine or ryanodine) whereas P2 is responsive to agents which affect transmembrane cation influx (eg. manganese) (King and Bose, 1983). Therefore, this preparation allows us to distinguish the contributions of intracellular and extracellular activator cation for a given intervention.

ELECTROPHYSIOLOGICAL EXPERIMENTS

Muscles, obtained as above, were mounted in a 10 ml bath containing KH buffer maintained at 37°C. This bath formed part of a recirculating reservoir with total capacity of 40 ml. This design was employed so that continuous gassing of the buffer could occur external to the tissue bath which alleviated mechanical noise and assisted in the maintenance of microelectrode impalements. The level of the bath could be altered by varying the height of the tube which drained the chamber. The bath level was maintained at levels just covering the muscle, in order to minimize stray capacitance. One end of the

muscle was pinned to the silicon base of the muscle chamber. The other end was attached to a stainless steel extension attached to a Grass FT-O3C force transducer. This in turn was fed to a home-built amplifier from which the signal was split with one output feeding a Hewlett-Packard 4-channel storage oscilloscope and the other, after suitable amplification, was sent to a Gould Brush model 440 4-channel chart recorder and a Vetter FM instrumentation tape recorder. Stimuli were applied to the muscle by platinum punctate electrodes which were attached to a micromanipulator. Stimuli were of similar characteristics as for mechanical experiments and were obtained by the same method.

Microelectrodes were made on a Brown-Flaming microelectrode puller from fibre filled glass capillary tubes obtained from Frederick Haer (Omegadot 1.0 mm o.d., 0.75 mm i.d.). These were filled with 3 M KCl solution and electrodes were selected with resistances between 25-50 megohms. A microelectrode was placed in an acrylic microelectrode holder which had a Ag-AgCl pellet in its base. This was filled with 3 M KCl and then attached to the headstage of a Neuroprobe model 1600 preamplifier (Transidyne General Corp.). The headstage was mounted on a micromanipulator which held the microelectrode vertically over the muscle. After determination of resistance and after appropriate negative capacity compensation, impalements were attempted with a motorized micromanipulator (Brinkman). Visual guidance was aided by an inverted Zeiss microscope which was mounted over the muscle bath. Additional stainless steel insect pins inserted in the muscle stabilized a region for microelectrode impalement. The quality and success of an impalement was monitored on the oscilloscope and by digital readout of membrane potential from the preamplifier. Interval changes or pharmacological interventions were not carried out unless a stable impalement was achieved. In at least half the experiments, the impalement was not maintained throughout usually due to the more vigorous contractions produced by the various interventions. When this occurred, a cell was reimpaled often with the same microelectrode. If action potentials similar to those preceding the disruption could not be reobtained, the experiment was discarded.

RAPID COOLING CONTRACTURE APPARATUS

The rapid cooling contracture apparatus was designed to permit rapid changes in temperature of the solution bathing the muscle. Muscles were obtained as previously described. One end was attached via silk thread to a stainless steel extension on a Grass FT O3C force transducer. The other looped end was attached to a steel rod on a micromanipulator which permitted length alteration. The muscle was placed in a bath of 2 ml volume and oriented in the same direction as the flow of solution. The bath formed part of two large recirculating reservoirs. Flow of either solution through the bath was controlled by manual switching of a solenoid. The volume of each separate reservoir was 50 mls of which a significant portion was contained in a water jacketed column. This permitted control of solution temperature which typically held one solution at $37^{o}C$ and one at $0\text{-}2^{o}C$. HEPES buffer was used in all experiments reported although some trials compared HEPES against bicarbonate buffer. No substantial difference was observed. HEPES was chosen as pH was more easily controlled. The solution not flowing through the muscle chamber flowed through a bypass system. This design permitted continuous circulation and oxygenation of both solutions. Total deadspace of the solution not bathing the muscle was about 3 mls (1.5 mls at both input and output ends of the bath) and therefore over 90% of the solution was being temperature controlled and oxygenated. Since solution flowing through the bath also flowed through the bypass system, the diameter of tubing for the bypass was controlled by a screw-clamp which permitted regulation of flow through both the bath and the bypass. In at least 4 separate determinations, flow through the bath was adjusted to about 300 mls per minute. Since switching between solutions by the solenoid was essentially instantaneous (relative to other processes influencing temperature change), total solution change occurred in less than 1 second. The actual temperature change would be somewhat slower given the small but significant heat capacity of both the muscle and the bath.

The apparatus holding the muscle (tension transducer and micromanipulator) was mechanically isolated from the remainder of the set-up (bath, solenoid, recirculation system). Therefore, transmission of mechanical noise by recirculation and the violent mechanical switching by the solenoid was severely

damped as it was only transmitted to the muscle by fluid in the bath. Provided that the flows between the two solutions was equalized, the transition between the two solutions was barely detectable in force tracings. Oxygenation of the two solutions was also achieved external to the bath to alleviate mechanical noise.

Recirculation of physiological salt solutions in both the rapid cooling contracture apparatus and the electrophysiology apparatus were similar. Solution flowed to the bath by gravity feed from a reservoir of adjustable height. Selection of tubing diameter to the bath and variable reservoir height were used to control flow rates. The oxygenation of the solutions was carried out in the reservoirs to minimize mechanical noise in both set-ups. Solution from the bath was withdrawn by negative pressure generated by a Cole-Palmer roller pump. This fluid then returned to the reservoir. Since the aspiration rate from the bath could be set above flow rates to the bath, constant fluid levels could be maintained as the pump would suck air if fluid was unavailable. This prevented the need for accurately matching input ant output flow rates.

STIMULATION PROTOCOLS

Muscles were stimulated by punctate or field electrodes as indicated. The stimulation protocol was programmed into an Apple computer to control the stimulus sequencer (Boyechko and Bose, 1984). The basic stimulation rate or basic cycle length (BCL) was 2000 msec unless indicated otherwise. For all experiments, trains of stimulation were created such that the first beat of a new train was the test interval being examined. Thus, the first beat of a new train could be joined to the preceding train either early to examine extrasystoles or late to examine rest responses. The remainder of the train was at the BCL for a variable number of beats (30-100) to return the muscle to steady state conditions prior to the next test interval. When the effects of rapid stimulation were examined, the BCL of the test train was either increased or decreased. Test intervals were usually inserted at random as the computer program accepted trains in any order. A signal from the computer drove a stimulator (Pulsar 6i, Frederick Haer & Co.) which sent square wave pulses of 4 msec duration at about 50% above threshold levels.

SOLUTIONS

In mechanical and electrophysiological experiments, Krebs-Henseleit buffer of the following composition was used (mM): NaCl 118.0; KCl 4.7; $CaCl_2$ 2.5; $MgSO_4$ 1.2; KH_2PO_4 1.4; $NaHCO_3$ 12.5; glucose 11.1. The solution was continuously gassed with 95% O_2 - 5% CO_2 to maintain a pH of 7.4. For rapid cooling contracture experiments, HEPES buffer was used with the following composition: NaCl 140.0; KCl 4.7; $CaCl_2$ 2.5; $MgCl_2$ 1.0; glucose 10.0; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 5.0. The pH was adjusted with NaOH with appropriate reduction in NaCl to achieve pH 7.4 at 37^oC. This solution was continuously gassed with 100% O_2. For both HEPES and KH buffers, reduction in sodium concentration was achieved with isosmolar sucrose substitution. When potassium or calcium were elevated or lowered, no corrections for osmolarity changes were made. Strontium replaced calcium in Krebs-Henseleit buffer for biphasic contraction experiments (Sr-KH).

BAY k 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) (Bayer AG) was a generous gift from Dr. A. Scriabine (Miles Institute of Preclinical Research) and was dissolved in 100% ethanol. The concentration of ethanol did not exceed 0.1% in the bathing solution. Ryanodine (Merck, Sharp and Dohme; courtesy of Dr. R. Rogers) and caffeine (Sigma) were dissolved in water and added directly to the bath. Ouabagenin and nifedipine (Sigma) were also dissolved in alcohol to make stock solutions and added directly to the bath. Again, the alcohol concentration did not exceed 0.1% in the final solution.

DATA ANALYSIS

Analysis of mechanical experiments (twitch tension and rapid cooling contractures) was made by direct measurement of data from the polygraph recordings. Calibration prior to experiments permitted calculation of exact tension levels. Muscles served as their own controls during ionic or pharmacological interventions. For electrophysiological experiments, recorded data were analysed using a Data Precision model Data 6000 waveform analyzer and hard copies of data were obtained from a Hewlett-Packard 7470

digital plotter. The waveform analyzer was programmed using an Apple 2e computer to measure the following tension and membrane potential parameters:

CONTRACTION PARAMETERS: time to peak tension, tension rise time (from 10-90% of total contraction height, onset latency (0-10% of contraction height, tension decay time (90-10% of contraction height) and relaxation compartments (90-60%, 60-30%, and 30-10%), and contraction width at 50% tension development.

ACTION POTENTIAL PARAMETERS: resting membrane potential, overshoot, Vmax, action potential duration at 80, 50 and 20% repolarization.

Statistical analysis, where appropriate, was conducted using Student's t-test for paired or unpaired observations for experiments with two sets of variables or a completely random design analysis of variance with multiple comparisons by Duncan's test (Steel and Torrie, 1960) for experiments with three or more sets of variables. Error bars on graphs indicate standard error of the mean in all cases.

RESULTS

EFFECTS OF BAY k 8644 ON THE INTERVAL-FORCE RELATIONSHIP

The purpose of this study was to examine the effects of the calcium channel agonist, BAY k 8644, on the interval-force relationship in isolated canine ventricular muscle. The paradigm of particular interest was rest potentiation although other interval alterations were examined to a lesser extent. In figure 1 (upper tracing), a control response to an extrasystole is shown. The basic cycle length (BCL) of the steady state train was 2000 msec and an extrasystole was imposed 600 msec after a steady state beat. Tension development of the extrasystole was less than that observed for beats of the regular train. However, the beat following the extrasystole is potentiated despite the normal 2000 msec coupling interval. This behavior is referred to as post-extrasystolic potentiation and has been observed in a variety of cardiac tissues from different species (Koch-Weser and Blinks, 1963). After the addition of 1 uM BAY k 8644, steady state tension increased about 100%. When an extrasystole with the same coupling interval (600 msec) was applied, it now developed more tension than the potentiated steady state beats. However, this was no longer followed by potentiation of the next normally coupled beat. That is, post-extrasystolic potentiation was no longer observed.

Figure 2 illustrates the effect of the coupling interval on extrasystolic force development. This is expressed as a percentage of steady state tension. In untreated muscles, extrasystolic force monotonically increases as the coupling interval increases. This increase reaches a plateau at about half the basic cycle length at which point the extrasystole develops the same force as beats in the normal train. Extrasystoles can occasionally be produced as early as 100-150 msec but this is normally an interval at which the muscle is refractory. In the BAY k 8644 treated muscle group, extrasystoles are usually not observed at intervals shorter than 250 msec. Several groups have demonstrated the ability of this agent to prolong the cardiac action potential (Thomas et al, 1985; Brown et al, 1984) and thus the refractory period would be increased. Once an extrasystole can be generated, tension increases at a greater rate with increasing interval than for untreated muscles. At longer coupling intervals (usually greater than 600 msec), tension development exceeded steady state levels. This was not observed at any interval in the control muscles.

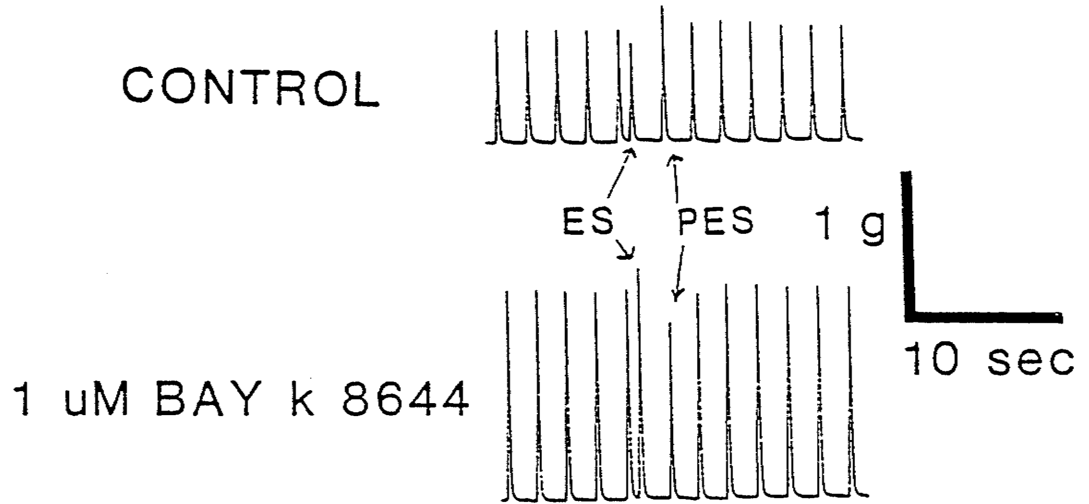


FIGURE 1. Typical tension tracings in response to a 600 msec extrasystole of a control (top) and 1 uM BAY k 8644 treated muscle (bottom). The extrasystolic (ES) and post-extrasystolic (PES) beats are indicated by arrows. Basic cycle length = 2000 msec.

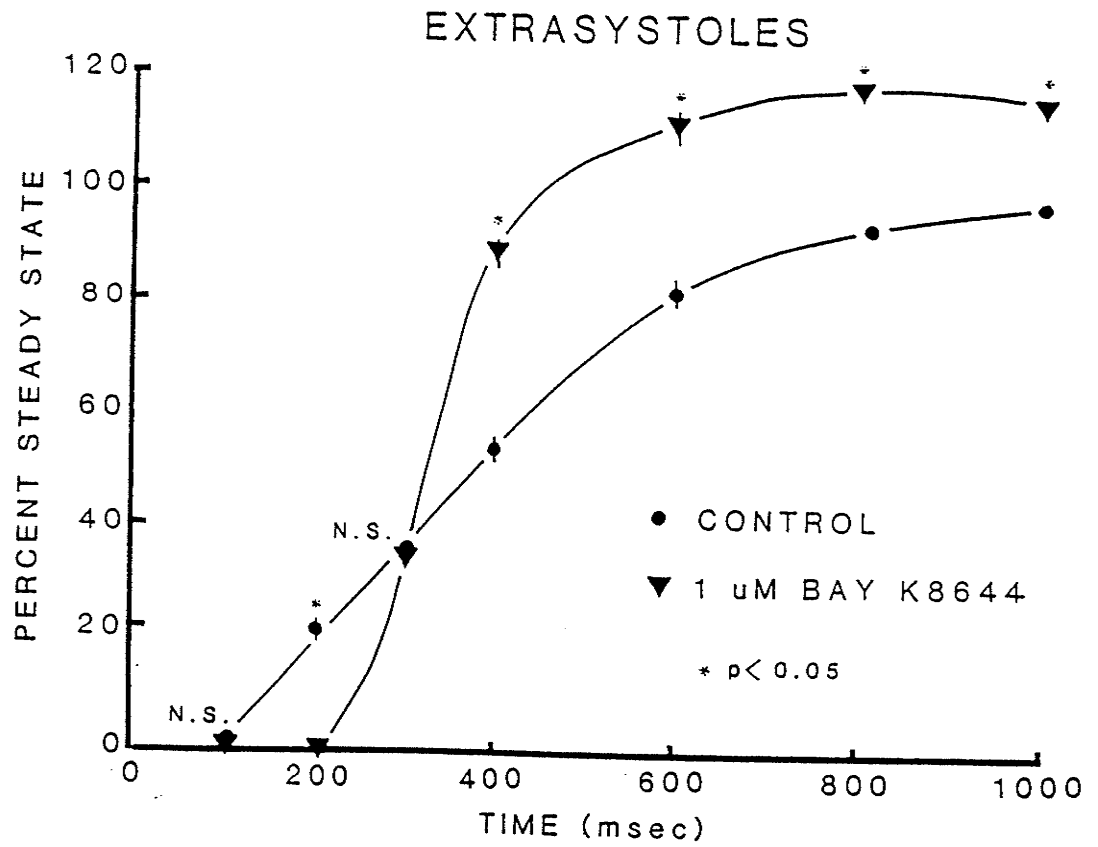


FIGURE 2. Extrasystolic tension responses of control and BAY k 8644 treated muscles over a range of coupling intervals. Tension is expressed as a percentage of the steady state tension preceding the extrasystole. n=5

The inotropic response to extrasystoles over a range of coupling intervals is shown in figure 3. Unlike extrasystolic tension development, the post-extrasystolic tension is inversely related to the coupling interval in control muscles. The greatest potentiation is observed after a 200 msec extrasystole and then gradually declines at longer coupling intervals. As the extrasystolic tension approaches steady state levels, so does the post-extrasystolic beat. In the presence of the calcium channel agonist, potentiation is severely reduced or not observed at all. This effect was observed at all coupling intervals and a relatively flat response was obtained. Thus, an apparent augmentation of calcium influx during the extrasystole (as tension is increased) is not translated into post-extrasystolic potentiation.

During alterations in stimulation frequency, many mammalian species show both a positive and negative staircase response. The short term change is often directionally opposite to that observed once tension has stabilized at the new frequency. This behavior is seen in a typical control muscle in figure 4 (upper tracing). The immediate response to the change in BCL from 2000 msec to 500 msec is a decrease in developed tension. This first beat is analogous to an extrasystole and thus this behavior should be expected. Over the next several beats, tension increases and approaches a new steady state level. Once stimulation at the original rate is resumed, the opposite effect is observed. That is, the first few contractions are potentiated before returning to the original tension levels. This response is analogous to rest potentiation as the original coupling interval is long relative to the high frequency train.. After BAY k 8644 treatment (lower tracing), a different response was observed. Similar to the effect on extrasystoles, the first beat of the fast train is not appreciably depressed. Tension increases rapidly and then declines. On resumption of stimulation at a BCL of 2000 msec, a depression of the first beat is observed. Small aftercontractions were also usually observed as the high frequency train was terminated.

Several mammalian cardiac tissues exhibit a positive inotropic response to periods of rest (Koch-Weser and Blinks, 1963; Lewartowski et al, 1978; Bers, 1985). The two tension tracings in figure 5 (left) show the response of canine ventricular muscle to two different rest intervals. After 2 min. of rest, a large potentiation of contraction is normally observed. At the longer rest interval of 8 min., a potentiation of lesser magnitude is observed. The potentiation at both intervals decays with successive contractions and usually dips to below steady state tension levels. Following the tension undershoot, the muscle gradually

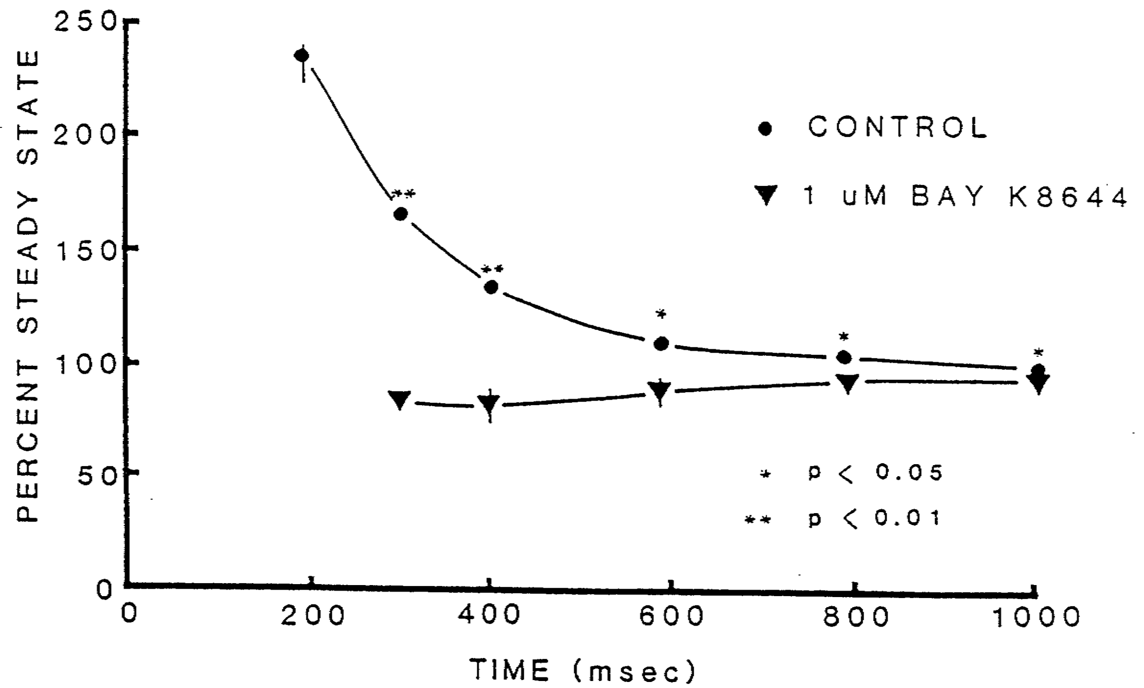


FIGURE 3. Post-extrasystolic tension responses of control and BAY k 8644 treated muscles. Tension is expressed as a percentage of the steady state tension preceding the extrasystole. n=5

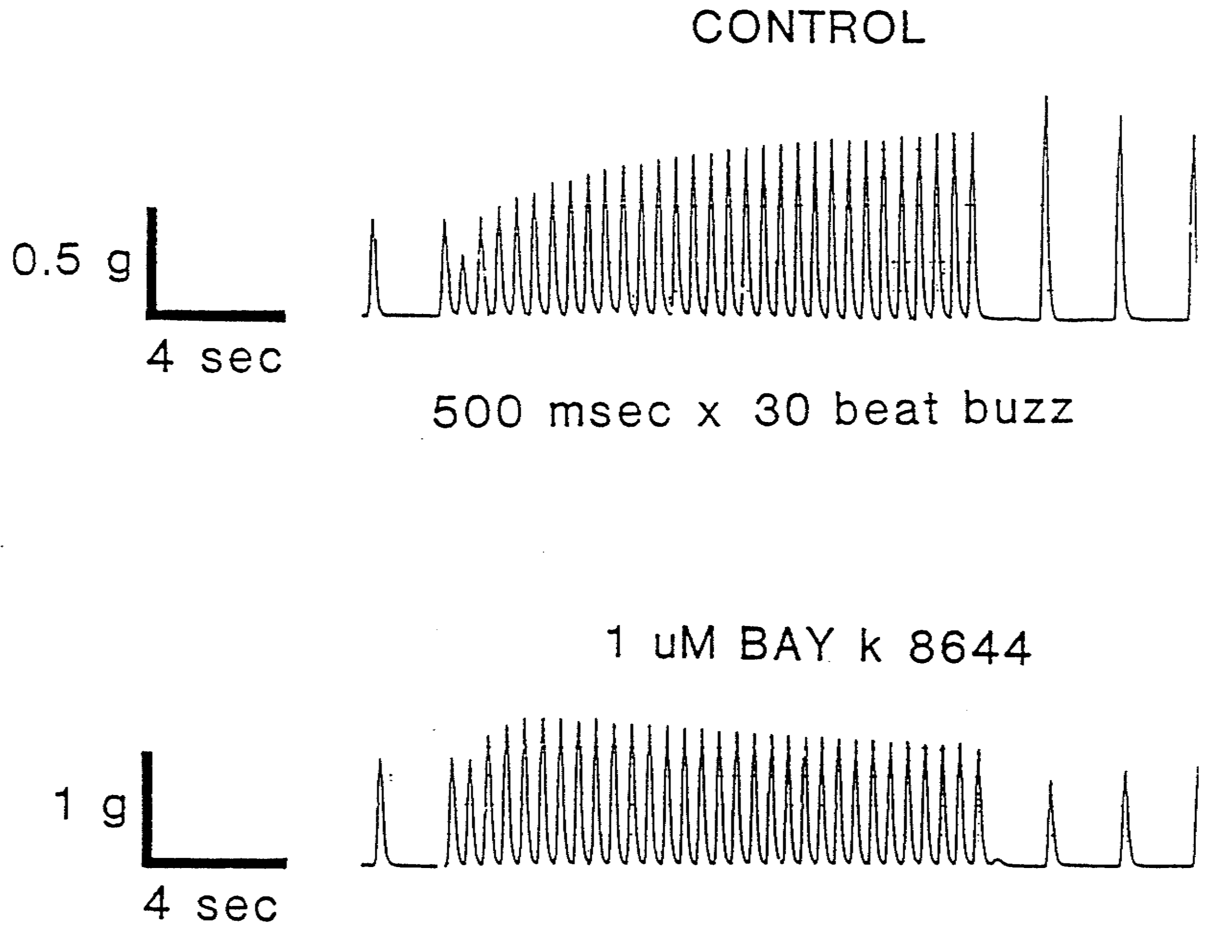


FIGURE 4. Tension tracings of control (top) and BAY k 8644 treated muscle (bottom) in response to a change in frequency from 0.5 Hz (BCL = 2000 msec) to 2 Hz (BCL = 500 msec). Following the high frequency train of stimulation (30 beats), the original frequency was restored.

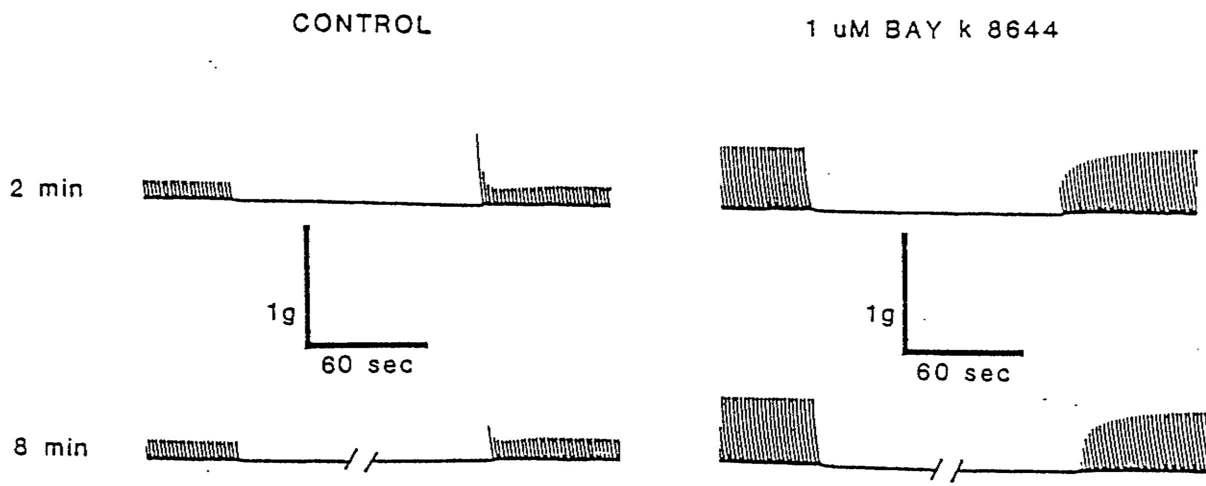


FIGURE 5. Tension tracings of control (left) and BAY k 8644 treated muscle (right) in response to rest intervals of two (top) and eight (bottom) minutes. BCL = 2000 msec.

recovers to steady state levels. In the presence of 1 uM BAY k 8644, rest potentiation is not observed at either interval. The post-rest beat is increasingly depressed as the rest interval is increased. Tension recovery is monotonic although tension overshoots were sometimes observed. Pooled results over a range of rest intervals are shown in Figure 6. In the control muscles, maximum augmentation of the post-rest beat usually occurred between 1-2 min. of rest. Potentiation rapidly increases as this interval is approached and then decreases gradually at longer rest intervals. If very long rest intervals were examined (15 min or greater), a post-rest depression was always observed. However, tension recovery after such an intervention was very slow and often incomplete. Therefore, "rested state" contractions were seldom examined. In the BAY k 8644 treated muscles, a rest depression was observed at all rest intervals. The degree of rest depression increased with increasing interval and in some muscles, tension was was not detectable after an 8 minute rest. However, recovery was always rapidly achieved on resuming stimulation, unlike recovery after prolonged rest (> 20 min) in untreated muscles.

ELECTROPHYSIOLOGICAL ALTERATIONS PRODUCED BY BAY k 8644

To gain insight into the mechanism by which BAY k 8644 altered the interval-force relationship, several accompanying electrophysiological parameters were investigated. The effects of BAY k 8644 and another calcium channel agonist, CGP 28 392, have been reported to be frequency dependent but this was only examined for steady state frequency changes (Thomas et al, 1985; Kennedy and Seifen, 1985; Kamp et al, 1985). Figure 7 compares the action potentials in control and BAY k 8644 treated muscle in response to an extrasytole with a coupling interval of 600 msec. In the control muscle (top), the extrasystolic and steady state action potential have similar durations and magnitudes. Both increases and decreases in action potential plateau height and duration have been reported and also observed in this laboratory (see introduction). A more consistent observation is the decrease in these parameters for the post-extrasystolic beat. Decreases in plateau height and duration were consistently observed. Thus, the greatest tension is produced by the beat with an apparent decrease in extracellular calcium entry. While the magnitude of extracellular calcium entry is not known with surety (as other currents also contribute to

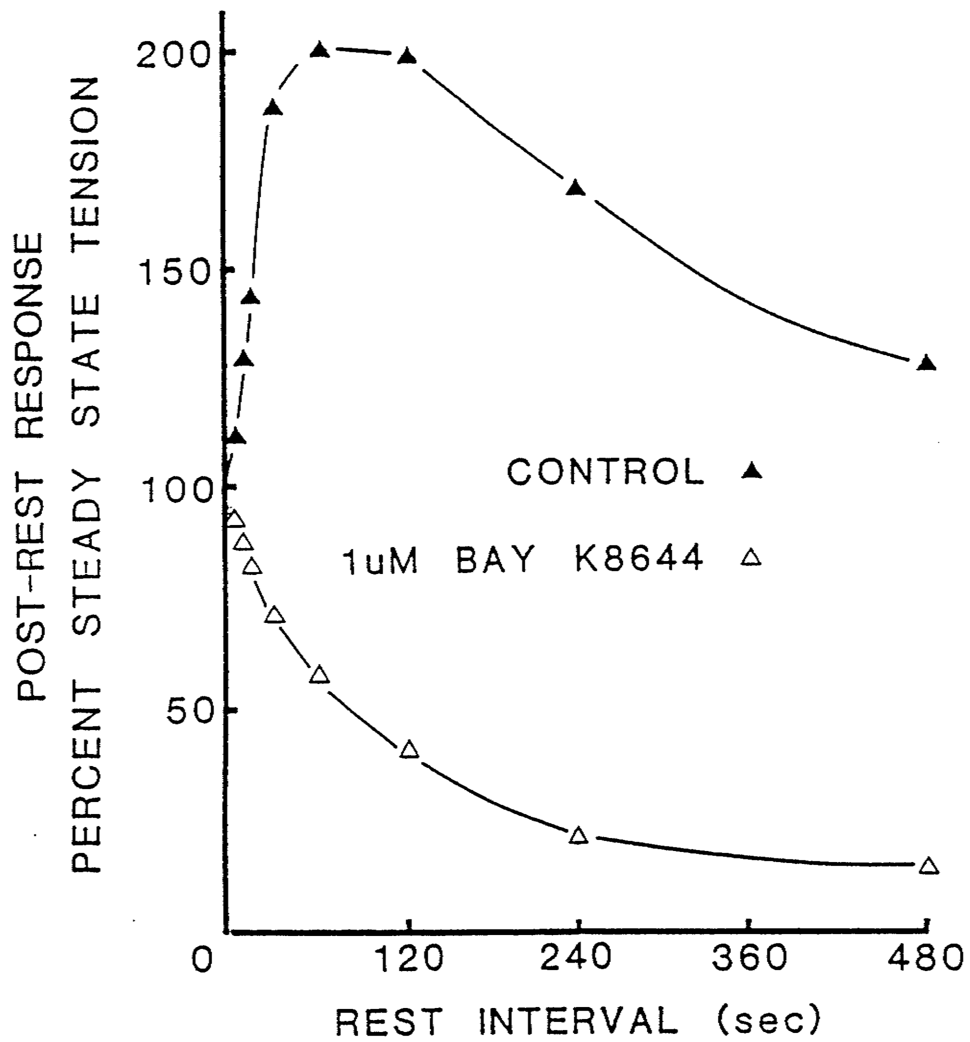


FIGURE 6. Post-rest tension development in control and BAY k 8644 treated muscles over a range of rest intervals. Post-rest tension was expressed as a percentage of the steady state tension preceding the rest interval. A significant difference was observed between control and treated muscles at all intervals. n=7

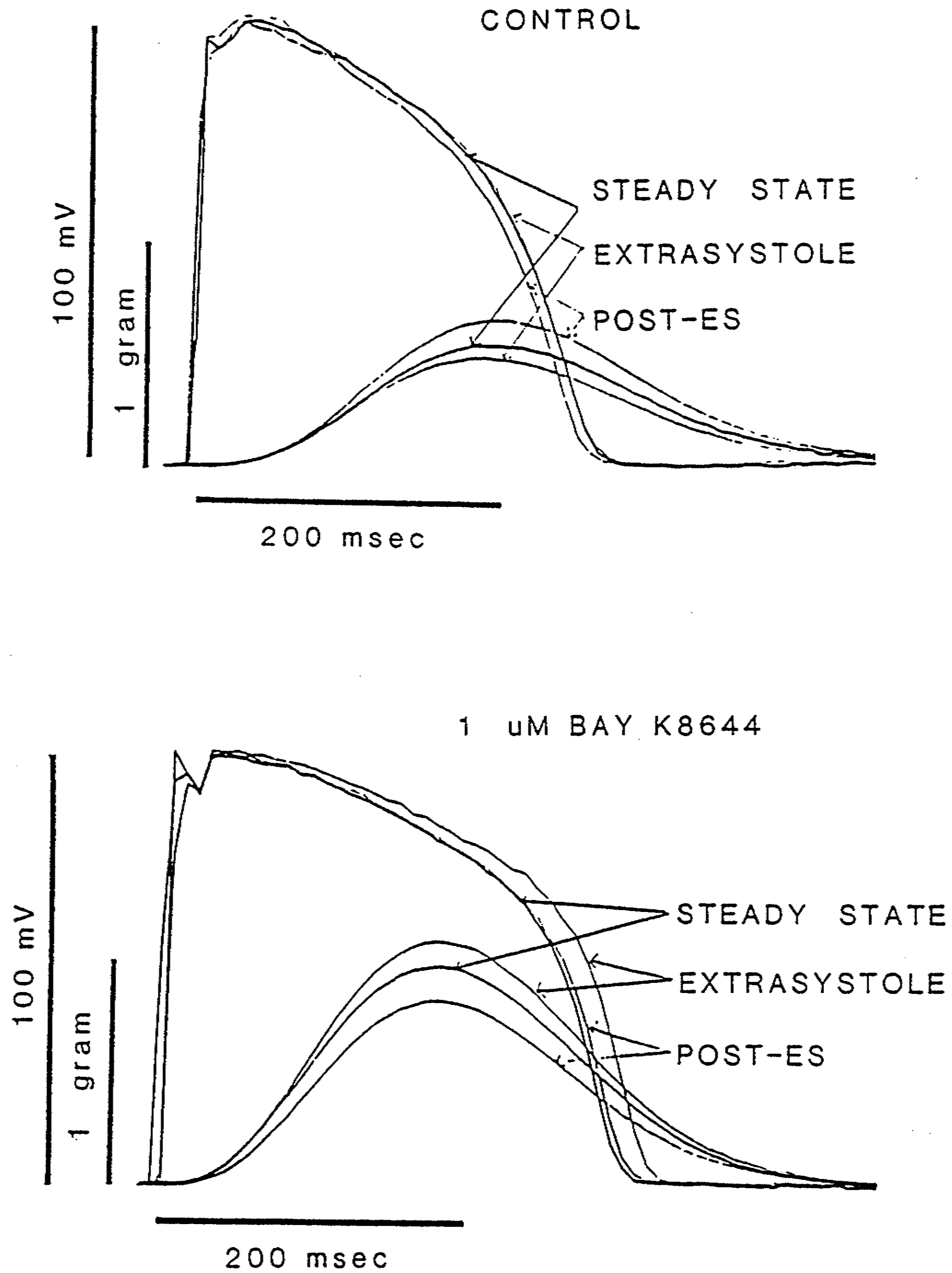


FIGURE 7. Action potentials and contractions for a 600 msec extrasystole in control (top) and BAY k 8644 treated muscle. The steady state, extrasystolic and post-extrasystolic beats are indicated by arrows. Tracings are overlayed.

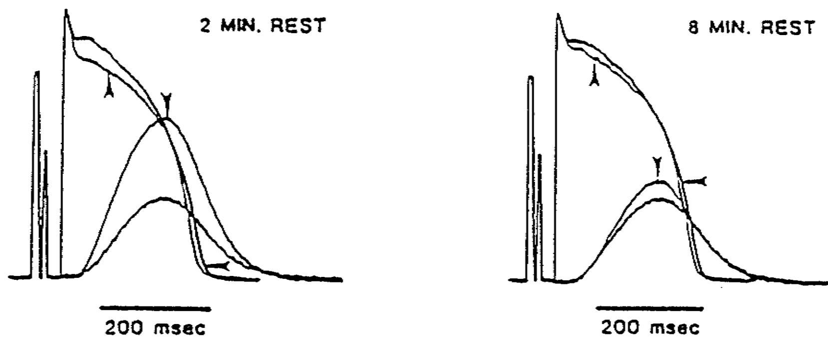
the plateau phase), this result might be expected if increased intracellular calcium release accelerates the inactivation of calcium channels (Lee et al, 1985). The BAY k 8644 treated muscle consistently showed an enhanced plateau and duration for the extrasystole. Despite an apparent increase observed for the extrasystole, the post-extrasystolic beat is not potentiated. As shown by Wood et al (1969), increasing the plateau height by voltage-clamp techniques led to a potentiation of the next several beats. However, the possibly analogous effect produced by BAY k 8644, does not result in a similar potentiation.

Extracellular calcium entry during a post-rest beat is reportedly decreased (Bers, 1985). Our results are also suggestive of this as the post-rest action potentials shown in figure 8 (top tracings) have a reduced action potential plateau height. This was observed at both rest intervals of 2 and 8 min. Again this result may be caused by an enhanced intracellular calcium release (Bers, 1985) with subsequent acceleration of calcium channel inactivation (Lee et al, 1985). As reported by Hilgemann (1986), terminal repolarization in atrium is usually prolonged for these beats as well. The post-rest action potentials in the BAY k 8644 treated muscle suggest an augmentation of extracellular calcium entry during this beat. Plateau height and duration were increased with increasing rest interval. After an 8 minute rest, tension was barely detectable despite a very large apparent increase in extracellular calcium entry.

BIPHASIC CONTRACTIONS

As discussed in the introduction, considerable evidence exists which supports the idea that interval-dependent potentiation is due to an augmentation of intracellular calcium release from the sarcoplasmic reticulum. The abolition of these potentiation phenomena due to the presence of BAY k 8644 led us to suspect that this agent was impairing SR function. To investigate this, we examined the effect of this agent on a biphasic contraction model which permits assessment of extracellular calcium entry and intracellular calcium release for individual beats. Extracellular calcium is replaced with strontium which leads to contractions that have slow onsets and rates of tension development, similar to "rested state" contractions (Lewartowski et al, 1978). At this point, the contractions are insensitive to agents which impair SR function but tension is extremely sensitive to agents affecting extracellular

CONTROL



1 uM BAY k 8644

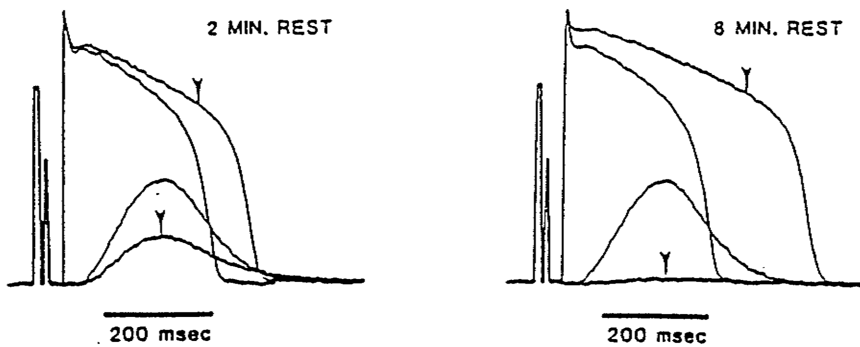


FIGURE 8. Action potentials and contractions for contractions before and after two (left) and eight (right) minute rest intervals in control (top) and BAY k 8644 treated muscle (bottom). Post-rest beats are indicated by arrows. The first vertical calibration bar indicates 0mV and the second calibration bar is a 50mV signal from the resting membrane potential.

calcium entry (King and Bose, 1983). Therefore this contraction is believed to result almost entirely from extracellular strontium entry. At this point, addition of small amounts of calcium to the bath cause reappearance of an early peak with normal time to peak tension.. By carefully titrating the added calcium (usually 0.05-0.2 mM), stable biphasic contractions result. The first peak (P1) has characteristics similar to the normal contraction. P1 is impaired by agents which affect the SR such as caffeine and ryanodine. P2 retains characteristics of contractions in strontium buffer alone. Figure 9 shows a post-extrasystolic contraction for a muscle in normal KH buffer (top) and during biphasic contractions (middle). During biphasic contractions, the extrasystole shows enhanced extracellular strontium entry relative to steady state beats whereas intracellular calcium release is relatively impaired. The ensuing potentiated beat shows marked augmentation of intracellular release supporting the contention of the role of the SR in this phenomenon. Extracellular calcium entry is similar during this beat but this may result from overlap with the large intracellular release. When BAY k 8644 was examined on the biphasic contraction model, steady state beats showed augmented P2 in accord with its known effect on extracellular calcium entry. P2 tended to increase continually with time but over short intervals (5-10 minutes) it was reasonably stable. A much larger P2 was now observed for the extrasystole (similar to the results shown in figures 1,2) and P1 showed a small decrease. A small potentiation of P1 was observed during the post-extrasystolic beat but it was greatly blunted compared to the biphasic control. Thus, BAY k 8644 again appeared to impair the augmented intracellular calcium release during post-extrasytolic potentiation.

In figure 10, rest potentiation for a control muscle is shown (top). The biphasically contracting muscle (middle) reveals that this potentiated contraction results from an augmented intracellular calcium release. In support of work by others (Bers, 1985), extracellular calcium entry is decreased during this beat as seen by a small decrease in P2. After the addition of BAY k 8644 (bottom), P2 was markedly enhanced for steady state beats and P1 was slightly depressed. Similar to our electrophysiological results, the post-rest beat showed a large and protracted increase in extracellular calcium entry. BAY k 8644 also abolished the usual enhancement of intracellular calcium release. The data in figures 9 and 10 are indicative of an inhibitory effect of BAY k 8644 on SR function.

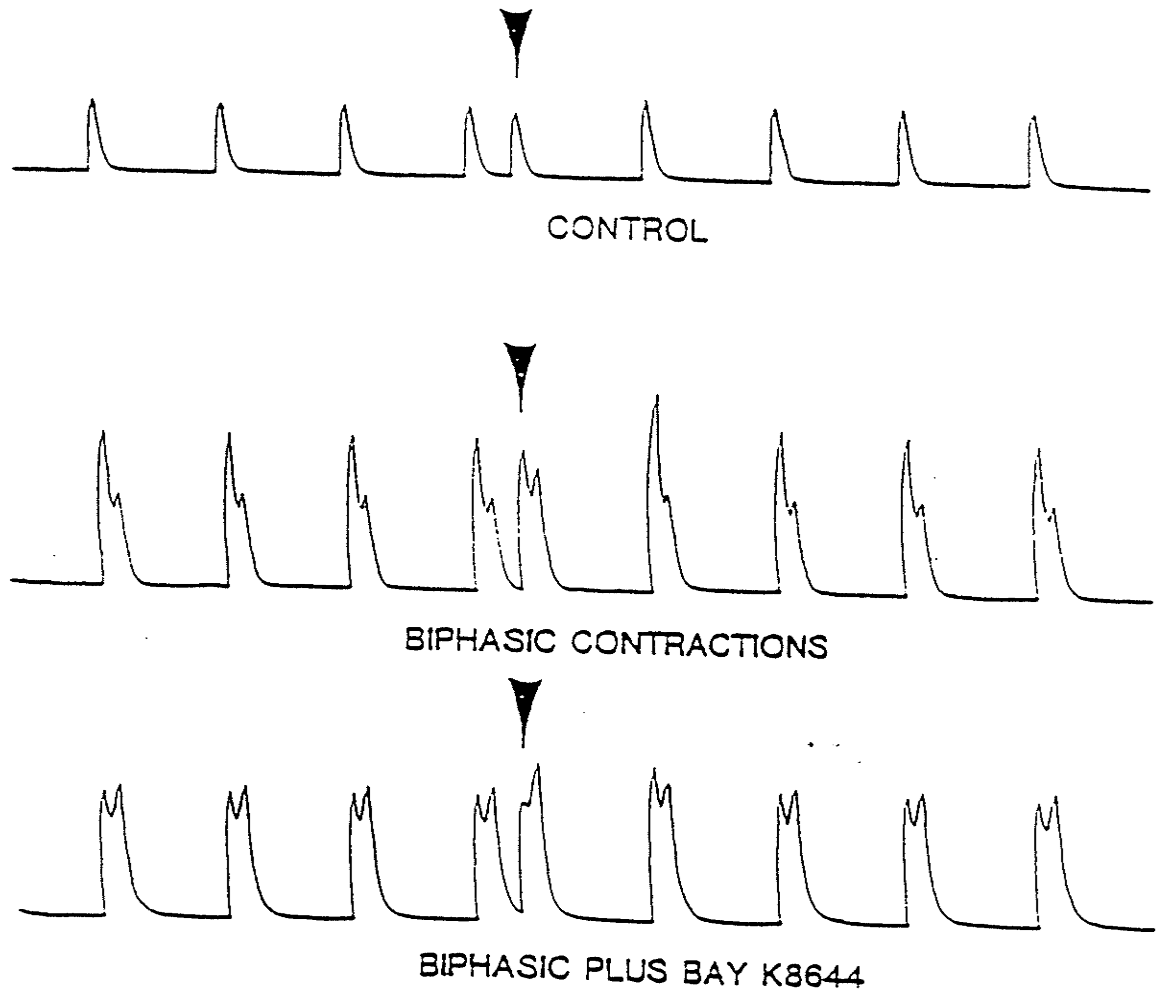
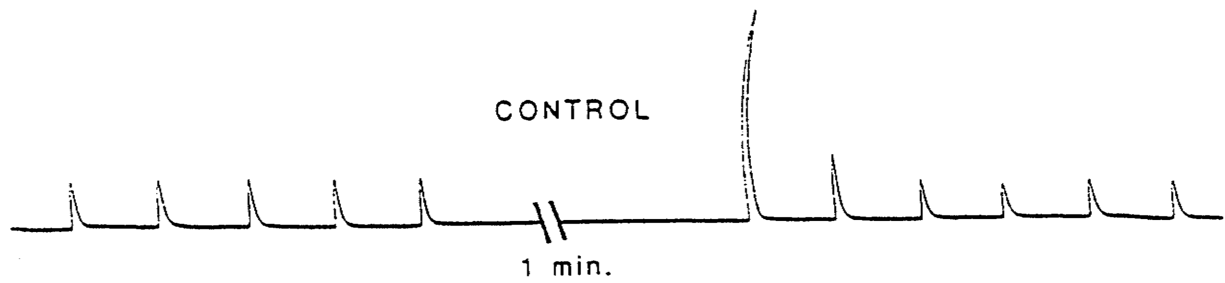
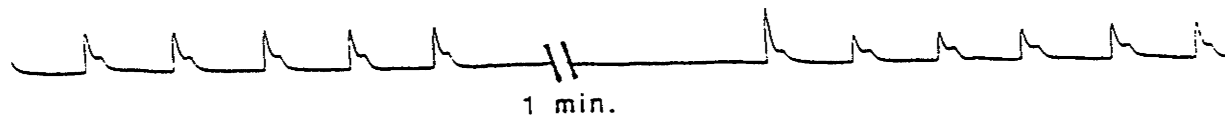


Figure 9. Response to an extrasystole (700 msec) in normal KH buffer (top), during biphasic contractions (middle), and during biphasic contractions in the presence of 1 uM BAY k 8644 (bottom). BCL = 2000 msec.

REST POTENTIATION





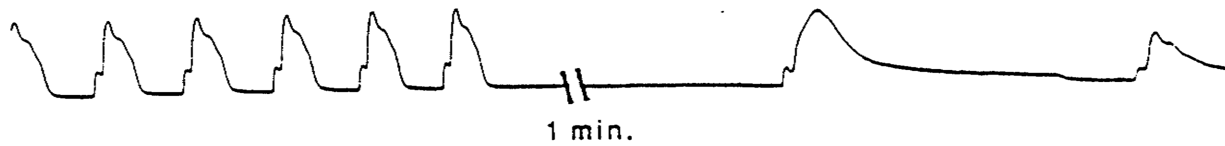


Figure 10. Response to a one min. rest in normal KH buffer (top), during biphasic contractions (middle), and during biphasic contractions in the presence of BAY k 8644 (bottom). BCL = 2000 msec.

INVESTIGATION OF POSSIBLE MECHANISMS OF ACTION OF BAY k 8644 ON THE INTERVAL-FORCE RELATIONSHIP

Several approaches were taken to investigate possible mechanisms by which BAY k 8644 might impair SR function. We considered the possibility that the effects produced by BAY k 8644 were due to calcium overload of the SR. As reported by Allen et al (1985), this would impair tension development by causing nonhomogenous calcium release from the SR. Cells or portions of would then act as series compliances and impair overall tension development. To examine this, the effects of BAY k 8644 on rest potentiation was examined with different calcium concentrations in the bathing medium. As shown in figure 11 (right), control muscles exhibit decreased rest potentiation as extracellular calcium is elevated. These tracings show post-rest recovery following an 8 minute rest interval. This is likely due to mechanical limitations as well as the increase in spontaneous intracellular calcium oscillations (Lappe and Lakatta, 1980). In many muscles, post-rest tension was depressed at higher calcium concentrations (7.5 mM). With 1 uM BAY k 8644 present, elevation of extracellular calcium had an opposite effect. Post-rest contractions were enhanced by elevated extracellular calcium. This occurred despite the negative effect of elevated calcium on steady state tension (due to calcium overload). Pooled results from 6 muscles are shown in figure 12 for an 8 minute rest interval. Diametrically opposed results are obtained in control and BAY k 8644 treated muscles. This observation indicates that calcium overload is unlikely to be the mechanism for BAY k 8644 induced rest depression.

The effect of variable extracellular calcium on action potentials during rest recovery is shown in figure 13. In control muscles (top), the steady state action potential was shortened in the presence of higher extracellular calcium. However at both concentrations, the post-rest action potential generally had a similar configuration. Similar effects were observed in the presence of BAY k 8644. That is, elevated extracellular calcium caused some shortening of the action potential but the configuration of all beats was maintained. Thus, elevated extracellular calcium entry does not appear to reduce BAY k 8644 induced rest depression by converting electrical characteristics toward control levels. An alternative hypothesis is

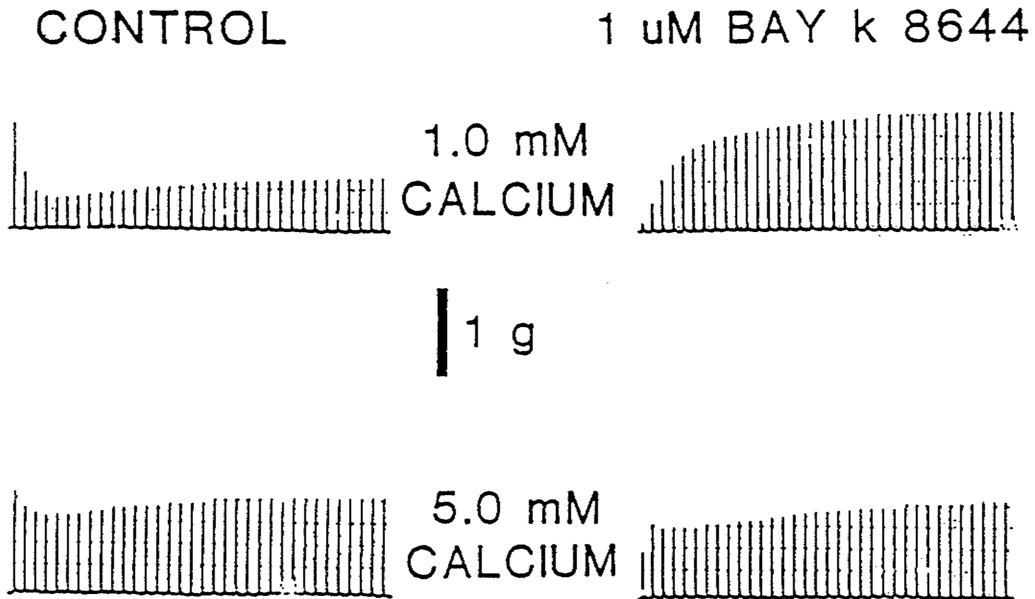


FIGURE 11. Effect of different extracellular calcium concentrations on the post-rest recovery after an eight minute rest interval in a control (left) and BAY k 8644 (right) treated muscle.

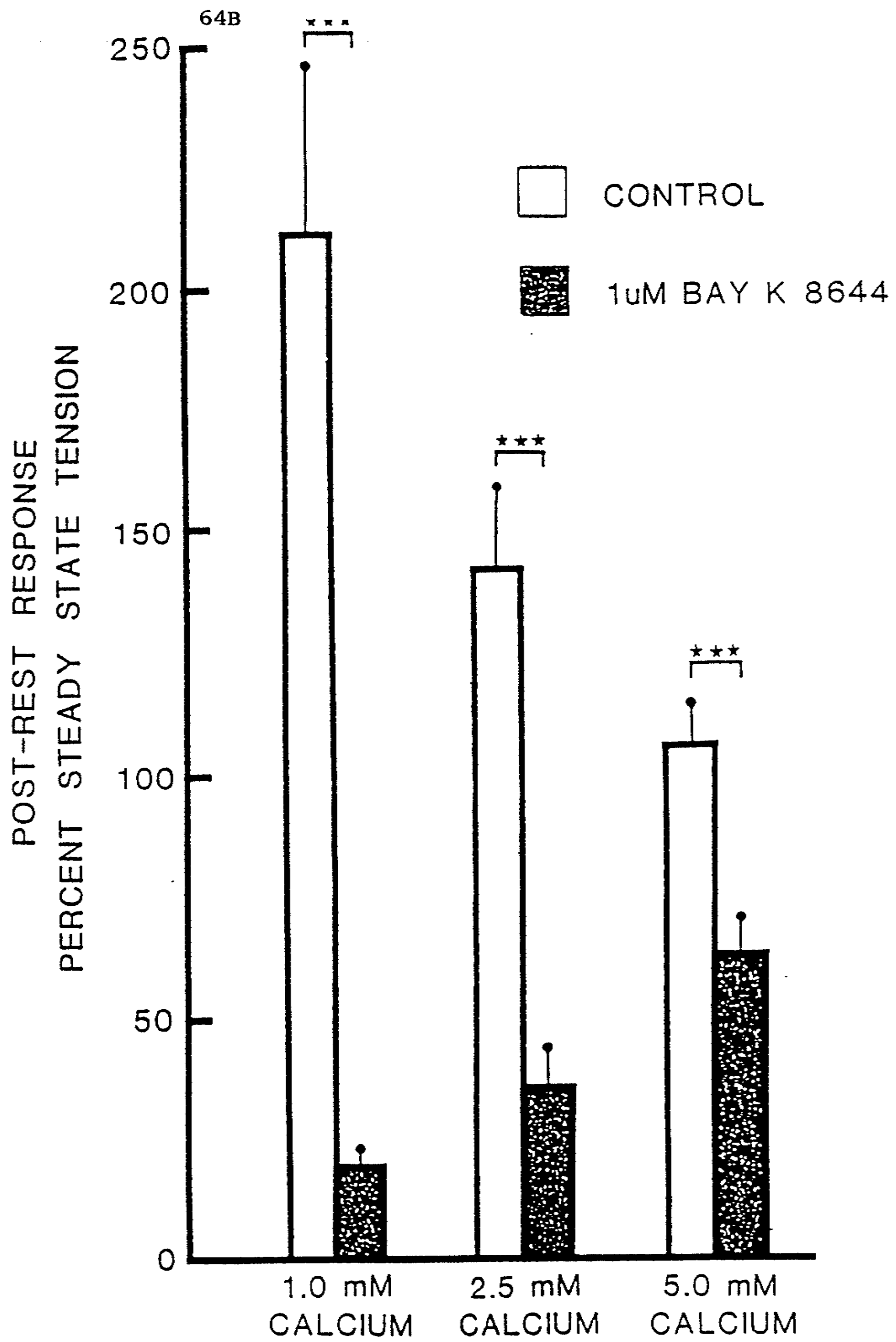
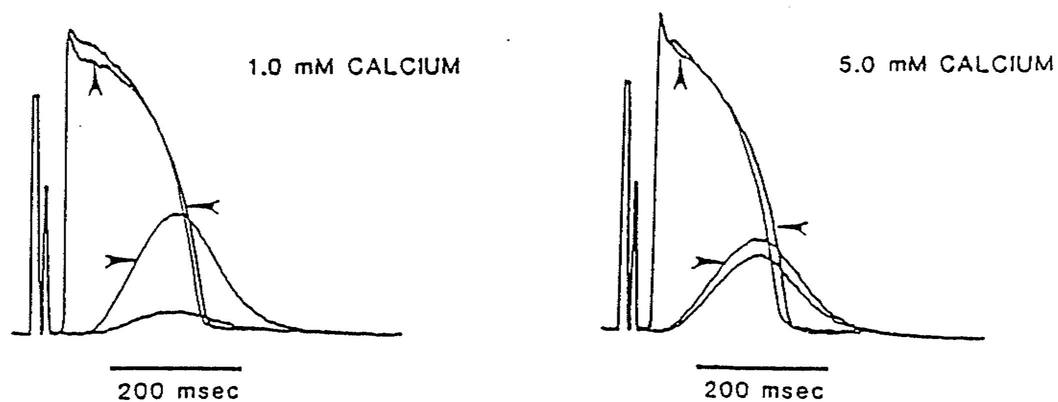


FIGURE 12. Response to three different rest intervals in the presence of different extracellular calcium concentrations. Pooled results from 6 muscles before and after BAY k 8644 treatment. Tension expressed as a percentage of steady state tension preceding the rest interval.



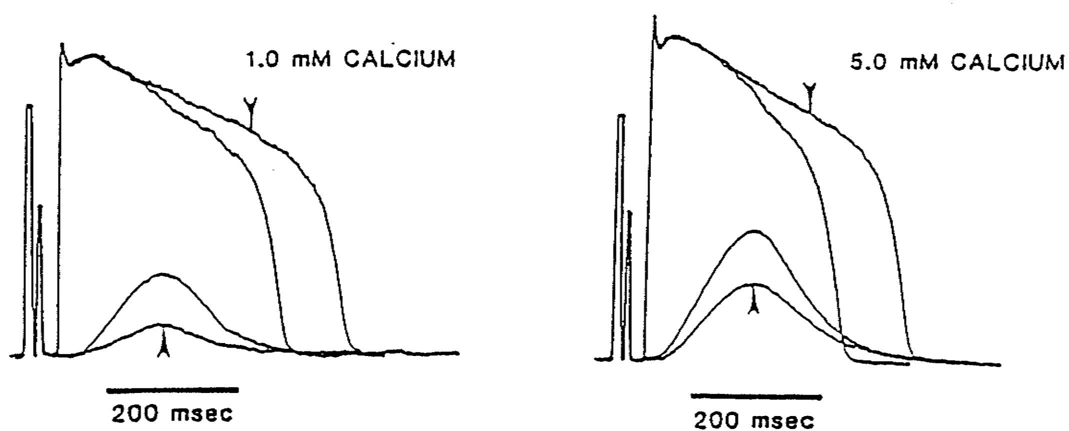


FIGURE 13. Action potentials and contractions for an eight minute rest before and after BAY k 8644 addition. Effect of different calcium concentrations. The post-rest beats are indicated by arrows. Voltage calibration signals as indicated before (Figure 8).

that elevated extracellular calcium decreases (due to an effect on the energy barrier for calcium export) the diastolic efflux of calcium from the cell promoted by BAY k 8644.

The depressant effect of BAY k 8644 on post-rest tension is not easily reversed by washing the drug out (complete recovery was never achieved). Therefore, reversibility was examined by pharmacologically competing for the calcium channels with nifedipine. This agent has been shown to compete with BAY k 8644 in receptor studies (Janis et al, 1984) and in biological assays (Schramm et al, 1983; Freedman and Miller, 1984). As seen in figure 14, equimolar concentrations of nifedipine reversed the effects of BAY k 8644 on post-rest tension development. This competition lead to steady state tension levels below the untreated muscle. However, if nifedipine was titrated in the presence of 1uM BAY k 8644 to produce pre-drug tension levels, the depressant effect of BAY k 8644 was not reversed. Thus, the actions of BAY k 8644 might involve more than simple occupation of its receptor on calcium channels.

RAPID COOLING CONTRACTURES

To examine the possibility that rest potentiation was abolished by BAY k 8644 due to enhanced diastolic efflux of SR calcium, rapid cooling contractures (RCC) were studied. This technique has previously been used to assess the relative size of the SR calcium pool (Kurihara and Sakai, 1985; Bridge, 1986). In response to a rapid cooling step from 37°C to between 0-2°C, a large slowly developing contracture was observed in the control muscle (Figure 15, top tracings). This was larger if the muscle was cooled immediately after steady state stimulation than after a 2 minute rest interval. This results shows that intracellular calcium is lost during periods of quiescence. Despite a decrease in the amount of intracellular calcium following rest, potentiation of these contractions are observed if twitches were invoked (due to augmented intracellular release). This apparent anomaly can be explained by proposing the SR contains more calcium than is released during an individual beat. Thus calcium release as opposed to calcium content would control tension development. This appears to be the case for both guinea pig (Kurihara and Sakai, 1985) and dog (this study) as steady state RCC shows greater tension development

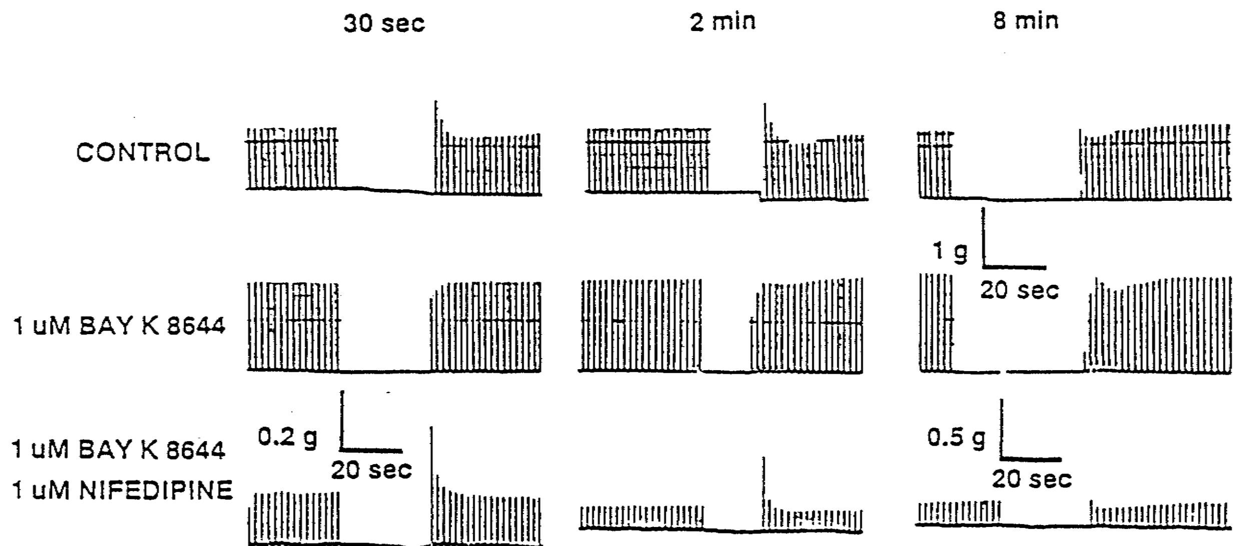


FIGURE 14. Reversal of the effects of 1 uM BAY k 8644 by 1 uM nifedipine for three different rest intervals (30, 120, 480 sec). Control (upper tracing), 1 uM BAY k 8644 (middle tracing), and 1 uM BAY k 8644 + 1 uM nifedipine (lower tracing) are shown for a typical muscle. BCL = 2000 msec

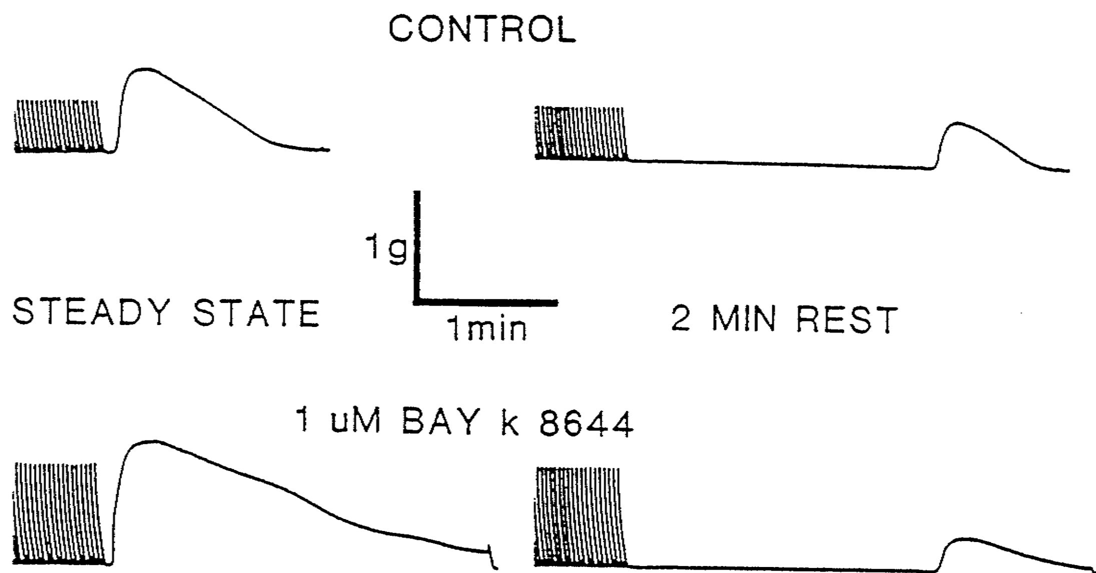


FIGURE 15. Effect of BAY k 8644 on rapid cooling contractures (37°C to 0-2°C) immediately after steady state stimulation and after a two minute rest interval. BCL = 2000 msec.

than evoked twitches. With BAY k 8644 present, the size of the RCC generated after a steady train of stimuli is markedly increased. However, following a period of rest, the RCC is reduced more than control despite a much larger initial pool size. Figure 16 shows pooled results of RCC tension development for control and BAY k 8644 treated muscles. Post-rest RCC were compared with RCC measured from preceding trains of steady state stimulation. This permits analysis of decay behavior. While both muscle groups show a progressive diastolic decay in RCC magnitude, this effect is considerably accelerated in the presence of BAY k 8644. Therefore, the simplest explanation for the depressant effects of BAY k 8644 on post-rest tension development is that diastolic calcium efflux is promoted by this agent.

COMPARISON WITH CAFFEINE

Since our results demonstrated an inhibitory effect of BAY k 8644 on the SR, we compared the effects of this agent with a compound classically used to impair SR function. Caffeine impairs the function of the SR by sensitizing the calcium release process (Blayney et al,1978) and inhibiting the re-uptake of calcium into the SR (Weber, 1968). Since it has a much smaller negative inotropic effect than ryanodine, it was used in the majority of studies. Typical effects of caffeine on post-extrasystolic potentiation are shown in figure 17 for two different extrasystolic coupling intervals. At both coupling intervals, caffeine increased the extrasystolic tension to above steady state levels. However, unlike BAY k 8644, the largest increases were observed at early coupling intervals and extrasystolic tension declined as this interval was increased. With BAY k 8644 treatment, extrasystolic force development increased at the longer coupling intervals. In the presence of caffeine, post-extrasystolic tension was usually augmented by a small amount. Depressions to the extent observed with BAY k 8644 were never seen. Similar results were observed in two additional muscles. The mechanism of action of caffeine on post-extrasystolic potentiation appears to be different from that due to BAY k 8644.

An examination of frequency potentiation in the presence of caffeine is shown in figure 18. Caffeine prevents the negative staircase observed in the control muscle during the initial change in frequency. A monotonic increase in tension is observed similar to that in species with sparse SR

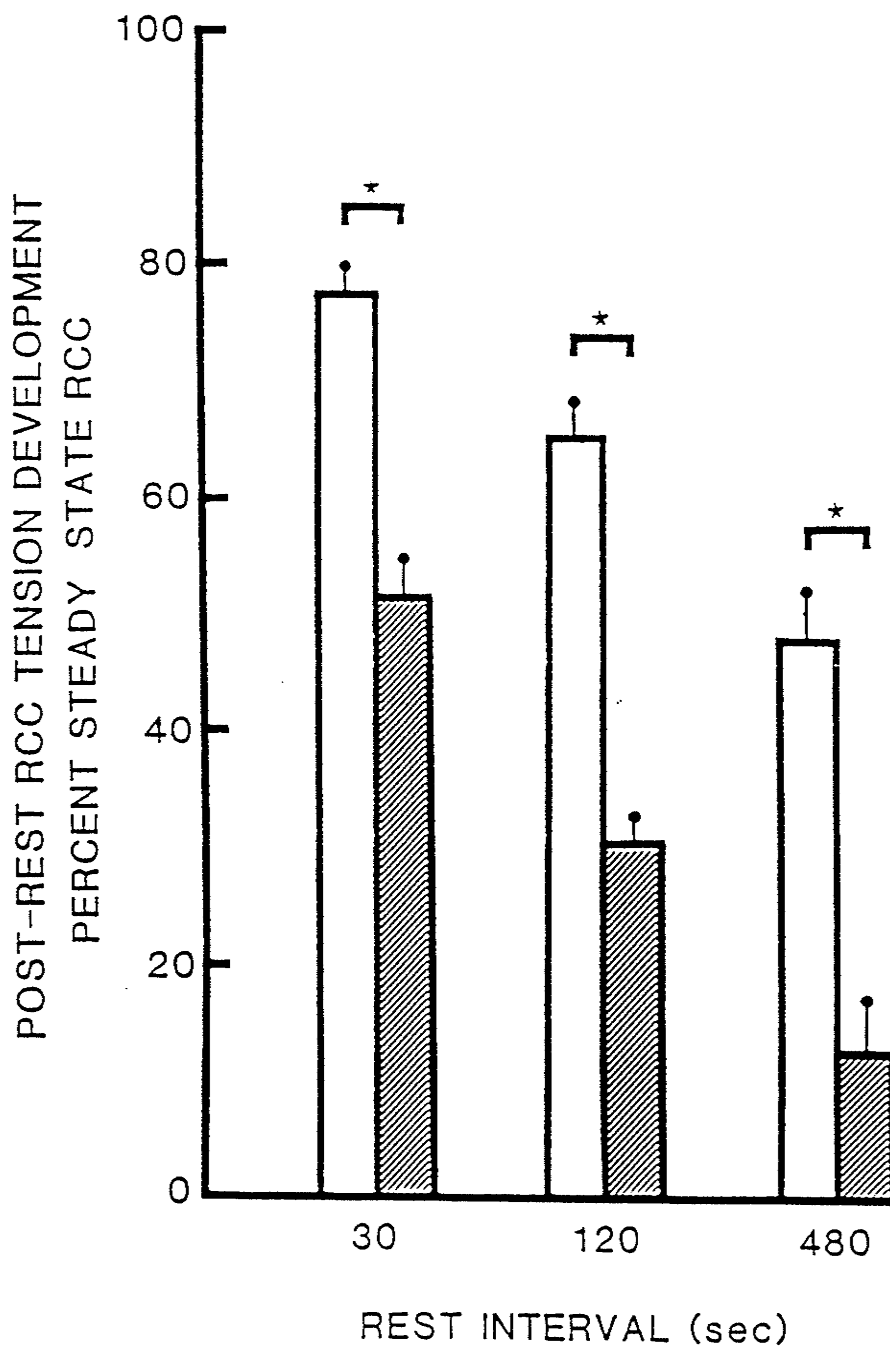


FIGURE 16. Pooled results of rapid cooling contractures from control (□) and BAY k 8644 treated (▨) muscles after three different rest durations (30, 120, 480 sec.). Data are expressed as a percentage of the post steady state RCC preceding the rest interval. n=14

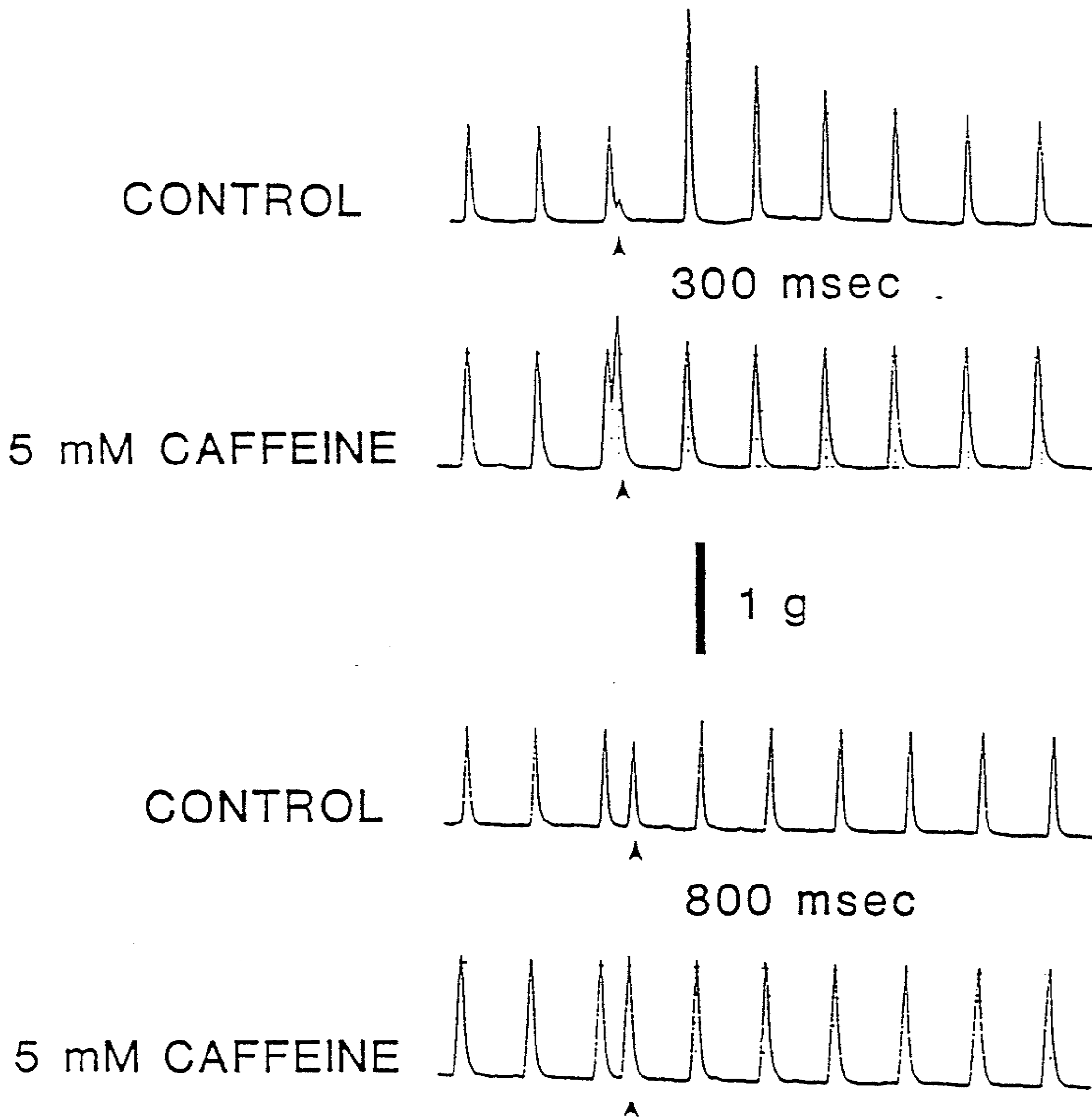
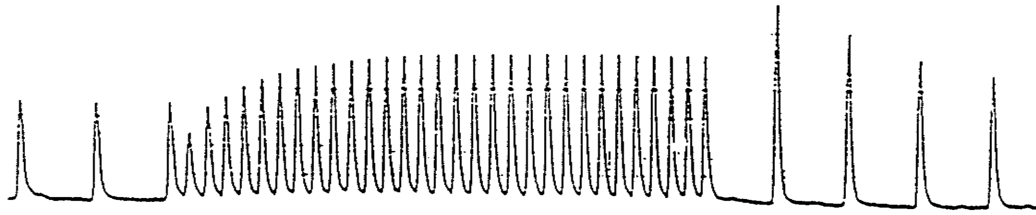


FIGURE 17. Effect of caffeine on post-extrasystolic force development for a 300 msec (top) and 800 msec (bottom) extrasystole. Control and caffeine treated muscles are shown. BCL = 2000 msec.



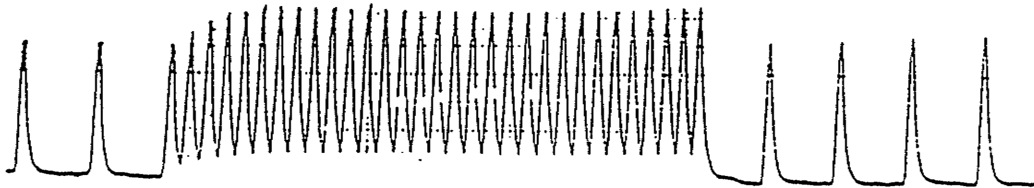


FIGURE 18. Effect of caffeine on high frequency stimulation. Control (top) and caffeine treated (bottom) muscle are shown. A 30 beat train (BCL = 500 msec) was inserted between two regular trains of stimulation (BCL = 2000 msec).

development such as the frog ventricle (Moreira et al, 1976). In the presence of BAY k 8644, tension would rise initially but a decline was observed towards the end of the high frequency train. This was not seen in caffeine treated muscles. After resumption of stimulation at the original frequency, no potentiation is observed. The tension response is relatively flat on restoration of the initial driving rate. The major differences between caffeine and BAY k 8644 with regard to frequency potentiation are the lack of a negative phase of contractility during high frequency stimulation in the presence of caffeine and the incomplete relaxation of tension during the high frequency train which is also only observed with caffeine.

Post-rest tension development is inhibited by both caffeine and ryanodine in several mammalian species (Bers, 1985). Figure 19 illustrates the rest response of canine ventricular muscle before and after the addition of caffeine. The control muscle exhibits typical rest potentiation whereas after the addition of caffeine, rest potentiation is no longer observed. The degree of post-rest depression is increased with increasing rest interval but is much less than that observed with BAY k 8644. Tension recovery is monophasic but not as rapid as that observed with BAY k 8644. Also, tension could always be detected for post-rest contractions after caffeine treatment whereas post-rest tension was occasionally abolished in the presence of BAY k 8644. Thus caffeine appears to have much less effect on post-rest tension development than BAY k 8644. This is evident when figures 6 and 20 are compared. Figure 20 illustrates pooled results from 5 muscles for three different rest durations. A depression is seen which progressively increases with increasing rest interval but this is to a lesser degree than that produced by BAY k 8644.

An examination of action potential alterations in the presence of caffeine provided further evidence for differences between caffeine and BAY k 8644. Figure 21 illustrates typical control responses for 2 and 8 min rest intervals. In the presence of 3 mM caffeine, the action potential duration is reduced in steady state beats and only a modest increase is observed following rest. Time to peak tension was also increased in steady state beats and further increases were observed with increasing rest interval. This is not seen in control muscles (unless a "rested state" contraction is produced) or after treatment with BAY k 8644.

Figure 22 illustrates the effect of caffeine on rapid cooling contractures. A typical response is

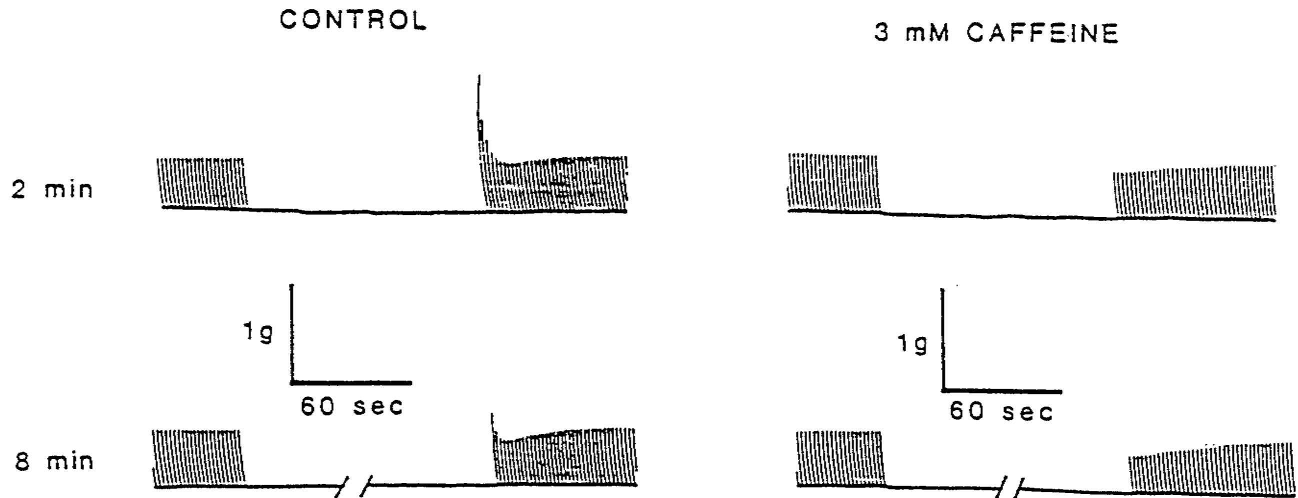


FIGURE 19. Typical tracings of control (left) and caffeine treated (right) muscle subjected to two (top) and eight (bottom) minute rest. BCL = 2000 msec.

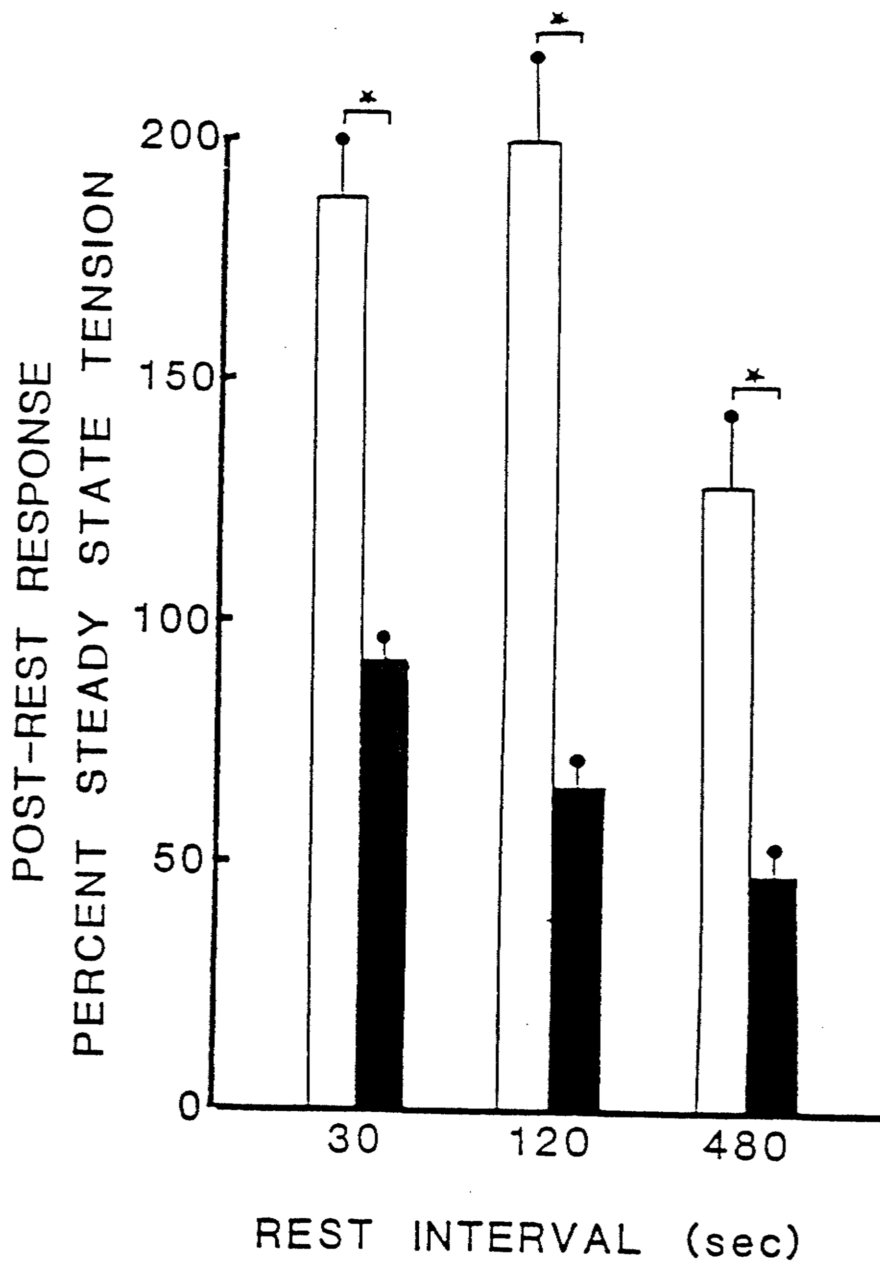


FIGURE 20. Pooled results from control (□) and caffeine treated (■) muscles. Response to three different rest intervals (30, 120, 480 sec). Tension is expressed as a percentage of steady state tension preceding the rest interval. n=8

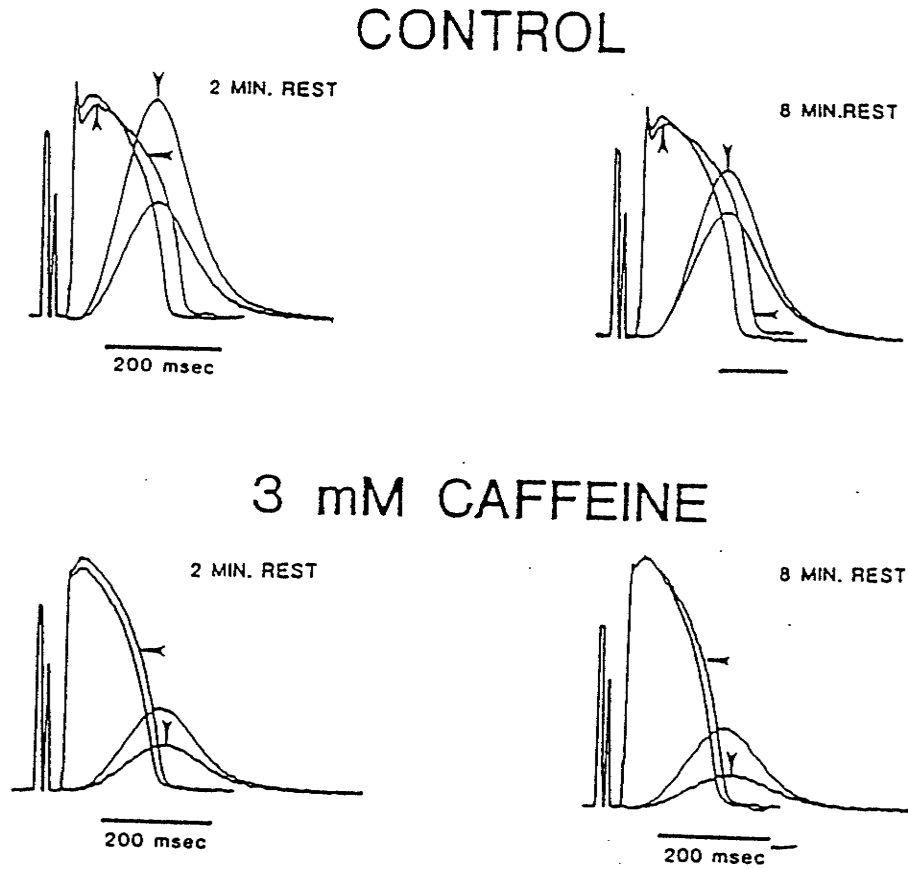


FIGURE 21. Superimposed action potentials and contractions of control (top) and caffeine treated (bottom) muscle. Responses to two (left) and eight (right) minute rest intervals. Post-rest beats are indicated by arrows. BCL = 2000 msec.

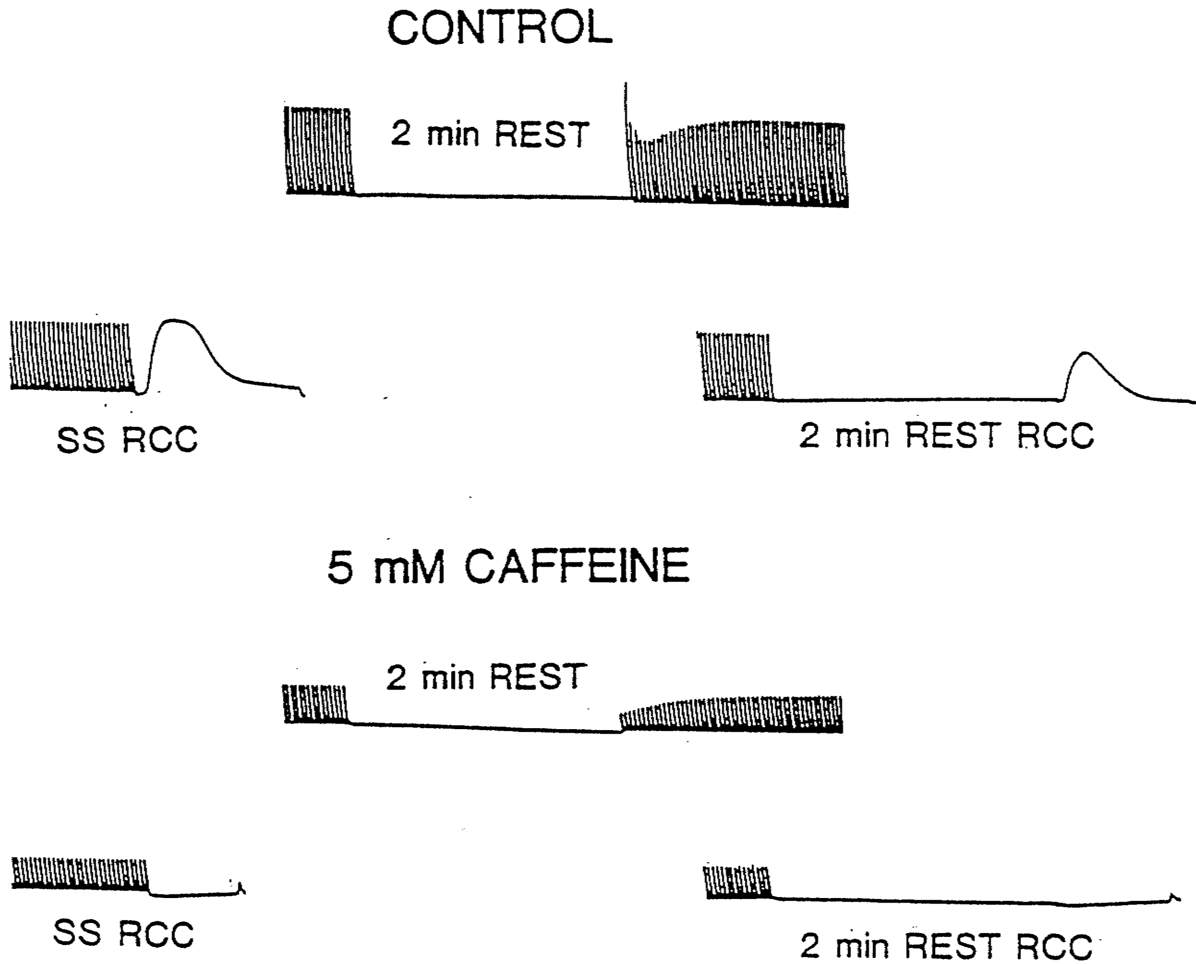


FIGURE 22. Effect of caffeine on post-rest tension and rapid cooling contractures after steady state stimulation and two minute rest. Control (top) and caffeine treated (bottom) muscle are shown. BCL = 2000 msec.

shown (upper tracings) prior to caffeine treatment. Note that the control muscle exhibits rest potentiation despite a decrease in intracellular calcium levels following rest. After caffeine treatment (lower tracings), RCC is abolished after both steady state stimulation and following rest. A very slow increase in tension was observed over the cooling period but this was unlike the relatively rapid responses obtained in control and BAY k 8644 treated muscles. This effect has been reported in rabbit ventricular muscle by Bridge (1986). The observation that the intracellular calcium pool is completely depleted by caffeine (as assessed by RCC) and yet post-rest tension development is impaired less than that in the presence of BAY k 8644 is very surprising. An essential corollary is that BAY k 8644 must inhibit rest potentiation by other means than simply augmented diastolic calcium efflux. If this explanation is not invoked, then the enhancement of myofibrillar calcium sensitivity by caffeine must be large enough to account for steady state and post-rest tension due to transsarcolemmal calcium influx in the absence of a contribution from the SR.

When the effects of ryanodine on rapid cooling contractures were examined (figure 23), a similar acceleration of calcium loss as produced by BAY k 8644 was observed. Steady state RCC was similar or only slightly reduced by this agent. This low concentration of ryanodine had only modest effects on steady state tension development but the post-rest beat was markedly impaired. At shorter rest intervals, RCC was also impaired but to a lesser degree than seen in figure 23. Thus ryanodine differs from caffeine which abolished RCC at all intervals. It may differ from BAY k 8644 only in the fact that the initial pool size is not altered by ryanodine or a small decrease is produced. BAY k 8644 markedly enhances the intracellular pool size and thus the starting points for diastolic efflux may differ.

We examined for interaction in the inhibitory effects of caffeine and BAY k 8644 by examining the effects of combined administration. Figure 24 illustrates typical tension tracings from a muscle initially treated with BAY k 8644 and subsequently with caffeine. Pronounced rest depression was observed in the presence of BAY k 8644 alone but when caffeine was added, rest depression was partially reversed. Pooled data from 4 experiments are shown in figure 25. At all intervals, addition of caffeine decreased the degree of rest depression produced by BAY k 8644. Caffeine also abolished RCC in the presence of BAY k 8644 (not shown). Thus, this effect may well reside in the ability of caffeine to augment myofibrillar calcium sensitivity. Another possibility is that in the absence of a contribution from the SR (as produced

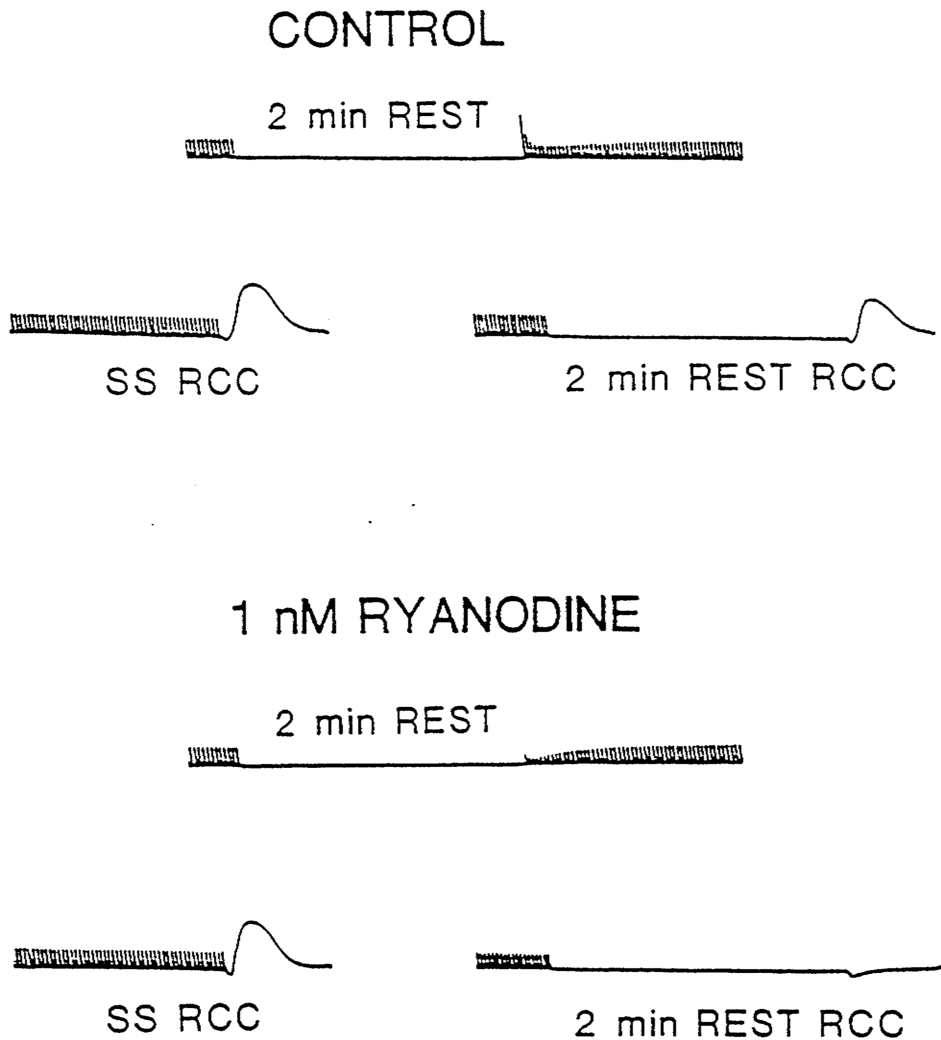


FIGURE 23. Effect of ryanodine on post-rest tension and rapid cooling contractures after steady state stimulation and following rest for two minute. Control (top) and ryanodine treated (bottom) muscle is shown. BCL = 2000 msec.

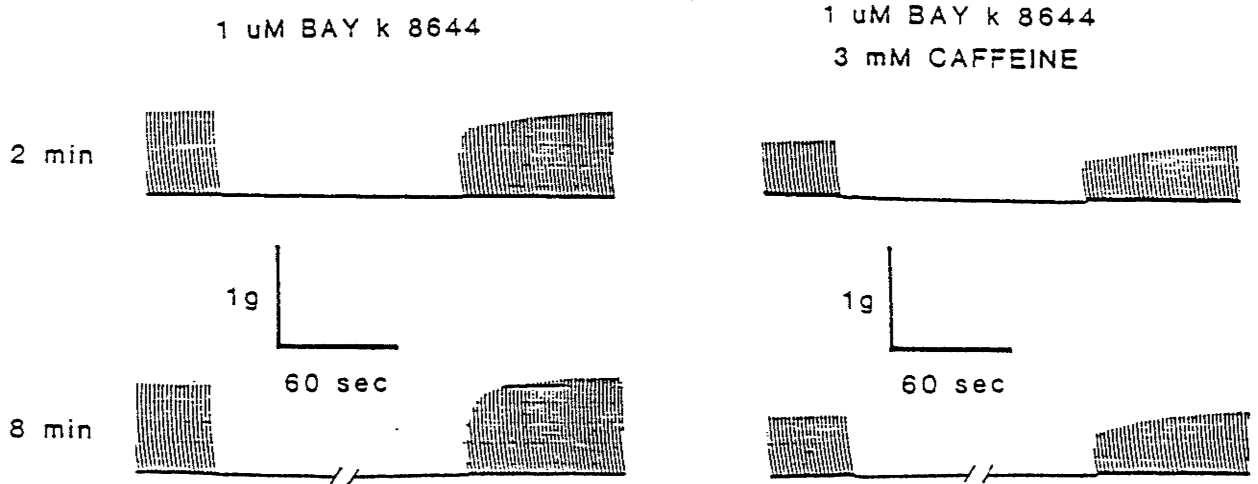


FIGURE 24. Effect of caffeine on BAY k 8644 induced rest depression after two and eight minute rest. A BAY k 8644 treated muscle is shown before (left) and after (right) caffeine treatment. BCL = 2000 msec.

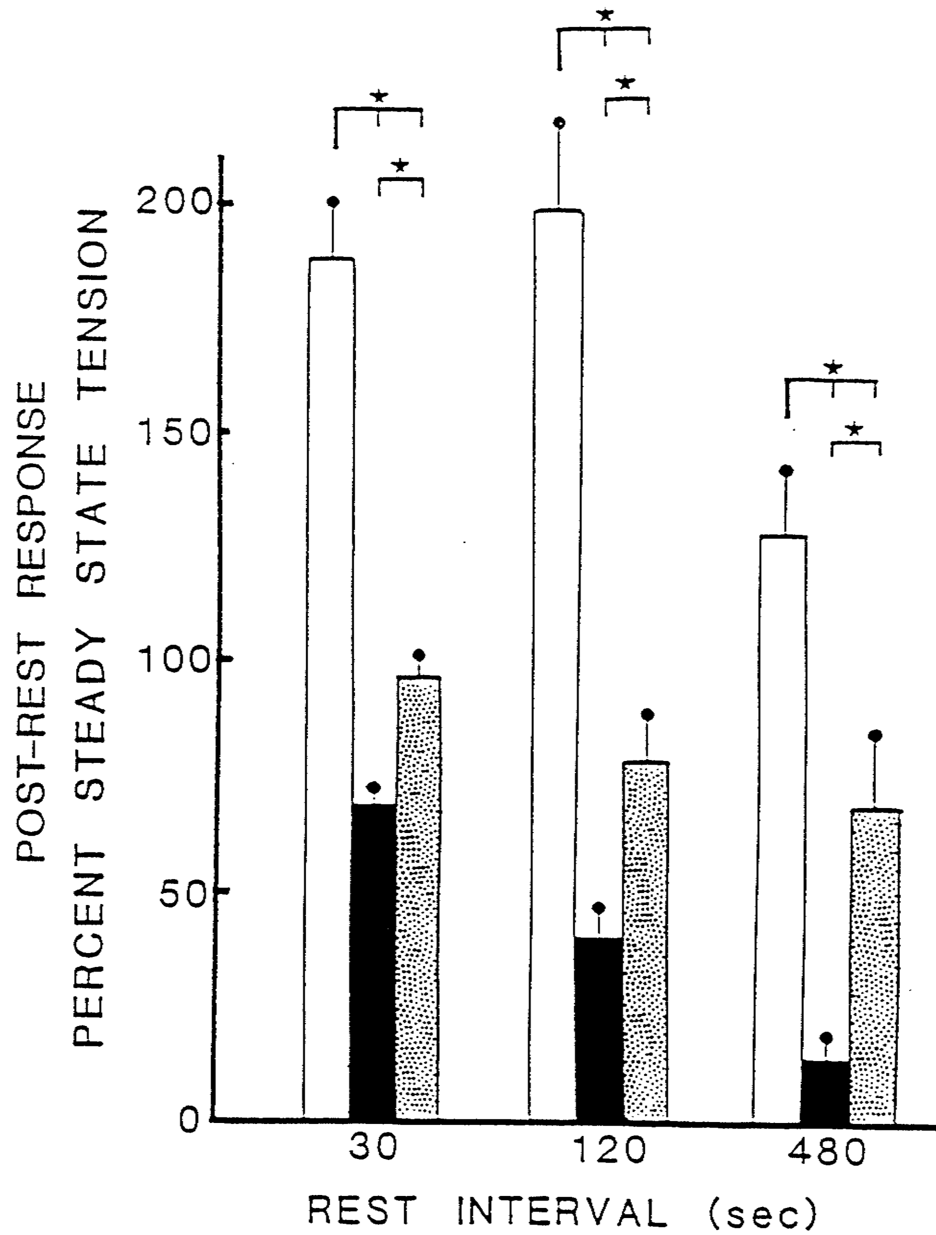
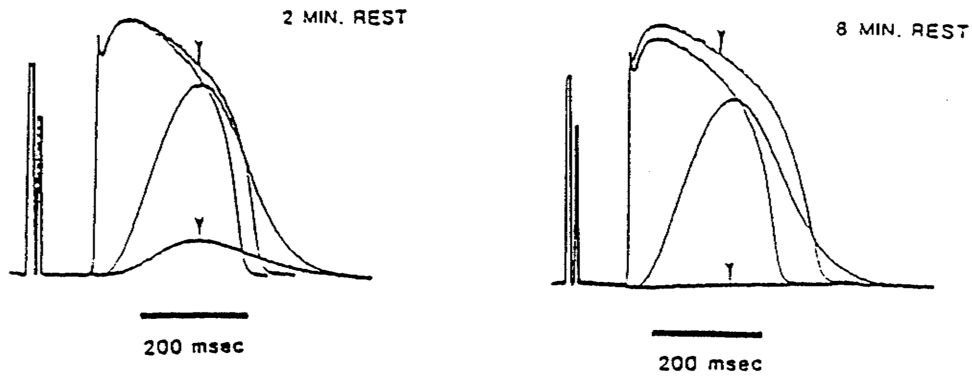


FIGURE 25. Pooled results of tension development following rest for eight minute expressed as a percentage of steady state tension preceding the rest interval. Control (□), 1 uM BAY k 8644 (■), and 1 uM BAY k 8644 plus 3 mM caffeine (▦). n=6

by caffeine alone), BAY k 8644 increases tension by augmenting extracellular calcium influx. Since the pool is already depleted by caffeine, BAY k 8644 has nothing to act upon. Support for this idea is provided by electrophysiological studies. Figure 26 shows the restorative effects of caffeine on BAY k 8644 induced rest depression. With both agents present, the action potential duration and plateau height are either unchanged or slightly increased. Thus caffeine does not affect the electrical alterations produced by BAY k 8644. However, the BAY k 8644 treated muscle does not show alteration of time to peak tension for either steady state or rested beats. With caffeine present, time to peak tension is greatly prolonged and further increases are observed following rest. Thus, BAY k 8644 appears to augment this contraction mediated entirely by extracellular calcium entry, similar to the results reported for "rested state" contractions in guinea-pig papillary muscles (Beyer et al, 1986).

Pooled results for action potential and mechanical parameters are shown in figures 27-29. In summary, control muscles do not show significant alterations in time to peak tension or tension rise time during post-rest beats. This suggests that the same calcium pools are involved in both steady state and post-rest beats. A small increase in action potential duration is seen with increasing rest interval. With BAY k 8644 present, time to peak tension and tension rise time values are also unchanged for both steady state and post-rest beats. A slight prolongation was observed at the 8 minute rest interval but this was due to the inclusion of data from muscles which exhibited a second phase of contraction. The appearance of this second phase in a few muscles supports the idea that the prolonged action potential duration reflects calcium entry but this is still inferential. BAY k 8644 treated muscles also show a progressive increase in action potential duration with increasing rest interval but to a greater degree than in control muscles. Conversely, caffeine consistently decreased action potential duration at all intervals although a similar trend of increase was observed as the rest interval was increased. The most notable effect of caffeine was an increase in time to peak tension and tension rise time which became greater after longer periods of quiescence. With both BAY k 8644 and caffeine present, the characteristic alteration in the rest response produced by each was maintained or increased. Despite this, less rest depression was produced. That is, time to peak tension and tension rise time was increased beyond that observed with caffeine alone. Action potential duration was similar or slightly greater than that observed with BAY k

1 uM BAY k 8644



3 mM CAFFEINE +
1 uM BAY k 8644

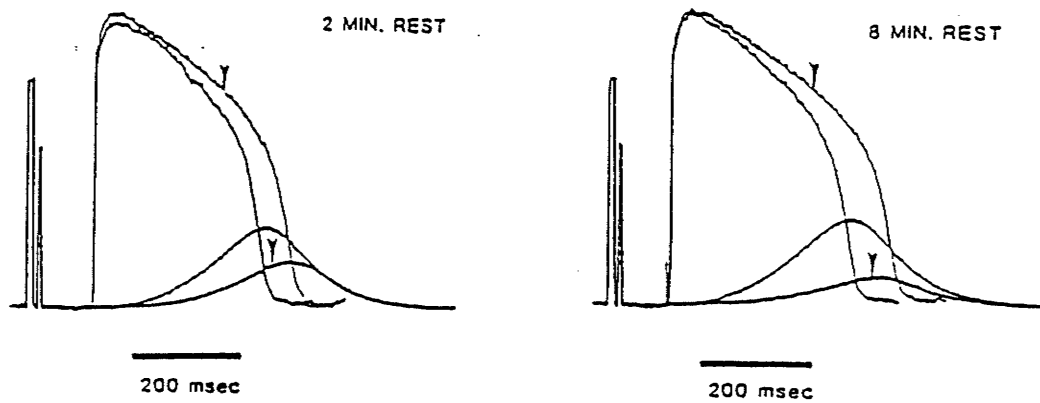


FIGURE 26. Superimposed action potentials and contractions in a BAY k 8644 treated muscle before (top) and after (bottom) the addition of 3 mM caffeine following two (left) and eight min. (right) rest. Post-rest beats are indicated by arrows.

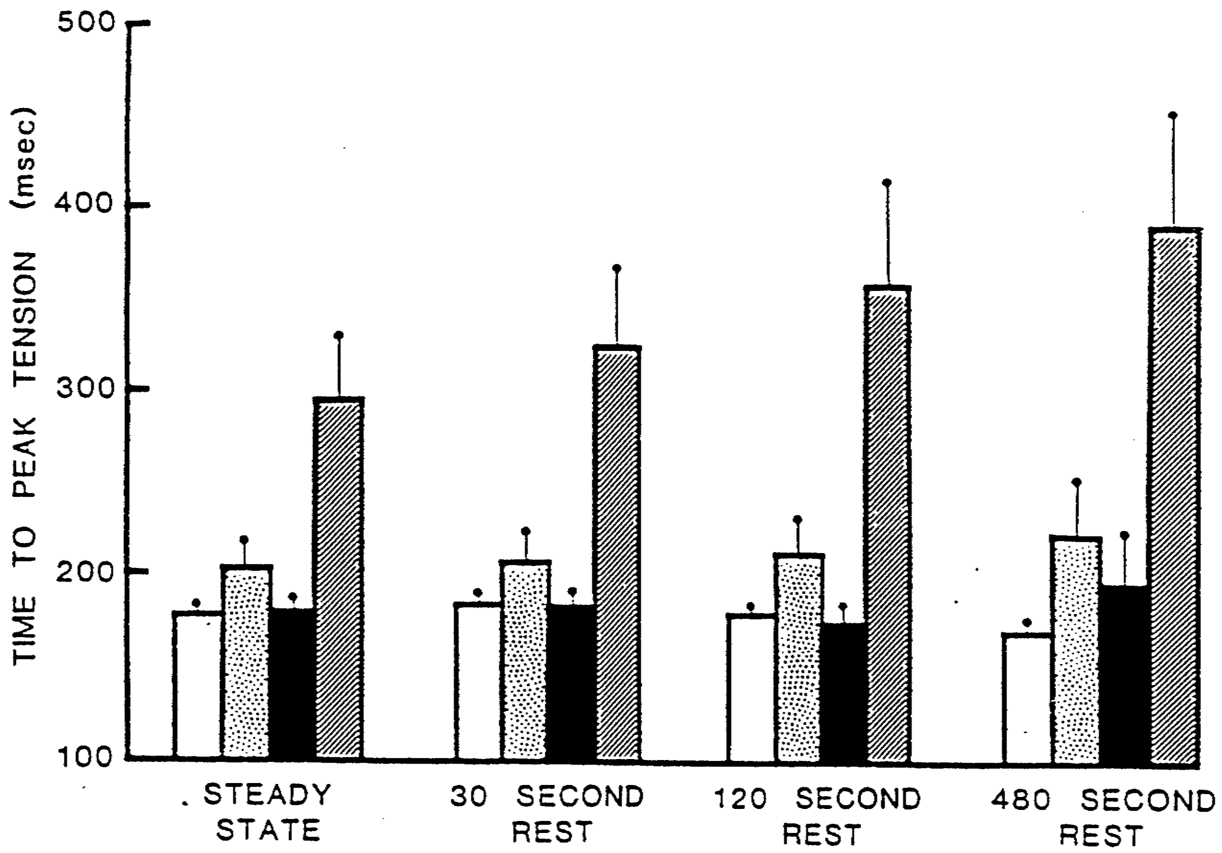


FIGURE 27. Time to peak tension of steady state and post rest beats in control (□), 3 mM caffeine treated (▦), 1 uM BAY k 8644 treated (■), and 1 uM BAY k 8644 plus 3mM caffeine treated muscles (▨). n=5

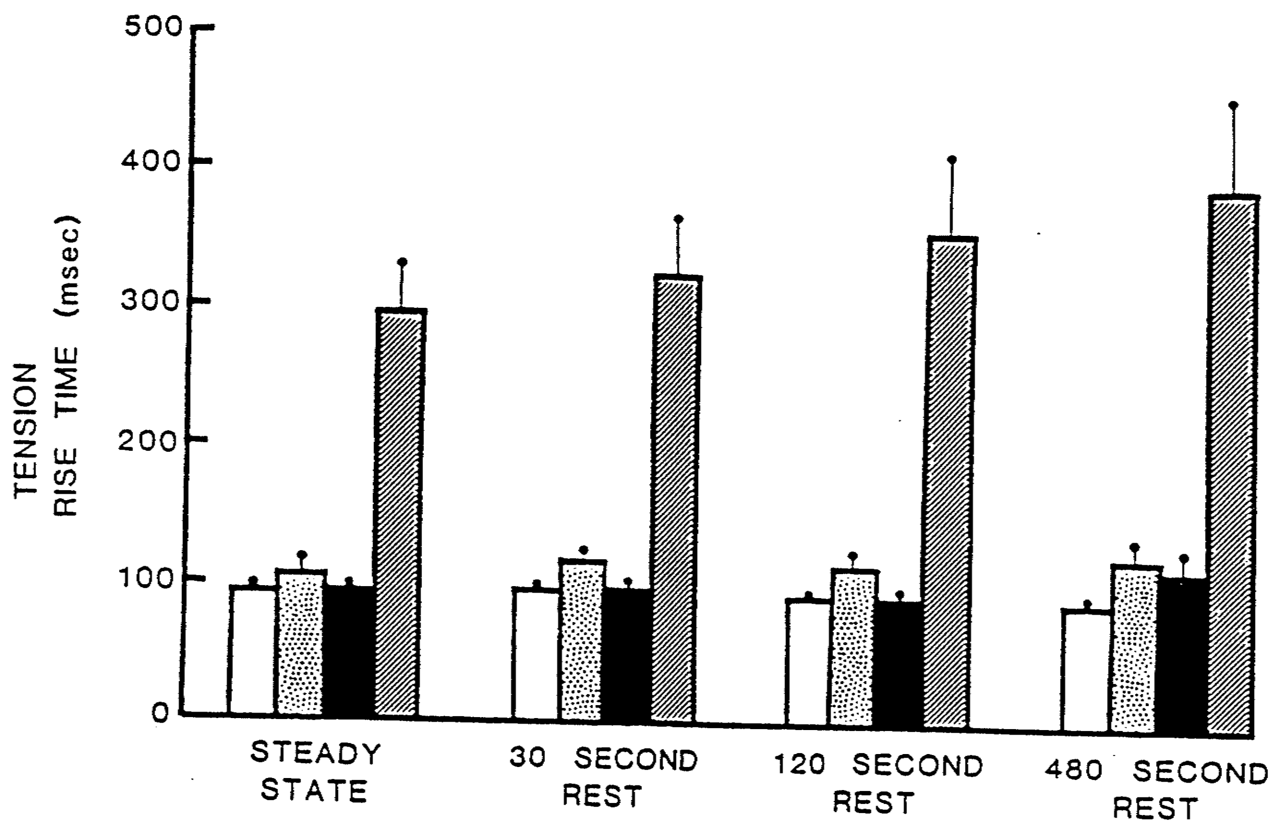


FIGURE 28. Tension rise time in steady state and post rest beats in control (□), 3 mM caffeine treated (▒), 1 uM BAY k 8644 treated (■), and 1 uM BAY k 8644 plus 3mM caffeine treated muscles (▨). n=5

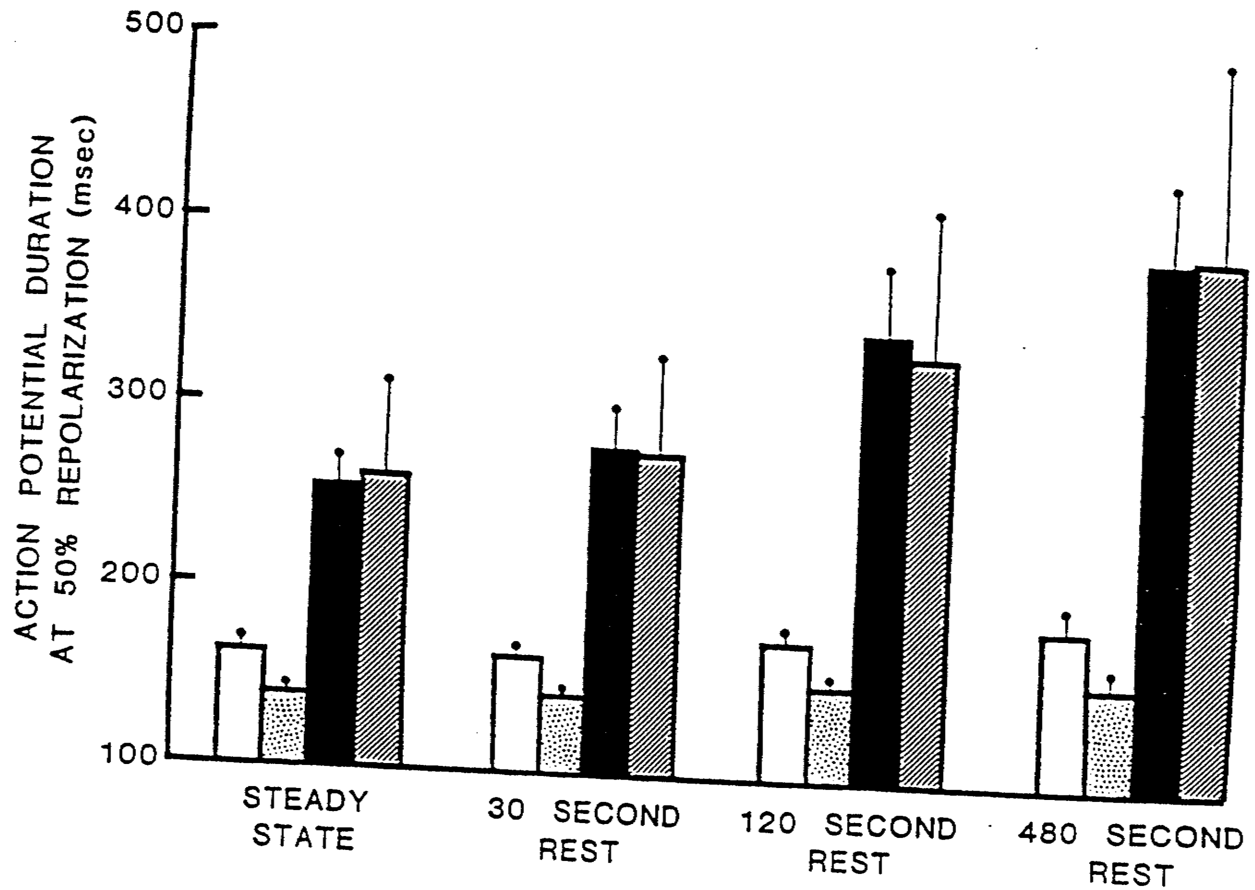


FIGURE 29. Action potential duration at 50% repolarization in steady state and post rest beats in control (□), 3 mM caffeine treated (▒), 1 uM BAY k 8644 treated (■), and 1 uM BAY k 8644 plus 3mM caffeine treated muscles (▨). n=5

8644 alone. Despite synergy or augmentation of a characteristic feature of each type of rest depression, post-rest tension was less impaired. It thus seems likely that the negative effects of BAY k 8644 on post-rest tension development are prevented by the fact that contractions are solely dependent on extracellular calcium entry in the presence of caffeine.

ROLE OF EXTRACELLULAR SODIUM

Electrogenic sodium-calcium exchange is involved in both calcium entry (Sheu et al, 1986) and removal (Barry et al, 1986) in myocardial cells. It has been shown to influence diastolic calcium movements both in terms of tension development (Sutko et al, 1985) and intracellular calcium pool size (Bridge, 1986; Kurihara and Sakai, 1985). Therefore, the role of sodium-calcium exchange in the rest depression produced by BAY k 8644 was examined by studying the effects of reduced extracellular sodium and ouabagenin on this phenomena. Figure 30 shows typical tension tracings for a two minute rest interval at two different sodium concentrations in the absence and presence of BAY k 8644. In control muscles, post-rest tension development was impaired when extracellular sodium was reduced. This result is in contrast to reports by Sutko et al (1985) in rabbit ventricular muscle but this tissue normally does not exhibit rest potentiation. This disparity will be addressed in the DISCUSSION. In the presence of BAY k 8644, reduced extracellular sodium produced an opposite effect. That is, post-rest tension development was increased with reduced extracellular sodium. This occurred despite definite signs of toxicity (aftercontractions, reduced tension, and slightly increased baseline).

Pooled results for the two sodium concentrations in the presence and absence of BAY k 8644 are shown in figure 31. This graph illustrates the opposite effects of impairing sodium-calcium exchange (operating in the calcium extrusion mode) on control and BAY k 8644 treated muscles. As BAY k 8644 appears to decrease post-rest tension by enhancing the diastolic efflux of calcium (Figure 15), the results obtained with reduced extracellular sodium suggest that sodium-calcium exchange may be important for this efflux. However, the opposite effect observed in control muscles is less easily explained. Possible explanations are given after the effects of reduced extracellular sodium on RCC are dealt with.

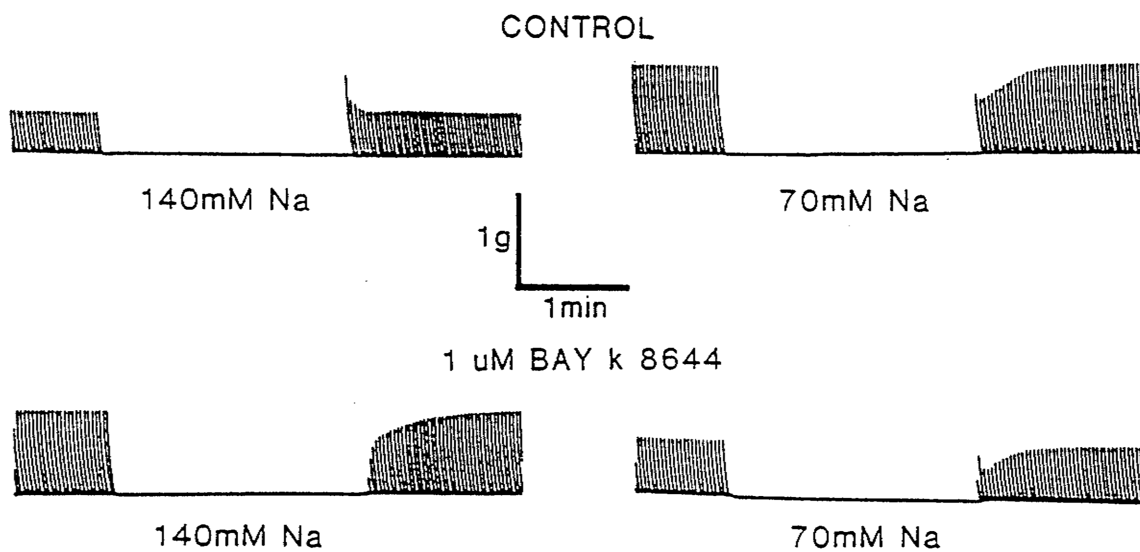


FIGURE 30. Effect of different extracellular sodium concentrations (140 mM and 70 mM) on post-rest tension development following rest for two minutes. Control (top) and BAY k 8644 treated (bottom) muscles are shown. BCL = 2000 msec.

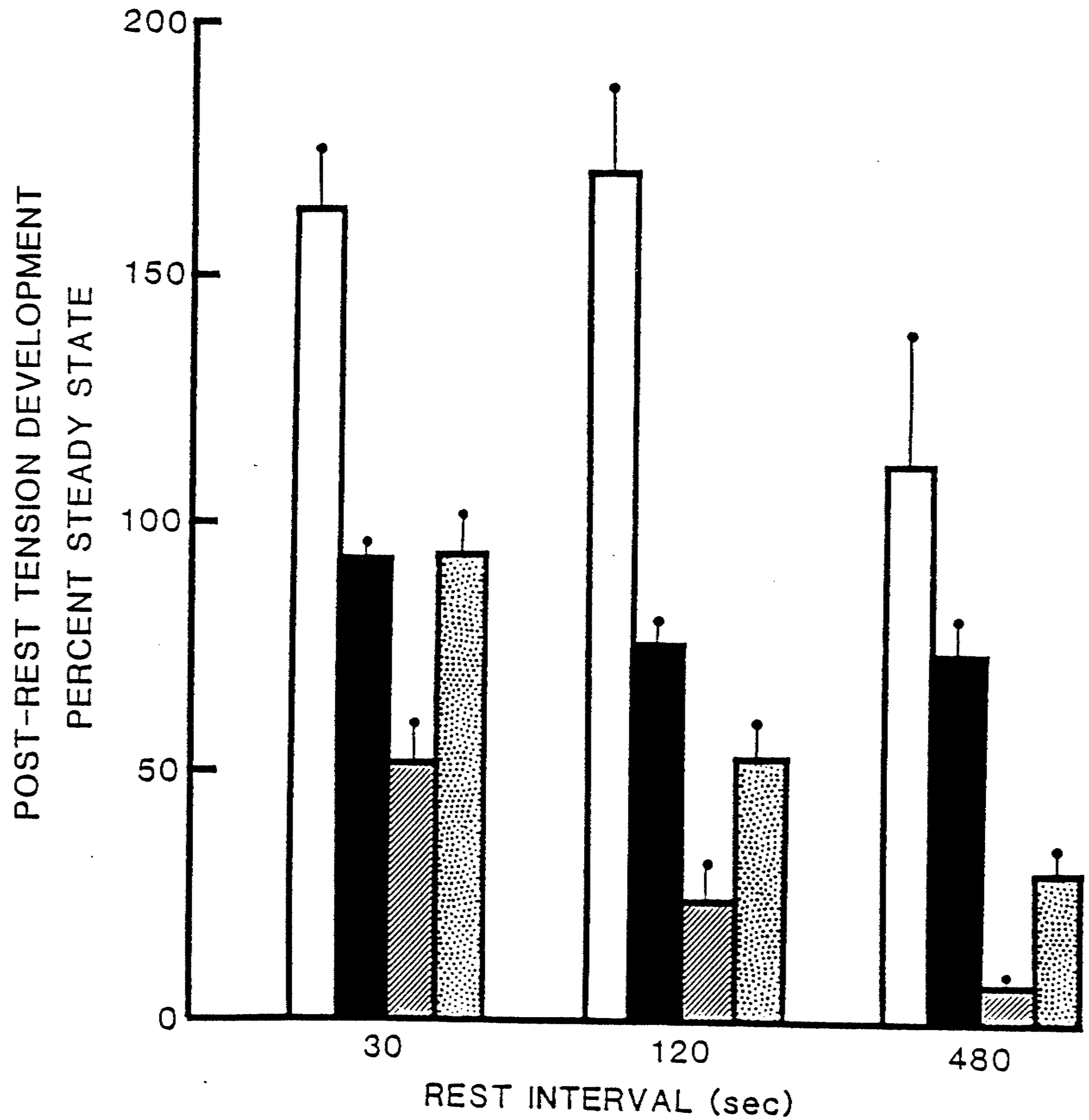


FIGURE 31. Post-rest tension development at three different rest intervals (30, 120, 480 sec) for control and BAY k 8644 treated muscles. Effect of different extracellular sodium concentrations (140 mM and 70 mM). Control (□), 70 mM Na (■), 1 uM BAY k 8644 (▨), 1 uM BAY k 8644 with 70 mM Na (▩). n=5

Figure 32 shows a typical example of the effects of lowered extracellular sodium on post steady state and 2 min. post-rest RCC before and after BAY k 8644 addition. Pooled results from 4 experiments are shown in figure 33 for three different rest intervals. These figures show that reducing the extracellular sodium concentration does not markedly alter diastolic calcium efflux in control muscles. The extent of SR calcium loss during diastole is increased somewhat at short coupling intervals whereas a decrease is observed for longer rests. However, only small changes are observed. The enhancement of diastolic oscillations (Lappe and Lakatta, 1980) produced by this intervention is likely countered by the decreased efficiency of efflux through sodium-calcium exchange (Barry et al, 1986). If sodium concentration is reduced even further (35 mM), diastolic efflux is severely impaired or a net uptake is observed (not shown, see results with ouabagenin). Similar behavior has been reported by Bridge (1986) who observed an enhancement of post-rest RCC at very low sodium levels (2.0 mM). It was not possible to examine BAY k 8644 treated muscles with further reduction in extracellular sodium (i.e. below 70mM) as this caused rapid muscle deterioration. BAY k 8644 treated muscles showed considerable toxicity even at 70 mM extracellular sodium and tension was consistently reduced. However, this intervention significantly decreased the diastolic efflux rate supporting the results of experiments where evoked tension was measured. Thus the diastolic efflux promoted by BAY k 8644 appears to utilize sodium-calcium exchange for calcium export.

Figure 34 shows electrophysiological studies examining the effects of ouabagenin on BAY k 8644 induced rest depression. Impairing sodium-calcium exchange by this mechanism led to augmentation of post-rest force development and a reversal of the action potential prolongation. It should be noted that steady state tension was reduced by this intervention and numerous signs of toxicity were observed. The tissue was depolarized and aftercontractions were usually present. However, the reversal of the action potential prolongation suggests that sodium-calcium exchange may serve a major role in the electrical effects produced by BAY k 8644. A highly speculative possibility is that electrogenic sodium-calcium exchange occurs during the plateau phase and enables calcium to be continuously extruded. This might limit tension during post-rest beats where the action potential plateau height and duration are markedly increased. These possibilities are for future exploration. However, the importance of sodium-calcium

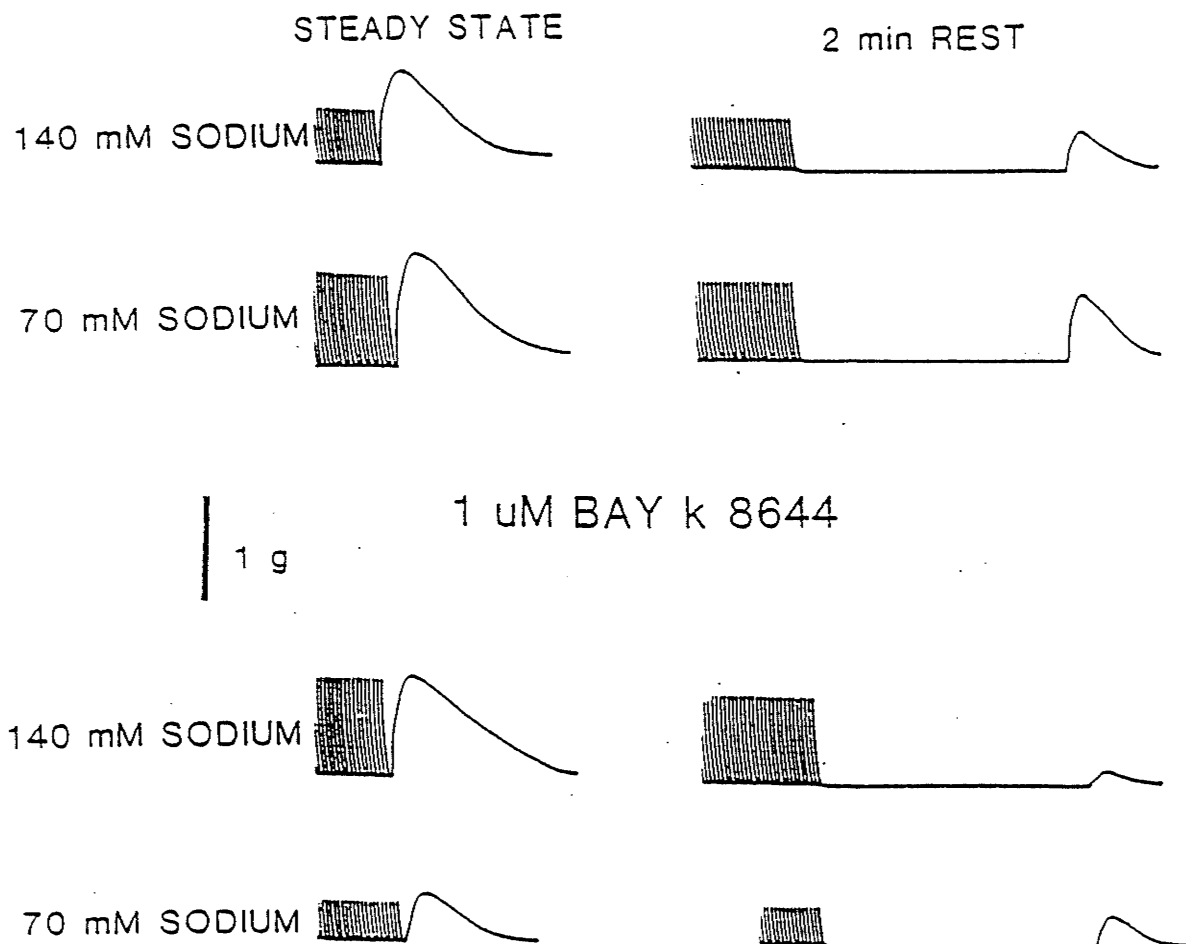


FIGURE 32. Effect of different extracellular sodium concentrations (140 mM and 70 mM) on post steady state and post-rest (two min) RCC in control (top) and BAY k 8644 treated (bottom) muscles. BCL = 2000 msec.

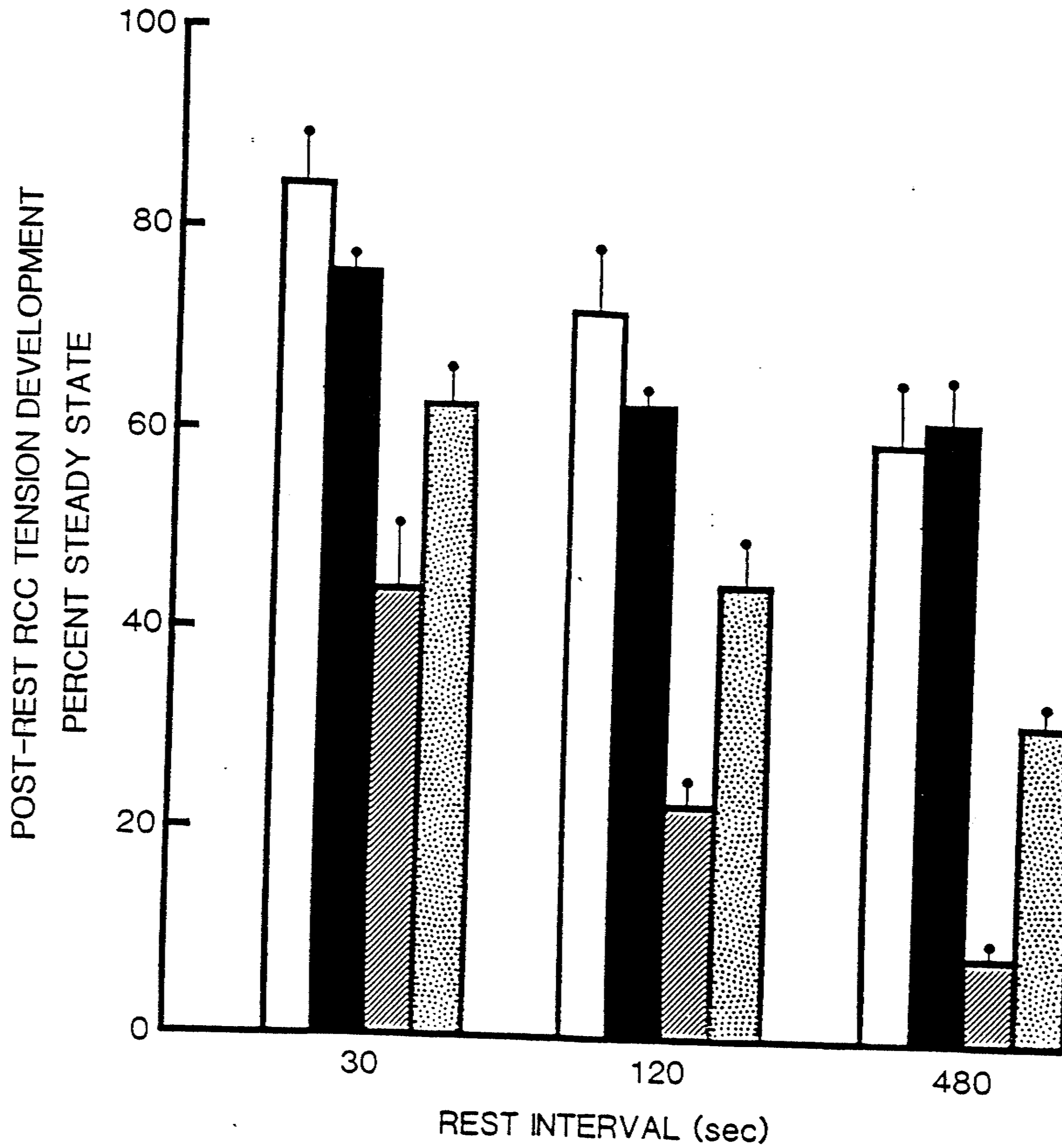


FIGURE 33. Pooled results for post-rest rapid cooling contractures after rest for three different intervals (30, 120, 480 sec) with different extracellular sodium concentrations (140 mM and 70 mM). Control 140 mM (□), 70 mM Na (■), 1 uM BAY k 8644 in 140 mM Na (▨), 1 uM BAY k 8644 with 70 mM Na.(▩). n=5

1 uM BAY k 8644

1 uM BAY k 8644

3 uM OUABAGENIN

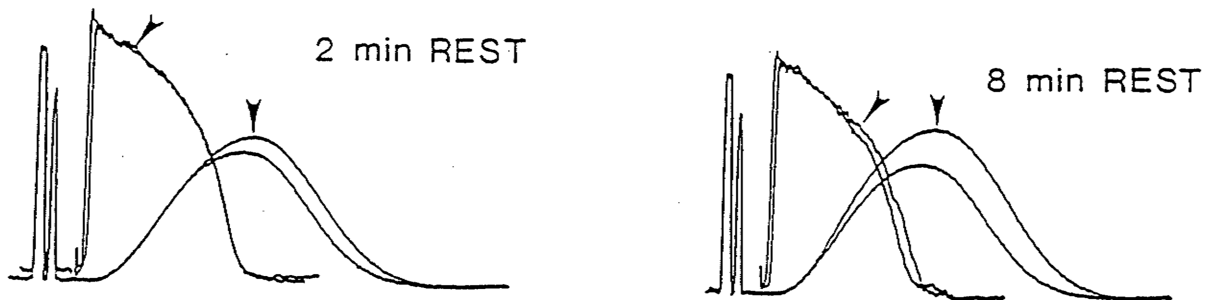


FIGURE 34. Superimposed action potentials and contractions for BAY k 8644 treated muscle before (top) and after (bottom) the addition of 3 uM ouabagenin. Post-rest beats are indicated by arrows.

exchange for the diastolic efflux of intracellular calcium is easily demonstrated. In control muscles, ouabagenin at toxic concentrations abolished or even reversed diastolic calcium efflux as shown in figure 35. Thus the role of sodium-calcium exchange is critical in diastolic efflux and outwardly directed Ca-ATPases appear to be insufficient for this process. This same conclusion was reported by Barry et al (1986).

EFFECTS OF EXTRACELLULAR CALCIUM

The effects of elevated extracellular calcium on the diastolic efflux of calcium were studied using rapid cooling contractures. These experiments were conducted to compliment the previous results which suggested that BAY k 8644 did not cause rest depression by producing calcium overload. Figure 36 illustrates the effects of elevated extracellular calcium on a typical muscle before and after treatment with BAY k 8644. Pooled results from three experiments are shown in figure 37. Elevated extracellular calcium significantly enhanced the rate of RCC decay in control muscles. This is in striking contrast to the effects of lowered extracellular sodium, where only small changes were observed (Figures 32 and 33). In the presence of BAY k 8644, elevated extracellular calcium reduced post-rest depression but this effect was also less than that produced by lowered extracellular sodium. Possible mechanisms for this behavior are now discussed.

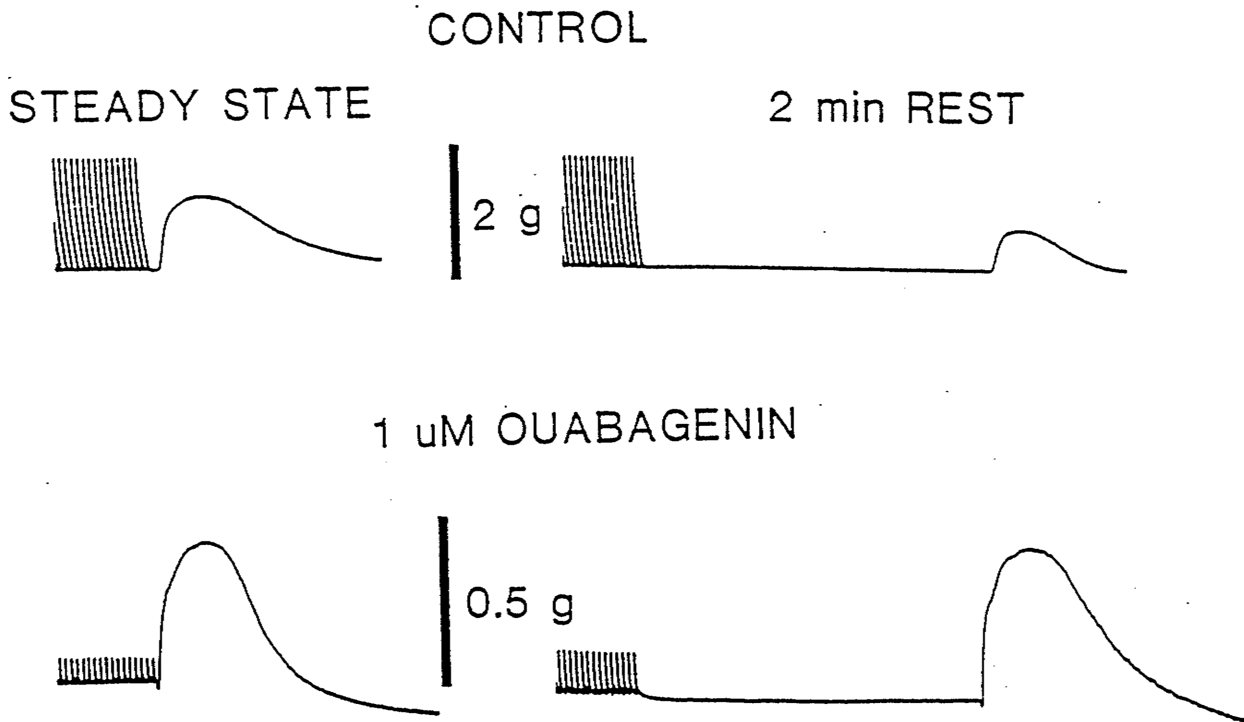


FIGURE 35. Effect of ouabagenin on post steady state and post-rest rapid cooling contractures. Control (top) and 3 uM ouabagenin treated (bottom) muscle are shown. BCL = 2000 msec.

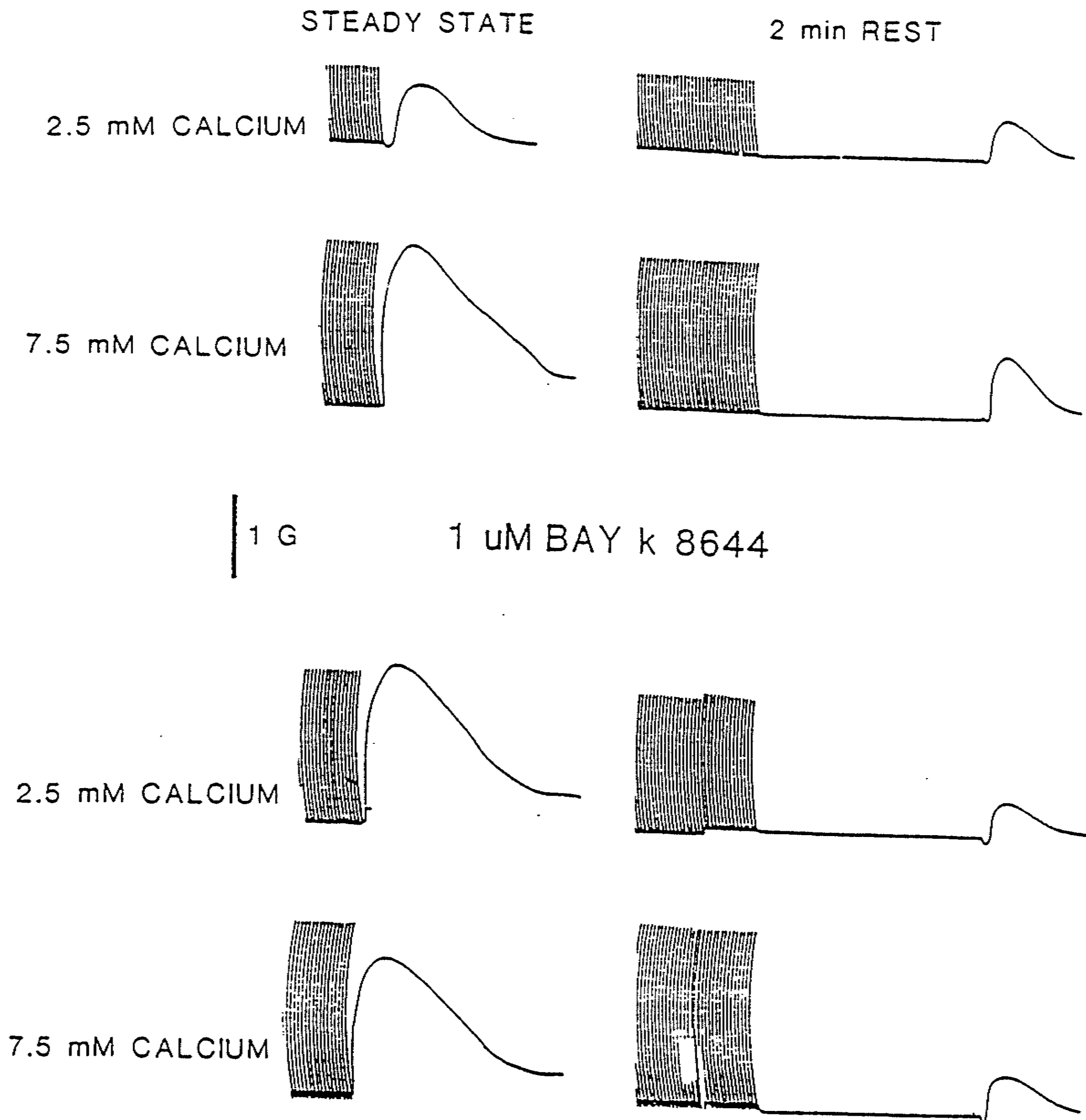


FIGURE 36. Effect of elevated extracellular calcium concentration (7.5 mM) on post steady state and post rest rapid cooling contractures in control (top) and BAY k 8644 treated (bottom) muscle. BCL = 2000 msec.

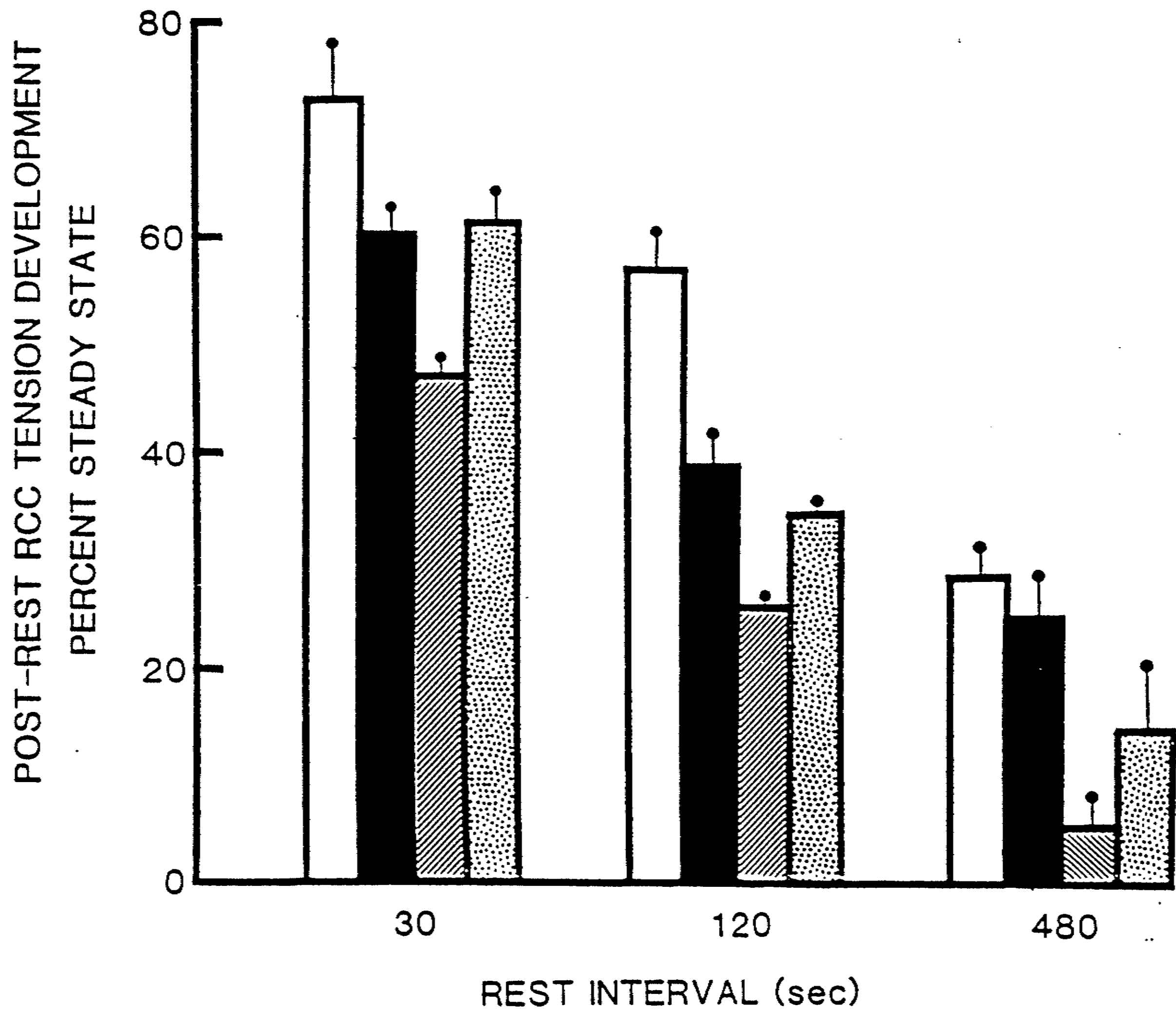


FIGURE 37. Effect of elevated calcium on post-rest RCC in control and BAY k 8644 treated muscle for three different rest intervals. Pooled results for control (□), 7.5 mM Ca (■), 1 u M BAY k 8644 (▨) and 1 uM BAY k 8644 with 7.5 mM Ca. (▩). n=3

DISCUSSION AND CONCLUSIONS

The primary focus of this study was to investigate the effects of the calcium channel agonist, BAY k 8644, on rest potentiation in canine ventricular muscle. To achieve this, the rest response was characterized by a number of approaches. These included tension measurements, electrophysiological studies, and rapid cooling contracture measurements. As well, a number of ionic and pharmacological interventions were employed to assist in the interpretation of results obtained with BAY k 8644. The effect of BAY k 8644 was also examined on other interval-alterations to gain insight into possible mechanisms of action. We have tried to interpret these results in terms of a model of excitation-contraction coupling for which the basis was discussed in the INTRODUCTION.

THE REST RESPONSE

In response to periods of quiescence, canine ventricular muscle contraction was potentiated following the resumption of stimulation. The degree of potentiation was dependent on the duration of rest. Maximum rest potentiation was typically observed after 1-2 minutes of rest and a rapid ascending phase preceded this. In contrast, potentiation declined gradually for longer rest intervals. Tension recovery almost always exhibited an undershoot prior to recovery to steady state levels. Within the confines of the model previously described, a postulated mechanism for rest behavior is now given. Several EC coupling models (Schouten et al, 1987; Adler et al, 1985; Wohlfart, 1982), including the one presented in the introduction, suggest that rest potentiation results from enhanced intracellular calcium release from the sarcoplasmic reticulum. These models postulate that calcium is taken up by the sarcoplasmic reticulum and must recycle or translocate to release sites. The necessity for translocation of SR calcium is evidenced by the fact that mechanical restitution takes considerably longer than electrical restitution. The relationship between the prematurity of extrasystoles and extrasystolic tension development is suggested to be an indication of the calcium recycling process within the SR. Rest potentiation results from the greater time allowed for this restitution process. The increased diastolic

interval permits a larger amount of calcium sequestered by the SR to be translocated to release sites. On resumption of stimulation, a larger than normal amount of calcium is released and potentiation results.

The interval dependency of rest potentiation is postulated to be a result of the translocation process of calcium in the SR. A rapidly ascending phase is observed which reflects a progressive increase in the size of the SR release pool. The slowly declining phase of rest potentiation is thought to result from two factors. At the point where rest potentiation is largest, the maximal amount of calcium is thought to be translocated to the release site. The hypothetical (but experimentally observed) "leak" or calcium removal process which results in diastolic loss of SR calcium (Bridge, 1986) continually depletes this pool. As calcium is no longer available for translocation, the pool size is continually diminished and the slow decline in rest potentiation is observed.

From action potential recordings, it appears that extracellular calcium entry is decreased during the post-rest beats. This is inferred from the decrease in action potential plateau height. This inference is also supported by previous literature which has examined net calcium fluxes during rested beats. Using different approaches, both Hilgemann (1986) and Bers (1985) have reached the same conclusion. Our results from biphasic contraction studies also support this notion. The second peak (P2) which is attributed to extracellular cation entry was depressed during rest potentiation. By deduction alone, this would necessitate an increased intracellular calcium release to support the augmented contraction. However, other results provide direct evidence to support this hypothesis. We have found that rest potentiation during biphasic contractions is mediated by the initial peak (P1). This peak is due to release of intracellular cation as documented by King and Bose (1983). Pharmacological studies by ourselves and others also suggest that the sarcoplasmic reticulum is responsible for the augmented calcium release. We have shown caffeine to inhibit rest potentiation as have Bers (1985) and Lewartowski et al (1978) in different tissues. Caffeine inhibits the sarcoplasmic reticulum by sensitizing the calcium release process (Blayney et al, 1978) and preventing the reuptake of calcium into the SR (Weber, 1968). Ryanodine also impaired post-rest tension development in canine ventricle. Studies in other tissues have shown similar effects (Bers, 1985). The exact mechanism of action of this agent is unknown but recent studies have shown that low concentrations of ryanodine enhance calcium release and loss from the SR. This has been

demonstrated in whole tissue by Hilgemann (1986) and in the isolated SR using the patch clamp technique (Meissner, 1986). At higher concentrations, ryanodine is thought to impair calcium release from the SR (Meissner, 1986). Regardless of mechanism, ryanodine impairs the contribution of the SR to contraction. Thus, the conclusion that rest potentiation results from enhanced SR calcium release is well supported.

The results from rapid cooling contracture studies suggest that total intracellular calcium levels are continuously decreased during diastole in the canine ventricle. Bridge (1986) has shown this in isolated rabbit ventricular muscle. RCC has also been shown to require previous stimulation or calcium loading in guinea-pig ventricle (Kurihara and Sakai, 1985). These results illustrate the diastolic calcium efflux or "leak" from the SR but the mechanism remains elusive.

Unlike rabbit ventricle which exhibits a negative inotropic response to rest (Bers, 1985), the canine ventricle exhibits marked potentiation over a certain range of rest intervals. However, both these tissues exhibit a continuous decline in intracellular calcium levels as assessed by RCC measurements (Bridge, 1986; my results). Therefore, tension development does not correlate with estimated relative intracellular calcium levels. This necessitates postulating that rapid cooling contractures are indicative of total SR calcium levels whereas post-rest tension reflects stimulus releasable calcium from the SR. These two parameters need not, and in fact, appear not to be the same. Thus, less intracellular calcium may exist in the cell during diastole but it is utilized more effectively (ie. greater amounts are released). The mechanism by which this is achieved is unknown.

The postulated mechanism for post-rest behavior is supported by other lines of evidence. For example, many cardiac tissues will exhibit a depression of tension development after very long periods of rest (Edman and Johannsson, 1976; Allen et al, 1976). This has been termed the "rested state" contraction (Koch-Weser and Blinks, 1963) and is characterized by a delayed time to peak tension and sensitivity to agents impairing extracellular calcium entry (Lewartowski et al, 1978). This occurs in tissues regardless of whether a post-rest potentiation or depression is observed. Thus, the diastolic calcium efflux process seems common to many tissue types (guinea-pig atria is a notable exception). Other recent experimental evidence also supports this model. Wendt-Gallitelli et al (1985) has shown using X-ray

probe microanalysis that the terminal SR cisternae are depleted of calcium under conditions which would result in a "rested state" contraction. Under conditions (eg. lowered extracellular sodium) where a contraction with an early time to peak tension would be observed, accumulated calcium can be measured in this region. Unpublished observations by A. Jorgenson have shown that Ca-ATPases are primarily located in the network SR whereas calsequestrin is found only in the terminal cisternae. Therefore, the longitudinal SR may represent the uptake sites while release occurs at terminal cisternae. Thus anatomical correlates may exist for the proposed model.

When the effect of the calcium channel agonist, BAY k 8644, was examined on post-rest tension development, a pronounced impairment was observed. The degree of rest depression was found to increase with increasing rest interval. In some muscles, tension was barely detectable at the longer rest intervals. The absolute magnitude of post-rest tension was smaller than both steady state and post-rest beats of the muscle before BAY k 8644 addition. Thus, this effect cannot be attributed to mechanical limitations restricting further tension development as it represents a true depression of tension (and not merely a decrease in potentiation due to a tension ceiling). Likewise, the rest potentiation curve was not shifted to the left (ie. to shorter rest intervals) as rest potentiation was not observed even for the shortest rest periods.

Rest potentiation is known to be relatively insensitive to agents which impair the slow inward current such as cobalt (Bers, 1985). Thus, the mechanism of action of BAY k 8644 is unlikely to be due to block of calcium channels. Though the racemic mixture of BAY k 8644 is reported to have calcium antagonist properties, these are observed at high frequencies of stimulation (Kamp et al,1985; Seifen and Kennedy, 1985; Thomas et al, 1985) or under depolarized conditions (Sanguinetti and Kass, 1986). Neither of these conditions would be expected to participate during rest intervals. Even if this occurred, tension development should not be substantially impaired. Rest depression produced by BAY k 8644 could also be reversed by equimolar concentrations of the calcium channel blocker, nifedipine. Results from biphasic contraction studies suggested that extracellular calcium entry was enhanced by this agent. This result was also supported by action potential measurements. The post rest beats exhibited large increases in plateau height and duration which is suggestive (but not proof) of enhanced extracellular

calcium entry. As post-rest tension development is sensitive to interventions which impair SR function, we concluded that the effects of BAY k 8644 must be mediated through effects on this organelle. The remainder of the study was directed at uncovering the mechanism by which this was achieved.

Using the biphasic contraction model, BAY k 8644 appeared to prevent rest potentiation by preventing the augmentation of intracellular calcium release. That is, P1 was no longer potentiated following a period of rest. Thus, BAY k 8644 inhibits rest potentiation by inhibiting SR calcium release as we originally were led to postulate. However this study did not provide much information with regard to mechanism. One possibility which we considered was that BAY k 8644 may have produced calcium overload in the muscle.

ROLE OF EXTRACELLULAR CALCIUM

As reported by Allen et al (1986), excessive intracellular calcium impairs tension by causing nonhomogenous calcium release from the SR. This reduces the amount of releasable calcium available during evoked twitches. The regions or cells where calcium release was reduced (due to previous asynchronous release) would then act as series compliances for the regions with full tension production. The net result would be a decrease in developed tension. Impaired contractile function might also be due to metabolic impairment secondary to calcium overload (Vassale and Lin, 1979). To examine this, extracellular calcium levels were altered and the rest response was re-examined. In control muscles, an increase in extracellular calcium was found to depress post-rest tension and increased potentiation was observed in lower extracellular calcium. As raising extracellular calcium increases diastolic asynchronous calcium mediated oscillations (Lappe and Lakatta, 1980), the mechanism proposed by Allen et al (1985) may account for this behavior. Increased diastolic calcium oscillations from the SR would increase the levels of myoplasmic calcium. In turn, this would increase the opportunity for sarcolemmal calcium transporters to extrude calcium. The net result would be an enhanced diastolic efflux of SR calcium as well as decreased tension due to nonhomogeneity of SR calcium release. However, diametrically opposite effects were observed for this intervention in BAY k 8644 treated muscles. That is, less rest depression

was produced as extracellular calcium was elevated. This result negates the possibility that the rest depression produced by BAY k 8644 is due to calcium overload. If such were the case, then post-rest tension should have been progressively impaired as extracellular calcium was increased.

Studies examining the effect of BAY k 8644 on scattered light intensity fluctuations (SLIF) have demonstrated that this agent actually reduces these (Bose et al, 1987). This result further negates the possibility that calcium overload might be responsible for rest depression observed in the presence of BAY k 8644. The effects on SLIF also distinguish BAY k 8644 from other means of augmenting inotropy such as elevating extracellular calcium (Lappe and Lakatta, 1980) or employing cardiac glycosides (Bose et al, 1987). Both of these interventions increase force development but predispose heart muscle to cellular toxicity. Thus BAY k 8644 might provide insight into safer means of increasing cardiac inotropy.

The explanation for the effects of elevated extracellular calcium on the control rest response was substantiated by examining rapid cooling contractures. While augmented RCC after steady state stimulation are observed with elevated extracellular calcium, the rate of diastolic decay is accelerated. This demonstrates that increased calcium export from the cell occurs presumably due to the increased diastolic calcium oscillations expected from this intervention (Lappe and Lakatta, 1980). Again, this would cause a partitioning between SR reuptake and efflux pathways. However, the increased rate of diastolic decay of RCC due to BAY k 8644 was slowed by elevated extracellular calcium. This explains the positive effect of increasing extracellular calcium on post-rest tension development but the mechanism is not clear. Perhaps because the diastolic efflux of calcium is already accelerated by BAY k 8644, the additional effect of increased extracellular calcium on diastolic efflux is not substantial. The small reduction in calcium export by sodium-calcium exchange and calcium ATPases due to the increased energy requirements caused by increased extracellular calcium may offset the effect of spontaneous oscillations on diastolic calcium efflux. Admittedly, this explanation is based on limited experiments. Further studies will be required to increase our understanding of this behavior. However, increased extracellular calcium did not reduce the depressant effects of BAY k 8644 by converting electrical responses towards normal. The action potential configurations were similar at both calcium concentrations. It could be postulated that elevated extracellular calcium results in greater triggering of

releasable SR calcium but this seems unlikely as the action potential remains similar and a larger SR pool already exists.

ROLE OF SODIUM-CALCIUM EXCHANGE

The role of sodium-calcium exchange was examined by lowering extracellular sodium and by application of the Na,K-ATPase inhibitor, ouabagenin. In the presence of reduced extracellular sodium, untreated muscles showed a decrease in post-rest potentiation. Again, the explanation of Allen et al (1985) may be invoked. This intervention would enhance diastolic calcium oscillations (Lappe and Lakatta, 1980) and impair tension by increasing the series compliance between cells. Rapid cooling contracture measurements revealed a small increase in diastolic decay but RCC generated immediately after steady state stimulation was potentiated. Thus, more calcium is available in the intracellular pool when extracellular sodium is reduced but the release process is less effective. Again, the effects of BAY k 8644 were altered by these interventions. Post-rest tension was increased and the diastolic decay of RCC was reduced. While these two observations correlate, the mechanism remains elusive. The reduction in calcium extrusion by sodium-calcium exchange must be of greater magnitude than the effect of this intervention on diastolic calcium oscillations. While these ad hoc assumptions provide possible explanations, further studies will be required to elucidate the mechanisms of increased extracellular calcium and reduced extracellular sodium on increasing post-rest tension development in the presence of BAY k 8644. Ouabagenin was also found to reverse the negative inotropic effects of BAY k 8644 on post-rest tension development. This occurred with concomitant reduction in the action potential duration and plateau height. Unlike calcium elevation which did not markedly alter the action potential configuration, impairment of sodium-calcium exchange show reversal of both electrical and mechanical alterations produced by BAY k 8644. As reported by Sheu et al (1986) sodium-calcium exchange is involved in calcium entry during depolarization of cardiac cells. If this exchange process is electrogenic as postulated by Mullins (1979), then it may also contribute to current flow across the sarcolemma. The direction of sodium-calcium exchange will depend on the prevailing electrochemical gradients for both cations.

Therefore, it might operate in either direction and even switch directions depending upon these conditions. Several recent studies have suggested that sodium-calcium exchange mediates calcium efflux during the plateau phase of the cardiac action potential (Hume, 1987; Hume and Uehara, 1986a,b). This exchange contributes to the plateau phase of the action potential and can be suppressed by intracellular EGTA administration or ryanodine (Mitchell et al, 1984). The fact that ouabagenin can prevent the effects of BAY k 8644 on electrical parameters might be indicative of an effect of this agent on sodium-calcium exchange. If sodium-calcium exchange was enhanced by BAY k 8644 in the calcium extrusion direction (with net positive current entry due to 3 Na\1 Ca exchange) both electrical and mechanical effects can be explained. That is, BAY k 8644 increases calcium efflux during rest by augmenting sodium-calcium exchange in the calcium extrusion direction. With ouabagenin present, this process is impaired as the sodium gradient is decreased by this agent and may be insufficient to extrude calcium. Another less likely possibility is that ouabagenin causes sufficient depolarization to convert BAY k 8644 into a calcium channel antagonist. Voltage-clamp studies will be required to address this issue.

COMPARISON WITH CAFFEINE

A comparison of the effects of caffeine and BAY k 8644 on the interval-force relationship was made. The intention was to gain insight into the mechanism of action of BAY k 8644 by comparing it with an agent classically used to impair SR function. The inhibitory effects of caffeine on SR function are mediated by inhibition of calcium uptake by the SR (Weber, 1968) and sensitization of the release process (Blayney et al, 1978) which results in rapid depletion of the SR calcium pool. Caffeine depressed post-extrasystolic potentiation but the effect had characteristics different from that observed with BAY k 8644. Extrasystolic tension was greatest at the shortest coupling intervals whereas longer coupling intervals resulted in more tension in the BAY k 8644 treated muscles. Thus, caffeine addition resulted in behavior similar to amphibian heart where extrasystolic twitches can summate on the previous contraction. Caffeine impairs the reuptake of intracellular calcium and slows relaxation. Thus the possibility for summated twitches is enhanced.

The effects of caffeine on frequency potentiation were also similar to that observed in amphibian heart. That is, tension increases directly with increases in frequency. In contrast, BAY k 8644 treated muscle showed a similar positive staircase initially but then tension began to decrease. During high frequency stimulation, the caffeine treated muscle failed to relax completely between individual beats. This results from the inability of the SR to take up calcium and therefore relaxation is entirely dependent on sarcolemmal efflux processes. This was not observed in the BAY k 8644 treated muscles suggesting that inhibition of SR calcium uptake is not a property of this compound. Both agents abolished the potentiation of the initial beats after frequency was restored to initial levels but only BAY k 8644 consistently produced an aftercontraction prior to this. This also indicates that the SR remains functional (though altered) in BAY k 8644 treated muscles.

Caffeine produced rest depression at all intervals examined but to a lesser degree than that observed with BAY k 8644. The marked prolongation of action potential duration observed during post-rest beats in the presence of BAY k 8644 was also not observed. The unique characteristic of caffeine induced rest depression was the prolongation of time to peak tension. While caffeine prolongs time to peak tension of steady state beats, a further increase is observed following rest. This behavior is not seen in control or BAY k 8644 treated muscles. This observation is extremely important as it provides an insight into the mechanism of action of BAY k 8644 mediated rest depression. Since time to peak tension is not altered by BAY k 8644, the post-rest contractions must still utilize the SR calcium pool (albeit less effectively). If these contractions depended solely on extracellular calcium entry, a prolongation of time to peak tension would occur. This is well documented for contractions known to depend on extracellular calcium entry such as after caffeine treatment (this study; Bers, 1985) and for "rested state" contractions (Lewartowski et al, 1978). Caffeine was also found to abolish rapid cooling contractures. RCC were not observed after steady state stimulation or following a rest interval. This effect is similar to the report by Bridge (1986) in rabbit ventricular muscle. Thus, steady state and post-rest tension in the presence of caffeine are entirely due to the extracellular entry of calcium. On the other hand, BAY k 8644 treated muscles appear to use the same intracellular calcium pool during both types of beats.

When BAY k 8644 and caffeine were present simultaneously, less rest depression was observed

than with either agent alone. RCC in the presence of both retained the characteristics of caffeine alone (ie RCC was abolished). A larger increase in time to peak tension was also observed and the action potential remained prolonged. These three observations provide a reasonable explanation for this result. Since caffeine abolishes the SR contribution to the intracellular calcium transient, BAY k 8644 can no longer act on or affect this pool. Thus, only the positive inotropic effect is seen which is due to augmented calcium influx. Since this contraction is supported entirely from extracellular calcium entry, BAY k 8644 causes an increase in tension development by its actions on sarcolemmal calcium channels.

The actions of BAY k 8644 on post-rest tension and RCC are similar to those of ryanodine. Both agents increasingly impair post-rest tension as the rest duration is increased. This is also true for post-rest RCC. However, ryanodine does not increase steady state tension or the RCC generated immediately after steady state stimulation. It therefore shares only the negative inotropic effects with BAY k 8644. Conflicting reports exist regarding the effects of ryanodine on calcium currents. An increase has been reported by Mitchell et al (1984) in rat ventricular cells whereas no effect was observed by Nishio et al (1986) in guinea pig ventricular cells. Regardless, the effect on extracellular calcium entry is unlikely to be of the magnitude produced by BAY k 8644. Therefore the difference observed between these two agents may result from differences in the calcium pool size which is being depleted. Whether the mechanism or rapidity of pool depletion is similar for both agents remains to be determined.

SUMMARY

1. BAY k 8644 produces rest depression in canine ventricular muscle.

2. This effect is achieved by inhibiting the augmented intracellular calcium release which normally produces rest potentiation.

3. Calcium overload is not responsible for this effect.

4. BAY k 8644 accelerates the diastolic efflux of calcium from the sarcoplasmic reticulum.

5. Sodium calcium exchange is involved in the export of intracellular calcium during diastole.

6. The effects of BAY k 8644 on the interval-force relationship are not similar to those produced by caffeine.

7. Ryanodine has similar negative inotropic effects as BAY k 8644 on the size of the SR calcium pool but lacks the positive inotropic effects.

8. The exact mechanism of BAY k 8644 induced rest depression is unknown.

IMPLICATIONS

The interval-force relationship is not merely a phenomenon of laboratory interest. The *in situ* heart also shows frequency dependent mechanical alterations (Kavaler et al, 1971) although the response is generally less dramatic. This may be due, in large part, to the numerous compensatory mechanisms (eg. Starling mechanism, matching of metabolic needs to work performed) and neuroregulatory systems operative in the whole animal (Wohlfart, 1982). In isolated tissues, these limitations are removed and greater insight into mechanisms of EC coupling can be revealed. Regardless of physiological correlates to the interval-force relationship, it is most assuredly beneficial to increase our understanding of heart function. Serendipity seems a poor way to access new therapeutic approaches.

We have shown that the calcium channel agonist, BAY k 8644, has profound effects on the ability of the sarcoplasmic reticulum to augment its output for post-rest beats. It is interesting to note that previous studies have examined for similar effects. In a study by Thomas et al (1985) BAY k 8644 had no

effect on the rested state contraction in guinea pig atrial muscle. The myofibrillar calcium sensitivity and sarcoplasmic reticulum calcium release process was unaffected by BAY k 8644 in skinned guinea pig ventricular fibres (Thomas et al, 1985). BAY k 8644 did not promote calcium release from rabbit skeletal muscle sarcoplasmic reticulum (Zorzato et al, 1985). The first of these observations may be due to differences in EC coupling between atrial and ventricular muscle as suggested by the authors. The last two reports are from skinned fibres or isolated SR. Thus the effects of BAY k 8644 may require intact cells for its demonstration. Skinning might impair a cellular process which mediates the effect of BAY k 8644. This may also be true for experiments using isolated SR.

Relative to other inotropic agents, BAY k 8644 produces considerably greater force increases and yet toxicity is seldom seen. The primary cause of toxicity in isolated muscles is calcium overload. It would be very exciting if BAY k 8644 was relatively safe due to an imperviousness to calcium overload as diastolic efflux was promoted. This combination of inotropy and safety might provide much better therapeutic agents in the future. Unfortunately, the utility of BAY k 8644 as a therapeutic agent is limited by the fact that it has pronounced pressor effects. Unacceptably high increases in vascular and coronary resistance prevent any clinical applications (Schramm et al, 1983; Hwa and Bevan, 1986; Wada et al, 1985). However, if cardioselectivity could be achieved with similar compounds, it is quite likely that digitalis would no longer remain the mainstay of inotropic therapy.

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