Studies on contractility of cat splenic capsular smooth muscle

A Thesis Presented to
The University of Manitoba

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

by

Deepak Bose

1971

ABSTRACT

Mechanisms governing contractility of cat splenic capsular muscle were investigated. Isometric contraction in this tissue in response to agonists such as catecholamines and histamine consisted of a fast and a slow phase while the response to potassium consisted of a single phase. The slow phase of the response to noradrenaline and the single phase response to potassium were more sensitive to reduction of external calcium than the fast phase of the response to noradrenaline.

It was postulated that the fast phase of the response to noradrenaline depended upon a tightly bound store of calcium whereas the slow phase was due to a loosely bound pool of calcium. Differential blockade of the slow phase by the calcium chelator, EGTA, or by manganese, diazoxide or clonidine, and of the fast phase by zinc, procaine, metabolic inhibition, papaverine, aminophylline or sodium nitrite, suggested that noradrenaline could mobilise calcium from each pool independently. These results were given additional support by the observation that noradrenaline released ^{45}Ca from labelled tissue stores. This release was inhibited by zinc or procaine but not by manganese. Surprisingly, noradrenaline did not increase ^{45}Ca influx in the spleen strips, although external calcium concentration did regulate the slow phase. Compartment analysis of the 'desaturation' curve revealed two ^{45}Ca washout components. The faster component was lost by the strips at a rate faster than the loss of an extracellular marker, sucrose, suggesting some binding of calcium with tissue constituents. The slower component of ^{45}Ca desaturation was released faster in the presence of ^{40}Ca in the external medium,

suggesting that it is self exchangeable. The amount of ^{45}Ca released by ^{40}Ca is large in comparison to the amount released by noradrenaline and also differs in not being sensitive to the blocking action of procaine or zinc.

Additional experiments to elucidate the arrangement of calcium pools showed that the two functional pools of calcium were arranged in a 'series-parallel' fashion, i.e. each pool could provide calcium to the contractile apparatus independently but the tightly bound pool derived its content from the loosely bound pool. This was shown by the ability of manganese to prevent refilling of the tightly bound pool as well as the ability of extracellular strontium to release bound calcium when the muscle was depolarised with potassium.

Depolarisation of the muscle cell membrane with potassium resulted in a preferential inhibition of the slow phase. Similarly when the sodium pump was stimulated in a manner which has been found in other tissues to cause hyperpolarisation, the slow phase was preferentially decreased. The activity of an electrogenic sodium pump was demonstrated in the spleen by blockade of the inhibitory effects of this mechanism by ouabain, substitution of sodium chloride by lithium and changes in ambient temperature.

In addition to being regulated by the concentration of extracellular calcium, the slow phase also appeared to be modulated by factors which determine the steady state concentration of noradrenaline in the biophase. Inhibition of neuronal uptake of noradrenaline by the potentiating agents cocaine or 6-hydroxydopamine on the one hand and the alpha-adrenoceptor blocking agent phenoxybenzamine on the other hand

increased the proportion of slow phase in any given response to noradrenaline. The ratio of slow to fast phases in responses due to noradrenaline after inhibition of uptake resembled the ratio in the case of a poorly transported catecholamine, isoprenaline, in normal tissue. Another agent, reserpine, which produces potentiation of noradrenaline-induced responses without blocking neuronal uptake, did not have significant effect on the ratio of the two phases.

The spleen strip normally behaves as a multiunit preparation, showing no rhythmicity in response to noradrenaline. Single unit behaviour, characterised by rhythmic contractions and also contractions in response to rapid stretching of the preparation, was induced by treatment with cocaine, reserpine or 6-hydroxydopamine which cause supersensitivity to catecholamines and, in the case of cocaine and reserpine, to other agonists also.

Dedicated

to

Ratna, Nandini

and

my parents

ACKNOWLEDGEMENT

This work was made possible due to

- the able guidance and constructive criticisms of Dr. I.R. Innes. His kind understanding and patience made my task easier and enjoyable;

- the friendly collaboration of Dr. D.L. Woodward during the initial stages of this study.

- the help of W.J. Davidson, J. Elbrink, R. Mailhot, V.S. Vishnubhakta, S. Vivian and S. Whittaker in the form of consultation, technical aid and as sounding boards;

- the technical assistance of S. Lewis, D. McPhee, D. Montgomery and R. Weselake during various stages of this study;

- the able efforts of B. Barnett, C. Elbrink and R. Simpson towards giving this thesis its present appearance;

- Marge Turner for her concern and help on numerous occasions to make my life as a graduate student, happier;

- the award of a fellowship by the Medical Research Council of Canada.

I wish to convey my thankfulness to the above.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Contraction of any muscle, be it skeletal, cardiac or smooth, represents the end result of a chain of events which begins as a result of 'excitation' of the limiting membranes of the muscle cell either on account of physiological (humoral) stimuli or due to electrical or chemical stimuli under experimental conditions. The ultimate biophysical mechanism and manifestation of 'excitation' are as yet unknown. However, excitation of the cell membrane most commonly results in a decrease in resting membrane potential. This is due to changes in the relative permeability to the ions distributed on either side of the membrane. The resulting ionic shifts in turn make available a stimulus (calcium) for the activation of the contractile proteins (actin and myosin) in the muscle. This results in their sliding against each other (Hanson and Huxley, 1955; A.F. Huxley, 1957). Since actin and myosin are arranged longitudinally, in an orderly fashion, the above process results in shortening of the muscle or increase in tension. Any discussion on muscle function should take into account the following steps, as proposed by Bohr (1964):

(a) Membrane phenomena

(b) Coupling process (excitation-contraction coupling)

(c) Protein contraction

Work is performed during muscle contraction. The ultimate fuel for this is adenosine triphosphate (ATP). Hence this discussion should also include:

(d) Energy metabolism

The above four processes are intimately connected and are invariably present in all types of muscles even though quantitative differences may exist.

In view of the more organised structure and a relatively lesser degree of heterogeneity in cardiac and skeletal muscle compared to smooth muscle more is known about their function. A great deal of structural as well as functional heterogeneity exists not only between smooth muscles obtained from different species but also between smooth muscles obtained from different regions of the same organism. For example, one can detect differences in behaviour between gastrointestinal smooth muscle and those obtained from the genitourinary system or the vascular system. Even among vascular smooth muscles, anatomical and functional differences exist between muscles of larger conduit vessels and those of smaller arterioles or large veins (Somylo and Somylo, 1968b). The following review of the above mentioned four important facets of muscle function will pertain mainly to smooth muscle although it may be necessary to consider what happens in skeletal or cardiac muscle for the sake of comparison or for bridging gaps that exist in the knowledge of smooth muscle contraction.

"PROTEIN CONTRACTION"

Since the ultimate cause of muscle contraction is shortening of the contractile proteins, an analysis of this will clear the way for further discussion of other equally or more complex events occurring before this step.

1) Experimental models

One of the landmarks in muscle physiology was the demonstration by Szent Györgyi (1951) that treatment of fresh bundles of skeletal muscle fibres with aqueous glycerol destroyed the muscle membrane and extracted cell components of metabolic importance. The filaments of actin and myosin were, however, preserved in their natural state. Glycerinated muscle fibres when placed in buffered KCl solution are relatively inextensible, as if the muscle is in rigor. Addition of ATP restores extensibility of the muscle. This has been referred to as the 'plasticising action' of ATP. This action occurs only if manganese is present. Addition of minute quantities of calcium to the plasticised muscle results in contraction. The stimulant action of calcium can be elicited only in the presence of an active ATPase system in the contractile protein and is blocked by mersalyl or by the removal of Mg (Portzehl, 1952). Contraction is accompanied by hydrolysis of ATP. The contracted muscle can still be stretched back to its original length. Eventually when all the ATP has been hydrolysed the shortened muscle again becomes poorly extensible (Szent Györgyi, 1951; Filo et al., 1965; Bendall, 1969). Similar results have also been obtained in the smooth muscle (Briggs, 1963; Filo et al., 1965). The conclusion that can be drawn from these results is that ATP has a dual effect on myofilaments and that contraction can be initiated by calcium due to its stimulating effect on ATPase. As we shall see later this action of calcium is indirect.

Results similar to those in glycerinated muscle have been obtained in another very useful model of muscle contraction - the isolated myofibrils. These fibrils 10 - 100μ long, contain actin and myosin fila-

ments, normally arranged. They are obtained by homogenising muscle in weak buffers at pH 7 and an ionic strength of 0.1 - 0.15. The fibrils are then separated from connective tissue and intact fibres either by differential centrifugation or filtration through butter muslin. Contraction of myofibrils occurs in the presence of ATP,Mg and Ca and is measured by a decrease in the packed volume of myofibrils after centrifugation at 3000 x g. (Bendall, 1961; Perry and Grey, 1956).

ii) Chemistry and ultrastructure of contractile proteins

Further insight into the chemical basis of the interaction between actin and myosin was obtained when it was possible to extract contractile proteins from muscle and study their chemical nature (Ivanov et al., 1959; Laszt and Hamoir, 1961). They found that the major contractile proteins of smooth muscle can be extracted with solutions of low ionic strength. This contrasts with skeletal muscle where a solution of high ionic strength is needed.

The protein, extracted from vascular and other smooth muscles by neutral solutions of low ionic strengths in the presence of minute amounts of ATP, contains an actomyosin which resembles that obtained from skeletal muscle. It has similar viscosity, exhibits superprecipitation reaction and possesses ATPase activity. A curious feature of the vascular smooth muscle is its ability to generate a greater tension/unit amount of actomyosin than does skeletal muscle. Another interesting feature of the vascular smooth muscle contractile protein is that it is more readily extracted at pH 7 than at pH 6 (Schirmer, 1965). Needham and Shoenberg (1964) in their electromicrographic studies on uterine smooth muscle found it difficult to identify thick filaments which correspond to myosin

even though the latter has been shown by biochemical technique to exist in this smooth muscle (Needham, 1962). Actin in the form of thin filaments could be readily identified by these workers. Somylo and Somylo (1968) have suggested that the inability to see myosin in electron-micrographs maybe because of greater extraction of this protein at the pH employed for preparing the tissue for histological examination. A second possibility is that the myosin filament in smooth muscle may consist of fewer myosin molecules, therefore being thinner and indistinguishable from actin filaments. A third possibility (Nemetschek - Gansler, 1967) is that contractile proteins in relaxed muscle occur without orientation of filamentous form and that under the influence of calcium, polymerisation takes place. This results in contraction of the colloidal mass through a three dimensional interaction (cf. parallel sliding movement in skeletal muscle). A more recent work shows that myosin-like thick filaments can indeed be demonstrated in smooth muscle of guinea-pig taenia coli (Rice et al., 1970). These workers found ordered arrays of thin filaments (6.5×10^{-9} meter diameter) along with other apparently random arrangements of thin and thick filaments (10^{-8} - 10^{-7} meter diameter) in contracted muscles. The thin filaments were arranged hexagonally or circularly. Few examples of rosettes with regular arrangements of thin filaments surrounding thick filaments were seen. The thick filaments varied in thickness and were fewer in relaxed muscle. This prompted the workers to propose that thick muscle filaments aggregate prior to contraction and disaggregate during relaxation. Lowy and Small (1970) found thick filaments even in relaxed taenia. However, Rice et al. (1970) have called their method of fixation 'unphysiological'

as it was done at 0° C. Further work is required before a clear final picture of the arrangement of contractile proteins in the smooth muscle can be obtained.

The glycerol extracted muscle was mentioned above as a popular model for studies of the behaviour of the actomyosin system. Another model is actomyosin extracted from muscle. When Mg and ATP are added to the system in the total absence of Ca, the actomyosin is dissociated to its individual components (Weber, 1956). Associated with this is a reduction in the viscosity and double refraction of flow. This is analogous to the 'plasticising action' of ATP in the glycerinated muscle. However, if calcium is added even in small amounts to the actomyosin in ATP system, a rapid reaction occurs in which the actomyosin instead of being dissociated, actually shrinks ('superprecipitation' if actomyosin was originally a sol and 'syneresis' if actomyosin was a gel), accompanied by vigorous splitting of ATP (Szent Györgi, 1947).

On biochemical examination native actomyosin exhibits a Mg. sensitive ATPase activity. Actomyosin can be dissociated into myosin and F-actin combined with tropomyosin B. Myosin exhibits a Ca-activated ATPase activity which, unlike actomyosin ATPase, is inhibited by Mg. Hydrolysis of smooth muscle myosin by trypsin results in formation of two fragments : L (light) meromyosin and H (heavy) meromyosin (Cohen et al., 1961). ATPase activity is believed to reside in the heavy fragment.

For a long time it was believed that calcium participated in bringing about actomyosin ATPase activation and muscle contraction, through its action on actomyosin. This was implicit in the theory proposed by Davies (1963). A snag was seen in the above scheme when

'artificially prepared actomyosin' was studied. Actomyosin prepared from purified actin and myosin superprecipitated with ATP and Mg even in the total absence of Ca (Ebashi, 1963; Mueller, 1966). In contrast, native actomyosin, under similar conditions, became less viscous. However, the prepared actomyosin regained its sensitivity to calcium when another protein, tropomyosin, obtained from the muscle was added to the experimental system.

It was reasonable to conclude that tropomyosin exerted an inhibitory effect on the actin and myosin filaments and addition of calcium to the complete system resulted in a removal of this inhibition. Subsequently studies by Ebashi's group have revealed that tropomyosin consists of troponin and tropomyosin B and is intimately associated with actin filaments. Troponin which appears to be the calcium receptor, can combine with Ca in a ratio of 100,000g/4M. The present belief about the mechanism of action of the tropomyosin - actin - myosin system is that tropomyosin is present within the helices of natural actin and it repels the Mg ATP^{-2} or ATP^{-4} which is attached to the myosin partner and thus prevents the inter action leading to splitting and onset of contraction. When Ca is added, it would then bind to negatively charged groups on the troponin part of the tropomyosin complex, neutralise the charge and thus allow interaction to occur between the actin monomers and the substrate-myosin complex (Ebashi et al., 1967). Thus calcium acts not as an activator, per se, but as an inhibitor of inhibition. The discovery of tropomyosin in the smooth muscle of chicken gizzard (Ebashi et al., 1966) points to the general applicability of the above scheme to different varieties of muscle. It may be mentioned here that in order to show the

inhibitory effect on actin and myosin bridging, the S-H groups of tropomyosin have to be protected from oxidation (Mueller, 1966). This may explain the curious observation that myofibrils stored in the presence of oxygen lost their need for calcium. However, when the myofibrils were stored in nitrogen atmosphere, the calcium sensitivity was preserved (Bendall, 1969). Similarly, Briggs (1963) noticed that after prolonged storage, glycerinated uterine muscle failed to respond to calcium. These experiments point out the labile nature of tropomyosin.

Several other proteins have also been obtained from smooth muscle. Alpha actinin stimulates network formation of F-actin and superprecipitation of actomyosin solutions (Ebashi et al., 1966). Beta actinin which inhibits network formation has been obtained from skeletal muscle but not from smooth muscle. Tropomyosin A has been extracted from some molluscan muscles and has been implicated in the prolonged maintainance of tone (catch mechanism) in these muscles. Several attempts to show this substance in mammalian smooth muscle have been unsuccessful (Needham and Shoenberg, 1967; Carsten, 1968).

It can be realised from the preceding discussion that two substances are very important for contraction and both have to be provided from outside the domain of the contractile proteins. These are - calcium and ATP. The detailed mechanism of how calcium is made available to the contractile apparatus during activity and how it is taken back to terminate activity will be discussed under the heading of 'excitation-contraction coupling'. Before dealing with that it may be necessary to consider the mechanism of ATP supply for contraction and also storage of energy in other chemical forms. It will also be necessary to consider the source

or fuel which can contribute to the energy that is stored and is ultimately utilised during contraction.

"ENERGY METABOLISM"

1) Role of high energy phosphate compounds

Like the skeletal muscle, smooth muscle also uses ATP as the immediate source of energy. The ATP content of vascular smooth muscle (Beviz et al., 1965) is very similar to that in a skeletal muscle, viz. the frog sartorius (Marechal and Mommaerts, 1963). Hydrolysis of ATP during muscular contraction can be more readily demonstrated in smooth muscle than in skeletal muscle. This is mainly because in skeletal muscle the abundant supply of creatine phosphate can replenish the ATP pools very easily, through the Lohman reaction. Smooth muscle on the other hand, contains about 50 times less creatine phosphate (Beviz et al., 1965). In smooth muscle, unlike skeletal muscle, much of the ATP required is directly synthesised through glycolysis and oxidative phosphorylation during contraction (Daemers-Lambert, 1964). Oxidative phosphorylation in the smooth muscle mitochondria is almost as efficient as in those obtained from skeletal muscle (Stephens and Wrogeman, 1970). The relatively large contribution of anaerobic glycolysis in ATP production in the intact smooth muscle is most likely due to the paucity of mitochondria.

The thermodynamic efficiency of smooth muscle contraction is difficult to assess. This is largely due to the fact that many of the smooth muscle stimulants, e.g. catecholamines, besides causing expenditure of ATP directly due to muscle contraction also possess metabolic effects. Arterial resynthesis of ATP (Beviz and Mohme-Lundholm, 1965) and the production of fructose-1,6-diphosphate (Beviz and Mohme-Lundholm, 1964)

which consumes ATP are both stimulated by adrenaline. Like catecholamines, potassium also increases hydrolysis of ATP, (Daemers-Lambert (1964). This can be shown only after blocking glycolysis, and thereby preventing resynthesis of ATP. Potassium can also stimulate ATP hydrolysis in subcontractile concentrations. This is reminiscent of the Solandt effect seen in skeletal muscle (Van der Kloot, 1967). It should be kept in mind that measurement of net changes in ATP levels and relating them to contractility without taking into account metabolic effects of agonists can be misleading.

In smooth muscle, carbohydrates are the major source of the energy which is eventually transformed into the universal metabolic currency, ATP. This is evident from the respiratory quotient of nearly one (Kosan and Burton, 1966). Preformed glycogen appears to be preferentially utilised during contraction because addition of glucose to media containing whole vascular segments does not increase respiration (Chattopadhyay, 1962). The importance of maintained synthesis of ATP, especially during activity of the muscle, is great since the total ATP content of the smooth muscle may be less than what can suffice for the energy expenditure of even a single large contraction (Lundholm and Mohme-Lundholm, 1962).

ii) Substrate requirement for contraction

Studies on the rabbit thoracic aortic strips by Coe et al. (1968) show that these muscles can perform adequately in the absence of glucose in the medium for a reasonably long time provided they are stimulated with 'physiological' (1-10 μg/l) concentrations of adrenaline. Contractility was diminished only after a prolonged contraction or repeated short

duration contractions due to large (1-10 mg/l) dose of adrenaline. Responses improved after glucose was restored. The spontaneous tone developed by bovine mesenteric artery as well as its contractile response to drugs can be maintained under aerobic conditions by endogenous glycogen for at least 8 hours but for only 1 hour under anaerobic conditions (Lundholm and Mohme-Lundholm, 1960, 1962). Contractility and lactic acid production due to adrenaline are reduced in the absence of substrate at a rate greater than that due to histamine or potassium (Lundholm and Mohme-Lundholm, 1962 and 1963). Adrenaline probably stimulates metabolic processes that compete with the contractile system for the limited ATP supply (Somlyo and Somlyo, 1968b). Lactic acid production increases during contraction produced by most agonists. However, this is not entirely indicative of the energy requirement of the contractile process. The contractile effect of drugs can be dissociated from lactic acid production (Lundholm and Mohme-Lundholm, 1963). For example, alpha-adrenoceptor blocking agents reduce the contractile response to adrenaline more than the lactic acid stimulating effect. Similarly histamine and adrenaline stimulate lactic acid production in the absence of contraction when bovine mesenteric artery is challenged in the absence of extracellular ions. Besides utilising the Embden-Meyerhof pathway and Krebs cycle, muscles can also metabolise carbohydrate through the hexose monophosphate pathway (Sbarra et al., 1960; Wertheimer and Ben-Tor, 1962).

Several studies (Furchgott, 1966; Coe et al., 1968) to determine substrate requirement for supporting contractility have been done on smooth muscle depleted of substrate by incubation in glucose-free medium. The effect of reintroduction of known substrates on contractility

was noted to assess the energy contribution of any particular metabolic pathway for contraction. Glucose and mannose restored contractions, suggesting that the embden-Meyerhof pathway was functioning. In spite of the inability of several Krebs cycle intermediates such as citrate, alpha-ketoglutarate, succinate, fumarate and malate to restore contraction, the presence of Krebs cycle in smooth muscle cannot be doubted because pyruvic acid and lactic acid are extremely effective as replacements for glucose. Recovery of contractility after fatty acids such as butyrate, oleate, acetoacetate and betahydroxybutyrate suggests the presence of beta oxidative pathway. The amino acids alanine, glutamate and aspartate do not support contractions. It may be mentioned here that absence of effect on contractility of a given substrate does not imply that it is of no importance, in vivo. The Krebs cycle intermediates are ineffective most probably because they do not cross the membrane barriers encountered under the experimental conditions. However, Furchgott and Wales (1952), working under the assumption that unionised di- or tricarboxylic intermediates would penetrate biological membranes better, tested them while the pH of the medium bathing the muscle was reduced to decrease ionisation. Under such conditions high concentrations of these substrates did become effective in restoring contractions. In experiments on substrate depleted muscles one has to be very cautious about interpretation of results for the following reasons: the fact that a particular substrate (which is not an intermediate in carbohydrate metabolism) reverses the inhibitory effect of glucose deprivation does not necessarily mean that the same substrate is normally being utilised by the muscle in vivo. The question of availability of this substrate

in vivo has to be considered before it is assigned a definite role. The second pitfall is in the assumption that decrease in contractility during substrate deprivation is solely due to a decrease in the energy supply at the level of the contractile apparatus. Membrane activity and the state of ion pumps, especially those regulating the intracytoplasmic calcium concentrations are of great importance in determining the ultimate extent of contraction. When one studies the end result of a series of reactions such as contractility of the muscle, the change seen can very well be due to the effect of decreased energy supply on earlier steps. Recent evidence presented by Kroeger (1970) shows that hypoxic trachealis muscle of the dog loses its responsiveness to acetylcholine while gaining in resting tension. The loss of responsiveness has been attributed to the loss of calcium from an intracellular pool probably as a result of inhibition of an active pump which normally serves to replenish this pool. The rise in resting tension of the muscle, which is probably due to increase in the normally low permeability of the muscle membrane for calcium, suggests that the energy requirements of the contractile apparatus are still being fulfilled. Increase of resting tension has not been universally seen in hypoxic smooth muscles but decrease in contractility in response to agonists during hypoxia is well known (Detar and Bohr, 1968a; Guyton et al., 1964). These and other findings previously described suggest strongly the importance of aerobic metabolism of carbohydrate fuel by the Krebs cycle in providing energy for contractility or cellular excitability or both. A curious phenomenon of adaptation to hypoxia has been reported by Detar and Bohr (1968b); responses to adrenaline in the rabbit aorta were depressed less after 15 hours of hypoxia

than after less than 2 hours. On restoring the former muscles to normoxic conditions the responsiveness to adrenaline paradoxically decreased at first and then increased towards the prehypoxic value. The acutely hypoxic tissue, however, responded to oxygen by a prompt increase in tension. The authors also observed that unlike normoxic muscles when the hypoxic (adapted) muscle was placed in a glucose free environment and its residual response to adrenaline depressed, restoration could not be achieved with pyruvate but could be readily attained with glucose. These workers speculated that hypoxic adapted muscles attained a greater ability to utilise the Embden-Meyerhof pathway for energy production. The inhibition of the adapted muscle after return to normoxic conditions was ascribed either to an inhibition of glycolysis by oxygen ('in a fashion reminiscent of the classically described Pasteur effect') or by stimulation of a sarcoplasmic reticular calcium pump. Obviously, as the authors have themselves mentioned, more biochemical investigations have to be done to clarify this phenomenon. It is also necessary to know if the inhibition seen in these muscles after oxygen can be related to changes in membrane potentials as a result of effect on ion pumps - specifically on an electrogenic sodium pump.

Future work will probably provide more information about the compartmentalisation of ATP and other high energy compounds in the cell for providing energy to the individual steps which ultimately lead to muscle contraction.

"MEMBRANE PHENOMENA"

The membrane of smooth muscle cells possesses many properties common to other excitable membranes. In their resting state a potential gradient exists across the inner and outer surfaces of the membrane and during activation or inhibition of the muscle, changes occur in the magnitude of this potential.

i) Resting membrane potential of the smooth muscle depends upon the transmembrane gradients of sodium, potassium and chloride along with the relative permeability of the membrane to these ions. Among these, the potassium concentration gradient and the relatively high potassium permeability of the membrane are the major determinants. Increase in external potassium concentration reduces the transmembrane gradient and causes depolarisation. The relatively low membrane potential of smooth muscle (50 mV for guinea-pig taenia coli, Bulbring, 1954) compared with frog skeletal muscle (90 mV, Adrian, 1956) has been explained by a relatively higher sodium permeability of the smooth muscle membrane (Kuriyama, 1963). Removal of sodium from the external medium, according to the above scheme, should cause hyperpolarisation. This has been found to occur by Brading and Tomita (1968) if sodium is replaced by sucrose. Low potassium permeability of the smooth muscle compared to that of skeletal muscle may also contribute to the smaller resting membrane potential in smooth muscle. This has been shown by the studies of Goodford and Hermansen (1961).

Two other factors may play some role in determining the resting membrane potential. They are the chloride potential and electrogenic sodium pump potential. According to Casteels (1970) the intracellular

chloride concentration is too high to fit a passive distribution (as assumed by many workers, e.g. Somlyo and Somlyo, 1968b). To explain this non-equilibrium distribution, one has to consider an active inward movement of chloride ions. That chloride ions contribute to the resting membrane potential is indicated by depolarisation of the membrane when external chloride is replaced with nonpermeant sulphate anion (Kuriyama, 1963).

Since the resting membrane potential is less than the calculated potassium equilibrium potential and more than the calculated sodium equilibrium potential, there is a tendency for these two ions to diffuse downhill across the membrane. The maintenance of dissimilar concentrations of sodium and potassium across the membrane is possible because of the sodium pump, which drives sodium out of the cell and brings potassium in a coupled fashion (Daniel and Robinson, 1960a,b). The pump can become electrogenic if it is uncoupled in such a fashion that sodium efflux exceeds potassium influx, which would result in an increase in membrane potential. Such a phenomenon has been recently demonstrated in uterine smooth muscle which, after sodium enrichment in cold potassium free medium, was made to recover in the presence of potassium in the external medium (Taylor, Paton and Daniel, 1970). This is similar to the events in the skeletal muscle after identical pretreatment (Cross, Keynes and Rybova, 1965). Hyperpolarisation, in both these instances could be reduced by drugs or procedures which inhibited the sodium pump, e.g. ouabain, substitution of lithium for external sodium and reduction of temperature. The internal sodium concentration of sodium enriched skeletal muscle is similar to that of normal smooth muscle (approx. 40 mM), and therefore

Holman (1969) feels that the possibility of the role of an electrogenic pump in the smooth muscle is attractive. Nevertheless there is no good evidence to substantiate this possibility. Axelsson et al. (1967) observed that the resting potential of rat portal vein decreased in K-free solution. While this can be explained by inhibition of the sodium pump (Somlyo and Somlyo, 1968b), it can just as well be explained by a decrease in potassium permeability as suggested for Purkinje fibres (Carmeliet, 1961) and for Carcinus axon (Keynes and Lewis, 1951).

The recorded resting membrane potentials of different smooth muscles vary considerably. Part of this is due to technical reasons, e.g. recordings with the sucrose gap technique may underestimate the true membrane potential because of the shortcircuiting effect of extracellular ions; tip potential of microelectrodes can be another cause of discrepancy, especially with high resistance electrodes (Adrian, 1956), which are essential for smooth muscle studies. Another difficulty is due to the spontaneous fluctuations in membrane potential that occur in many visceral smooth muscles. The maximum level of membrane potential, recorded in the absence of electrical activity, is around 75 mV (Burnstock et al., 1963; Su et al., 1964; Speden, 1967). Blood vessels exhibiting myogenic activity usually show smaller resting membrane potentials, e.g. small mesenteric arteries (Steedman, 1966) and anterior mesenteric vein of the guinea-pig (Nakajima and Horn, 1967). Whether this smaller membrane potential predisposes to spike activity characteristic of these muscles cannot be said with certainty.

ii) Action potentials

These are transient decreases in the membrane potential,

occurring either spontaneously or as a result of chemical influences or electrical stimulation. The controversy about whether these electrical changes (action potentials or spikes) are myogenic or neurogenic appears to be settled in favour of myogenic initiation since they cannot be abolished by atropine, hexamethonium, cocaine or tetrodotoxin (Bülbring, 1955; Bülbring et al., 1958; Bülbring and Kuriyama, 1963; Nonomura et al., 1966; Kuriyama et al., 1966).

The ionic basis of action potentials in the smooth muscle is not as clear as that in the squid axon or the skeletal muscle. Sodium appears to be less important for the production of spikes in smooth muscle than in nerves or skeletal muscle. Reduction of external sodium chloride to as little as 20 mM and replacement with choline does not change the spike amplitude. Further decrease reduces the rate of rise as well as spike amplitude (Holman, 1957, 1958). Bülbring and Kuriyama (1963), however, showed that when Tris chloride was used as a replacement, no decrease in amplitude but only a decrease in the rate of rise of action potential occurred. These results are not very easy to interpret. Reduction in rate of rise may not be simply due to lack of sodium ions and the replacing agents may themselves have an effect. Further, it is possible that the decrease in rate of rise during sodium deficiency may be an underestimation if compensatory changes take place during this procedure. For example, low sodium may facilitate the action of calcium, if sodium-calcium competition occurs in smooth muscle as it does in heart (Luttgau and Niedergerke, 1958). High calcium levels tend to increase the availability of membrane sites for inward sodium movement during the spike in the squid axon (Frankenhaueser and Hodgkin, 1957) and if a

similar process occurred in smooth muscle, spike amplitude and rate of rise will increase. Nevertheless the consensus is that the contribution of sodium to the smooth muscle spike is small. Spikes which can still be elicited in the absence of external sodium are reduced as external calcium is removed. This suggests that calcium may be an important contributor to the action potential. This is supported by the following evidence:

i) Since the transmembrane gradient of calcium is high (2.5 mM outside; less than 10^{-6} - 10^{-7}M inside, Caldwell, 1968) the equilibrium potential can be expected to exceed + 100 mV. If external sodium is reduced to 10 mM, the internal sodium concentration decreases from 35 mM to about 24 mM. Under these conditions the sodium equilibrium potential decreases from + 34 mV in the normal environment to - 22 mV. If the spikes are due to sodium entry, one would not expect them to exceed - 22 mV unless there is a significant contribution from another cation. In fact, when sucrose is used as a replacement for sodium, the spike potential exhibits an overshoot of + 20 mV (Brading and Tomita, 1968; Brading et al., 1969).

ii) In low sodium solutions, calcium deficiency causes a slight depolarisation. However, the decrease in amplitude and rate of rise of spike is far in excess of what can be accounted by the depolarisation. This suggests an increase in membrane permeability to calcium during the spike (Brading et al., 1969).

iii) The action potential in crustacean muscle is due to calcium entry (Hagiwara and Naka, 1964; Hagiwara and Nakajima, 1966). Several similarities exist between smooth muscle and crustacean muscle.

For example, like crustacean muscle, smooth muscle is resistant to tetrodotoxin, an agent which prevents sodium activation (Nonomura et al., 1966). However, manganese, which blocks calcium entry, in crustacean muscle also abolishes smooth muscle spikes (Nonomura et al., 1966). Finally, when the smooth muscle spikes are abolished by calcium depletion, substitution of barium can restore them (Bülbring and Tomita, 1969c).

Calcium can contribute to spikes in smooth muscle in two directly opposite ways. It may serve as a current carrier during excitation, and the magnitude of this effect will depend upon the concentration difference across the membrane as well as the amount of increase in membrane permeability. In addition to this, calcium adsorbed on the membrane surface acts as a stabilising agent (Frankenhauser and Hodgkin, 1957). This action results in a decrease of sodium permeability and secondarily a greater availability of sodium channels for activation during subsequent excitation. The stabilising action of calcium is not limited to the sodium-dependent squid axon but exists also in the calcium-dependent barnacle muscle (Hagiwara and Takahashi, 1967). Excitation, according to this theory, can occur due to displacement of calcium adsorbed to the stabilising site.

Bülbring and Kuriyama (1963) caution that the dependence of the spike on calcium in the absence of external sodium does not necessarily mean that calcium currents are important in the presence of normal amounts of sodium. The opposite situation has been shown by Golenhofen and Petranyi (1969) in the guinea-pig taenia coli. In a medium containing normal amounts of sodium and calcium the spikes were resistant to tetrodotoxin. However, in a calcium-free solution, preferably with

magnesium absent and sodium fluoride added, the spikes initially disappeared or decreased and then redeveloped to near the initial level. The new spikes were sensitive to tetrodotoxin thus indicating that sodium ions now were the most probable current carriers. In spite of the hazard of unquestioned faith in the specificity of tetrodotoxin, these results suggest that the smooth muscle may possess a more versatile means for utilising different ions as charge carriers in different situations than generally conceded.

iii) Functional classification of smooth muscles

Given a muscle cell membrane that is capable of altering its potential gradient, one next wonders as to how activation of a single muscle cell fits in the context of the contraction of a large mass of muscle consisting of numerous muscle cells. When a smooth muscle is stimulated via its nerves, are all the cells simultaneously activated by the nerves or is the activity originating from a few cells propagated to other cells?

Smooth muscles have been assigned single or multiunit status by Bozler (1948). Single unit muscle cells have very close electrical connections. Structurally, membranes of adjacent muscle cells are closely apposed. This may be seen either as abutment of adjacent cell membranes or bulbous projection of one cell against another cell or its process. The membranes are sometimes fused or lie very close to each other and this specialised region is called a 'nexus' (Dewey and Barr, 1962 and 1968). In the absence of an extracellular space between the two apposed membranes, electrical activity in one cell can readily affect the adjacent cell. In spite of difficulty in showing a nexus due

to shrinkage of cells during fixation, newer methods are bringing greater success to the histologist. Single unit muscles are generally present in the abdominal organs, such as in small intestine, taenia coli, and the uterus.

Several observations are consistent with the presence of low resistance intercellular junctions such as would occur with nexuses or tight junctions. For example, electrical excitation originating in one part of the muscle is propagated over long distances provided a critical number of cells is excited originally (Tomita, 1966). Also, Holman (1969) noticed that potential difference between a depolarised and polarised region of a smooth muscle separated several cell lengths apart can often be recorded by the sucrose gap technique. This can only be possible if the internal longitudinal resistance of several adjacent muscle cells is low.

Many smooth muscles can contract in a co-ordinated rhythmic manner. This has long been recognised in visceral smooth muscle but has only recently been demonstrated in vascular smooth muscle by Johansson and Bohr (1966) who showed that smooth muscle of the cutaneous artery of the dog's paw is rhythmically active when partially contracted by sympathomimetic agents or KCl. Bozler's earlier categorisation of vascular smooth muscle as multiunit, based on studies on large arteries appears now to have been premature (1948).

Another property of single unit muscles is their ability to contract on being stretched rapidly. This phenomenon depends upon stretch causing depolarisation. This in turn results in a propagated wave of excitation all over the muscle and also an increase in the

frequency of spontaneous action potentials (Cuthbert, 1966; Holman, Kasby and Suthers, 1968).

Single unit muscles have a resting active tone besides exhibiting spontaneous contractions. Both these are probably mediated by action potentials (Burnstock et al., 1963).

Multi-unit muscles have not been studied as thoroughly as the single unit muscle from the electrophysiological standpoint. Undoubtedly the small cell size and the large amount of tough connective tissue present in such muscles have not permitted successful intracellular potential recordings. In contrast to single unit muscles, the multi-unit muscles do not exhibit spontaneous rhythmicity, propagated electrical or mechanical activity and myogenic contraction. The muscle cells are extensively surrounded by connective tissue, thereby increasing intercellular separation and decreasing communication. Examples of multi-unit muscle are the large conduit vessels such as the rabbit aorta and pulmonary arteries, and the rat vas deferens. Another important difference between the single and multi-unit muscles is in the necessity for extensive nervous contact with cells in the multi-unit muscle. Single unit muscles do not need such extensive one-to-one innervation. This does not mean that single unit muscles necessarily possess a sparse innervation.

Most of the multi-unit muscles do not readily exhibit spike electro-genesis. Instead excitation of such muscles with KCl or drugs produces graded depolarisation (Somlyo and Somlyo, 1968a; Su and Bevan, 1965). In contrast, single unit muscles produce spikes accompanied by a slow depolarisation during excitation with drugs or potassium (Cuthbert and Sutter, 1965; Funaki, 1966; Johansson et al., 1967; Nakajima and

Horn, 1967; Speden, 1967; Holman et al., 1968; Somlyo and Somlyo, 1968a).

Classification of smooth muscles into the two types rests on additional criteria as well as on their ability to generate spikes or to respond by graded depolarisation. The spike generating muscles respond to electrical stimuli of shorter duration (10 msec) compared to the muscles depolarising in a graded fashion (Somlyo et al., 1965, 1968b). Also, the latter smooth muscles contract in a sustained fashion when depolarised by KCl, whereas spike generating muscles respond phasically (Somlyo et al., 1969). In an interesting experiment, Somlyo and Somlyo (1968a) showed that caffeine converts the behaviour of the smooth muscle of mesenteric vein from spike generation to graded depolarisation. Thus it is possible that smooth muscles may not always behave in a set pattern but may have dual properties.

It is still premature to say that the terms 'single unit', 'phasic' and 'spike generating' on the one hand and 'multiunit', 'tonic' and 'gradedly responsive' muscle on the other hand can be used interchangeably. Between these two extremes may lie muscles showing intermediate behaviour, e.g. although small arteries in vitro are normally quiescent (and hence should come under the category of multi-unit muscle, they can be made rhythmic under the influence of potassium (Johannson and Bohr, 1966).

The above description has painted a very consistent picture of the invariable decrease in membrane potential, with or without action potentials, when a smooth muscle is excited. Two studies are in discord with the above. Su et al. (1967), recording intracellularly, found no

change in the membrane potential of rabbit pulmonary artery during sympathetic stimulation while Shibata and Briggs (1966) employing extracellular electrodes recorded hyperpolarisation of the rabbit aorta during contraction induced by adrenaline.

While it is very hard to tell exactly why the two studies cited above were different from the rest one might take shelter under the concept of 'pharmacomechanical coupling' (Somlyo and Somlyo, 1968a) to explain the paradox. Increasing evidence is accumulating to suggest the possibility of a non-electrical process of excitation leading to contraction.

Although depolarisation is consistently seen during contraction produced by humoral stimuli, it is by no means absolutely essential. Experiments done by Evans and Schild (1958) and Waugh (1962), show that smooth muscle depolarised with potassium continue to contract in response to agonists without further change in membrane potential. Even though these results show that pharmacological agents can bypass the need for electrical activation of membrane one wonders if the prior depolarisation by potassium allows the other agonists to manifest their 'pharmacomechanical' effect. The critical proof will be to show contraction of a polarised muscle without any depolarisation. Until then it will be very difficult to quantify the relative roles of electrical and non electrical mechanisms in contraction produced by drugs under normal circumstances.

Just as smooth muscle stimulants act either by producing electrical changes in the membrane or by non electrical means, likewise smooth muscle relaxants also have effects on the muscle cell membrane. Agents influencing electrical properties of smooth muscle may do so

either by causing hyperpolarisation or by decreasing spike production. Bülbring and Tomita (1968; 1969a,b,c) have shown that inhibition of mechanical activity of guinea-pig taenia coli by adrenaline and noradrenaline is associated with hyperpolarisation. This is mainly due to an increase in potassium and to some extent chloride permeability. After blocking the alpha-adrenoceptors with phentolamine when only the effect of catecholamines on the beta-receptors remains, there is no hyperpolarisation but only a cessation of spontaneous spike activity. Others (Somlyo and Somlyo, 1970; Somlyo, Haeusler and Somlyo, 1970) have found that beta-receptor activation in the non-spike producing avian slow muscle or the rabbit main pulmonary artery can also produce hyperpolarisation by stimulating an electrogenic sodium pump. In the rat uterus, Diamond and Marshall (1969a,b) observed that small concentrations of adrenaline, papaverine, tetracaine or nitroglycerin, while reducing the amplitude of contraction, mainly reduced the frequency of spontaneous action potential discharge. Very slight or no hyperpolarisation occurred with the above drugs. Even though higher concentrations of adrenaline caused more marked hyperpolarisation, this was not believed to be the cause of the decrease in amplitude of contraction but rather of a decrease in resting tension of the muscle. All four agents were also capable of relaxing depolarised muscle without altering membrane potential, an observation which suggests that a 'pharmacomechanical' mechanism exists. The depressants were antagonised by raising external calcium concentration. Even though it appears on superficial examination that relaxants may be acting by interfering with calcium movements, the evidence is not very clear. For example, even though the relaxants tetracaine and cinchocaine decrease

radiocalcium influx (Feinstein, 1966; Northover, 1968) and papaverine inhibits lipid-facilitated transport of calcium between two solvents (Carpenedo et al., 1968), other relaxants like epinephrine and caffeine (Feinstein, 1966) and indomethacin and desipramine (Northover, 1968) do not seem to affect calcium movements. As mentioned elsewhere, this may reflect a true absence of effect or may indicate that the critical fraction of calcium movement related to contractility is very small.

"EXCITATION-CONTRACTION COUPLING"

It is clear from the previous discussion that electrical depolarisation of the smooth muscle, either spontaneous or evoked, is accompanied and to a great extent caused by inward movement of calcium ions. The questions that need be considered at this stage are the following:

a) Is the quantity of calcium that enters during depolarisation enough to account for the entire contraction or does the initial entry of calcium trigger further release of calcium from some internal site? Such a mechanism has been proposed for the skeletal muscle by Bianchi (1968) and Winegrad (1970). Goodford (1967) calculated that the calcium influx during a single action potential would barely be adequate to raise the intracellular concentration of calcium ions to a level which would activate contraction, although it was possible that several spikes could produce enough elevation of cytoplasmic calcium to activate the contractile proteins. Holman (1969) estimated that if the faster spikes of visceral smooth muscle were entirely due to calcium current, a single action potential could increase the cytoplasmic calcium concentration to

2 to 5 x 10^{-6}M, which is quite adequate for initiating contraction.

If the calcium current of the action potential is sufficient to trigger contraction, then several studies which show persistence of action potentials in the absence of contraction are difficult to explain (provided one assumes that the contractile apparatus remained functional after the various treatments). For example, Cuthbert and Sutter (1965) found that action potentials in rabbit anterior mesenteric veins continued for several minutes after mechanical activity had been abolished by calcium depletion. Axelsson (1961) found that action potentials continued although contractions were abolished when sodium in the bathing medium was replaced by lithium, hydrazine or choline. It has yet to be quantitatively estimated if the spikes which do not elicit contractions carry less calcium current than normal spikes.

b) If extracellular calcium is not the sole activator of the contractile proteins, where does the remaining supply of calcium come from? An answer to this question can come from analysis of the excitation contraction mechanism in smooth muscle.

The excitation-contraction coupling process links the excitation of the membrane with the shortening of the contractile proteins. Detailed studies in striated muscle have made it clear that the following events are intimately linked with one another:

a) The excitable membrane around the periphery of the muscle fibre depolarises, giving rise to a propagated impulse.

b) This impulse travels to the transverse tubular membrane which is an invagination of the surface membrane overlying the Z band region (Huxley, 1967).

c) The eventual effect of depolarisation of the transverse tubule is the release of sequestered calcium from the terminal cisternae. The latter are widened ends of the sarcoplasmic reticulum situated beside the 'Z' band of the sarcomere.

The mechanism by which depolarisation of the transverse tubule is coupled to the release of calcium from the terminal cisternae is not yet clear. Bianchi (1968) has proposed that extracellular calcium enters the muscle cell as a result of the depolarisation of the transverse tubules and this causes secondary release of calcium from the terminal cisternae. Calcium released from the lateral cisternae binds to troponin to cause contraction. Relaxation occurs when the calcium is pumped back actively into the longitudinal central part of the sarcoplasmic reticulum and then redistributed to the lateral cisternae. More direct evidence that an increase in free cytoplasmic content of calcium does occur during the active state of the muscle and these two may be causally related has come from the work of Ridgeway and Ashley (1967). These workers injected a chemiluminescent substance, aequorin, into barnacle muscle as an indicator of free calcium. An increase in luminescence (calcium transient) followed the activation of muscle membrane but preceded the increase in muscle tension. The peak of the calcium transient coincided with the maximum rate of development of tension and a decrease in the luminescence preceded relaxation. Even though this technique at the present stage of development can give an estimate only of the change in cytoplasmic calcium (rather than the absolute concentration of free calcium) and the inability of aequorin to penetrate muscle cell membrane precludes its use in small muscle cells (e.g. in smooth muscle) it is hoped that

brighter prospects for its application to other muscles will arise.

In skeletal muscle, the contraction-relaxation cycle has a much shorter time course than in smooth muscle. This is mainly due to a much better organisation of sarcoplasmic reticulum which functions not only as a site from which calcium is released to the contractile proteins during contraction but also as a pump for recapturing the calcium. In smooth muscle, sarcoplasmic reticulum is either not developed or at best poorly developed. It is therefore very likely that the cell membrane assumes the function of the sarcoplasmic reticulum. This would be aided by the small size of the smooth muscle cell, in which the ratio of membrane surface to cell volume is much larger than in skeletal muscle. It is possible that differences in the time course of contraction-relaxation cycle in phasic and tonic smooth muscles may be due to differences in the amount of sarcoplasmic reticulum. This has yet to be examined.

i) Sources and sinks for calcium in the smooth muscle

As mentioned earlier, most smooth muscles lack a well organised sarcoplasmic reticulum like the one described in skeletal muscle. Therefore the process of calcium release and re-uptake is probably not quite the same quantitatively as in skeletal muscle. The few reports existing on the presence of sarcoplasmic reticulum in smooth muscle and its ability to take up calcium actively (Carsten, 1969) reveal that the calcium binding is considerably lower than that of similar preparations from cardiac muscle (Carsten, 1967). Considering the smaller actomyosin content of the smooth muscle and the relative sluggishness of the contraction-relaxation cycle, it has been proposed that even this weak activity

of the sarcoplasmic reticulum may be physiologically adequate (Carsten, 1969).

Others, in view of the paucity of sarcoplasmic reticulum in most smooth muscles, believe that the muscle cell membrane and the extracellular millieu serve as the source of calcium for contraction (Peachey and Porter, 1959). There is some controversy regarding the mechanism of removal of calcium from the smooth muscle contractile proteins during relaxation. Because sarcoplasmic reticulum is poorly developed in most smooth muscles, the possibility of an active transport of calcium across the membrane to the extracellular space has to be considered (Schatzmann, 1961). Among the few studies done so far to test this possibility, no evidence has been obtained in support except by Nagasawa (1965) in the guinea-pig taenia coli, who found that radiocalcium efflux increased markedly when the ambient temperature was increased from 4°C to 37°C. In contrast Goodford (1965a,b) found that the increase in efflux of radiocalcium from guinea-pig taenia coli when the temperature was raised from 4° to 35°C was small (Q_{10} = 1.5). This was not significantly higher than the temperature-induced increase in calcium efflux from a heat-killed muscle. Goodford argued that an active process for 'extrusion' of intracellular calcium is not involved and suggested that removal of calcium from the cytoplasm may be by a process of 'exclusion'. Essentially similar results were obtained by Van Breemen et al. (1966) in the rat uterus. Definite conclusions cannot be drawn from the above results for the following reasons: Radio-calcium efflux from the tissue into the inactive bathing medium does not necessarily reflect extrusion from the cytoplasm. Instead, if most of the movement really is from the muscle

membrane, a small component of efflux from the cytoplasm can be completely masked. In Goodford's study, the tissues were loaded with radio-calcium at a low temperature, a procedure which was found to increase uptake of the isotope. If this was due mainly to a decrease in the permeability barrier of the membrane, and also due to the inability of a hypothetical externally directed calcium pump to keep pace with the calcium influx, then the rate of efflux in cold should also depend upon the above two factors. However, with an increase in external temperature, permeability should again decrease and this should, to some extent, counteract the increase in calcium pump activity and result in a spuriously low temperature coefficient for the increase in efflux. Nevertheless, Goodford's suggestion that calcium efflux is not limited by pump activity but by the rate of dissolution of calcium phosphate into the extracellular fluid is novel.

Smooth muscles possess spherical invaginations of the plasma membrane which are known as micropinocytotic vesicles. There is disagreement regarding their actual number as well as distribution. The functional significance of micropinocytotic vesicles is not clear. However, it is easy to realise that these vesicles cause at least a 25% increase in the area of the cell surface (Rhodin, 1962) and therefore make a larger area of membrane available for exchange or transport of electrolytes and nutrients. Recently Devine and Somlyo (1970) showed that in vascular smooth muscle the extracellular marker, lanthanum, not only entered micropinocytotic vesicles which communicated with the extracellular space but also into subplasmalemmal vesicles closed off from the exterior. This strongly suggests that the pinocytotic vesicles, which are exposed

to relatively high concentrations of calcium in the extracellular space can act as a reservoir for calcium that may be used for contraction. It is not known if these vesicles can also take part in the recapture of calcium from the cytoplasm (and contractile protein) and in its extrusion into the extracellular space.

ii) Functional pools of calcium

In spite of little direct information about the morphological sites which provide calcium for contraction of smooth muscle, a lot of indirect evidence has been obtained to indicate that calcium is available from more than one functional pool. Peachey and Porter (1959) suggest that extracellular calcium activates contraction because of the small amount of smooth muscle endoplasmic reticulum and the short diffusion distance in smooth muscle. This was supported by the observation made by Briggs (1962) that the magnitude of contraction of rabbit aorta produced by potassium sulphate was related linearly to the rate of uptake of radioactive calcium from the medium. The issue was somewhat confused by the fact that no increase in total calcium content of the tissue occurred even though potassium did not increase calcium efflux. The data of van Breemen and Daniel (1966) on the rat uterus differs from the above. They found that potassium increased the efflux of that fraction of radiocalcium which emerged slowly from the muscle. More recently, Krejci and Daniel (1970a) reported a decrease in radiocalcium influx in rat uterus when the isotope was allowed to penetrate after potassium depolarisation. This was attributed to a decrease in diffusion of tracer through the extracellular space due to contraction. However, when the isotope was added first and contraction with potassium produced subse-

quently, the influx was similar to that in an unstimulated tissue. Unfortunately, Briggs did not investigate the possibility of altered movements through the extracellular space during contraction, in his study. Despite these two opposite results, an increase in Ca^{45} influx during stimulation has been demonstrated in many smooth muscles (e.g. Sperelakis, 1962, in the cat intestine; Urakawa and Holland, 1964, in the guinea-pig taenia coli). In most of these studies no effect of potassium was seen on total tissue calcium levels (Briggs, 1962; Lüllmann and Siegfriedt, 1968). These differences in results are very difficult to explain. They may be related to differences in muscles, differences in the effect of potassium on the extracellular space of these tissues and differences in the extent of utilisation of extracellular calcium. An element of uncertainty is introduced by the assumption made in the above studies that the measured isotopic content reflects intracellular levels when it may actually be a combination of intracellular and extracellular (bound to the basement membrane and ground substances in the extracellular space) calcium. A translocation of calcium from one compartment into another by potassium need not necessarily be detected as increase in ^{45}Ca influx. This is also supported by the observation that total tissue calcium is not altered. Nevertheless, it is very likely that in most smooth muscles potassium causes an increase exchangeability between extracellular and tissue bound (? intracellular) calcium.

Complications were introduced into the earlier concept that only calcium derived from extracellular space was used for contraction when Hinke (1965) observed that the contractile responsiveness of rat

tail artery to potassium declined faster than that to noradrenaline when calcium was withdrawn from the perfusion medium. Addition of EDTA hastened decay of contractility but a difference still existed between potassium and noradrenaline. Several workers have subsequently shown differential effects of calcium removal on the contractile response of smooth muscle to drugs (e.g. noradrenaline or acetylcholine) or potassium (Durbin and Jenkinson, 1961; Edman and Schild, 1962; Sparrow and Simmonds, 1965; Hiraoka et al., 1968; Hudgins and Weiss, 1968). Others have succeeded in using pharmacological tools for differentiating between the responses to the two classes of stimulants. In the rabbit aorta, Hudgins and Weiss (1968) observed that procaine (1 mM) inhibited the responses to noradrenaline more than those due to potassium. Likewise Kalsner et al. (1970) found that SKF 525-A inhibited potassium responses more than noradrenaline responses in the above muscle. These results suggest, that unlike potassium, drugs like acetylcholine and noradrenaline utilise a more tightly bound pool of calcium. Studies done by Waugh (1964), Evans et al. (1958), Somlyo and Somlyo (1968a) and many others on depolarised muscle show that noradrenaline and acetylcholine exert their action in a manner different from that of potassium (i.e. without changing membrane potential further).

Further support for the role of a tightly bound calcium pool in smooth muscle contraction came from the experiments of Daniel and Irwin (1965) and Hurwitz and co-workers (1967a,b). These workers, especially the latter, found that guinea-pig intestinal smooth muscles could be made to contract transiently if they were quickly exposed to a calcium-free medium containing a small amount of EDTA. The amplitude of this

calcium washout contraction could be increased by loading the tissues with 36 mM prior to contraction. Since the contraction was produced presumably in absence of extracellular calcium, the source of contractile calcium was assumed to be tightly bound. It was hypothesised that in the calcium-free medium the muscle lost a superficial pool of calcium that served to stabilise the membrane and prevent the entry of bound calcium into the cytoplasm during rest. Addition of 1.8 mM calcium at the height of the calcium washout contraction produced prompt, reversible relaxation. However, acetylcholine produced a further shortening if added during a calcium washout contraction. Addition of 1.8 mM calcium now, surprisingly, produced additional shortening rather than relaxation. The workers proposed that acetylcholine by increasing membrane permeability to calcium nullified the stabilising action of the latter. One could also propose that acetylcholine acted by decreasing the affinity of the stabilising site for calcium. Even though the above demonstration of contractile effect in a calcium-free medium is important, several aspects of the work and conclusions are open to criticism. These workers have not eliminated the role of severe fluctuations in osmotic pressure (adding and withdrawing 36 mM $CaCl_2$) in the producing of the contraction. Also, using muscles labelled with radiocalcium during the initial incubation with 36 mM $CaCl_2$, Hurwitz et al. (1967a) found that ^{45}Ca efflux from the muscle increased during the calcium washout contraction. This increased undirectional flux of calcium represented to the workers an increase in membrane permeability. Alternate explanations can be provided for this observation, based on more recent observations of Hudgins and Weiss (1969) and Krejci and Daniel (1970b). These investigators found that

loss of radiocalcium from smooth muscle into the medium was slower when calcium was absent from the external medium but increased when calcium was introduced. This was explained partly on the basis of exchange diffusion and partly on the ability of external calcium to compete with the immediate re-uptake of ^{45}Ca released from the muscle, thereby causing an accelerated loss. EDTA added to the calcium free external medium increased the loss of radiocalcium. It is possible that the increased ^{45}Ca efflux measured by Hurwitz and co-workers (1967a) was not entirely due to release from a bound pool but due to the chelating action of EDTA present in their calcium-free washout medium.

From the foregoing discussion it is clear that at least two types of calcium pools are involved in contraction - one loosely bound and the other tightly bound. Calculating diffusion time in the uterus, Daniel (1963) postulated that the loosely bound calcium was not the one present freely in the extracellular water but was probably bound weakly to some 'superficial' site in the membrane. The tightly bound calcium pool was also named 'sequestered' calcium (Daniel, 1965). In addition there is a third, superficial pool of calcium which does not contribute to cytoplasmic calcium during contraction but simply serves to stabilise the muscle membrane during rest.

iii) Models of inter-relation of calcium pools

Considerable speculations have been made regarding the functional inter-relationships of these calcium stores (pools) in spite of the absence of histological evidence as to their physical inter-relationships (Daniel, 1965; van Breemen et al., 1966a; Hurwitz, et al., 1967b; Goodford, 1965). Goodford (1965) accounts for ^{45}Ca flux studies

in the guinea-pig taenia coli by proposing the presence of several energy barriers between calcium in the extracellular space, membrane, cytoplasm and storage sites. He proposes that the downhill movement of extracellular calcium into the cytoplasm is prevented by low membrane permeability. On the other hand, calcium from the cytoplasm is again moved uphill to a storage site by an energy dependent process. Inhibition of energy production by cold, anoxia or metabolic inhibitors increases tissue calcium (Goodford, 1965; Kroeger, 1970). Hypercapnia, while producing an increased resting tension in the trachaelis muscle of the dog, decreases the effectiveness of an agonist like acetylcholine, which presumably acts on bound calcium stores (Kroeger, 1970). These results support Goodford's model and also additionally suggest that the storage site not only acts as a sink for removing calcium from the cytoplasm but also as a source of bound calcium for contraction. Finally, Goodford proposes that the efflux of calcium from the stores is a passive physicochemical process and occurs at a rate similar to the rate of dissolution of calcium phosphate microcrystals.

The three simplest conventional arrangements of the two calcium pools are i) series, ii) parallel and iii) series/parallel (Daniel, 1965).

According to the 'series model', calcium from the superficially located pool has to pass through the more deeply located pool before reaching the cytoplasm. Interference with the deeper pool would affect inward movement of calcium from the superficial pool and vice versa for the outward movement of calcium. In the 'parallel model' the two pools communicate with the outside or inside of the cells independently. The rate of calcium movement from these pools, however, may be different.

In the 'series/parallel' model a part of the calcium movement occurs in series and a part in parallel.

On superficial examination, the series model appears rather attractive, since in none of the results mentioned so far could one demonstrate the effect of potassium (acting on 'loosely bound' superficial calcium) in the absence of responsiveness to noradrenaline (acting on 'tightly bound' calcium). Furthermore, after thorough depletion of tissue calcium, muscles lose their responsiveness to the agent acting on loosely bound as well as to the agent acting on tightly bound calcium pools. On addition of calcium to the medium, the responsiveness to the agent acting on the loosely bound pool returns faster than to the agent acting on the tightly bound pool of calcium (Daniel, 1963). However, if one postulated that calcium movement to and from the loosely bound pool was faster than from the tightly bound pool, the above data could just as well be explained on the basis of the parallel model. Additional evidence obtained by Hinke (1965) supports the parallel model. In the rat tail artery, when the responses to potassium and noradrenaline are abolished by calcium removal, addition of barium restores preferentially the contractile effects of potassium. Somlyo and Somlyo (1968b) have suggested that there is no need for postulating the multiple store ('site') hypothesis for explaining the differences between potassium and other agonist induced contractions. They propose that an 'external barrier, containing high affinity sites for calcium, could concentrate the cation from the external medium and, if fully utilised, support maximal contractions in the presence of very small amounts of tissue calcium. The basement membrane or some less specialised condensation of ground sub-

stance probably has the required calcium-binding properties. The inner barrier to calcium in the above model would be the plasma membrane whose permeability to calcium and other solutes would be controlled by the membrane potential, by labilising or stabilising drugs, and by calcium itself.' One can readily recognise that this model is a modification of that of Hurwitz et al. (1967b) and is functionally analogous to Daniel's 'series' model. Such a model, as mentioned earlier, cannot account for the ability of barium to restore only potassium but not noradrenaline contractions in a calcium-free medium.

Kinetic data to support the multiple pool hypothesis have generally been controversial. van Breemen et al. (1966a) and Goodford (1965a,b) failed to resolve the ^{45}Ca efflux curve in rat uterus and guinea-pig taenia coli, respectively, into clear cut compartments releasing ^{45}Ca at different rates. Others, such as Hudgins and Weiss (1969), Lüllman and Seigfriedt (1968) found that the efflux curve could be resolved into several components. In none of these latter studies were the rates of desaturation of tissue (^{45}Ca) compared with the rate of ^{45}Ca loss into the medium for parallelism. This is necessary to satisfy the assumption that ^{45}Ca emerges from a homogeneously mixed compartment (Persoff, 1960). In spite of difficulties in attaching great quantitative significance to these studies one can still get some idea about the behaviour of the calcium pools. Lüllman and Siegfriedt analysed the ^{45}Ca efflux curves of longitudinal muscle strips of guinea-pig small intestine at different external concentrations of nonradioactive calcium. In the presence of 0.6 mM ca/l, Fraction 1 of the efflux curve, which represented 31% of the ^{45}Ca coming out of the muscle, had a half time of 1 min.

Fraction 2 (45%) and Fraction 3 (20%) had half times of 7 and 39 min. respectively. However, when the experiment was repeated in the presence of 2.7 mM Ca/l, the results were as follows: Fraction 1 (34%) 1 min; Fraction 2 (52%) 4 min; Fraction 3 (14%) 22 min.

Attempts to attribute different rates of ^{45}Ca washout solely on the presence of different functional pools involve much over-simplification. Undoubtedly, the variable distance of various muscle cells from the muscle surface, presence of heterogeneous histological formed elements such as connective tissue, and the presence of multiple organelles in the muscle cell itself can probably influence the ^{45}Ca washout and make interpretations difficult. The solution of this problem may come from simpler preparations of muscle cells devoid of extraneous elements and probably also from purified viable membrane preparations. Alternatively the demonstration of potassium-induced or drug-induced alteration of ^{45}Ca movements and preferential blockade with agents which interfere with mobilisation of loosely bound or tightly bound calcium may be more promising. Relatively few studies have been done in this direction and results so far have not been conclusive (Schatzmann, 1961; van Breemen and Daniel, 1966; Von Hattingberg et al., 1966; Lüllmann and Siegfriedt, 1968; Hudgins and Weiss, 1969).

iv) Mechanical correlates of utilisation of calcium from different pools

Several independent studies have shown that contractions of a great variety of smooth muscles, produced by drugs, consist of fast (phasic) and slow (tonic) components.

Brodie et al. (1959) observed that the isotonic contraction of rabbit aorta produced by adrenaline consisted of an initial fast component

followed by a further slow component. Subsequently Bohr (1963; 1964a,b) showed that the slow phase could be easily decreased by reducing external calcium concentration, while the fast component decreased only when the reduction in calcium concentration was drastic and prolonged. In fact the acute effect of calcium deprivation was a slight increase in the magnitude of the fast component. Conversely increasing the external calcium concentration produced a decrease in the fast component. Bohr proposed that the magnitude of fast component depends upon membrane excitability while the slow component is indicative of the state of the excitation-contraction coupling process. He also suggested that membrane excitation and excitation-contraction coupling constitute two consecutive processes in a single sequence of events leading to tension development. If the calcium available for coupling becomes extremely low, coupling will then become the rate limiting factor for the fast as well as the slow component. This is supported by the decrease of both fast and slow components when calcium depletion was prolonged. The above proposed series arrangement of the two processes cannot be supported by the observation that high external calcium decreased both fast and slow components because one certainly does not expect the coupling process to be rate limiting under these conditions. It seems that a more plausible explanation for the two phases is that noradrenaline mobilises both a loosely bound (superficial) as well as a tightly bound calcium pool. The former contributes to the slow component whereas the fast component is produced by tightly bound calcium.

Two components of contraction have also been seen in the rat aorta (Godfraind and Kaba, 1969) and these two components show the same

type of dependence on external calcium as the two phases in the rabbit aorta. Very little is known of the electrophysiological correlates of these two components of contraction or about the other factors which modify these components of contraction. A very unexpected observation of Brodie et al. (1959) is the ability of the non-equilibrium alpha adrenoceptor blocking agent Dibenamine to block preferentially the fast component of adrenaline. If one assumes a single type of alpha receptor for adrenaline, blockade of this receptor will be expected to affect both phases equally. Similarly the observation that small concentrations of adrenaline activate mainly the fast components while higher concentrations produce both fast and slow components is difficult to explain on the basis of a single receptor. These authors therefore found it necessary to propose separate alpha adrenoceptors for fast and slow components.

Multiple components of contraction have also been observed in several visceral smooth muscles. Imai and Takeda (1967a,b), discovered that the guinea-pig taenia coli responded to potassium by a phasic (quick) component followed by a tonic (slow) component. Unlike the contractions in the aorta, the phasic response was more sensitive than the tonic component to a decrease in external calcium concentration. The phasic component was also preferentially decreased by manganese, cadmium and aminophylline while the tonic component was inhibited by papaverine. With the help of the sucrose gap technique, the phasic component was found to be associated with spikes in the membrane potential while the tonic component was associated with steady maintained depolarisation without any spike activity. Results similar to these have also been obtained by West et al. (1951) for responses to acetylcholine in the

rabbit intestine. These workers showed that the tonic component was inhibited by anoxia, cyanide or sodium fluoroacetate. Similar effects of anoxia, glucose lack, lithium substitution for sodium, ouabain and papaverine, on the tonic component of potassium was subsequently shown in the guinea-pig taenia coli by Pfaffman et al. (1965), Ferrari and Carpenedo (1968) and Urakawa et al. (1970). Very few studies have been made to correlate two components of contraction in smooth muscle with calcium fluxes. Thus, Urakawa and Holland (1964) found that both phasic and tonic components of contraction due to hypertonic KCl were associated with an increased ^{45}Ca influx and the tonic phase was accompanied in addition by an increase in total tissue calcium. These results suggest that the tonic phase depends more upon extracellular calcium and thus contradict the results of Imai and Takeda (1967a). However, Imai and Takeda employed isotonic KCl solution (KCl substituting for NaCl), while Urakawa and Holland employed KCl in a hypertonic solution (KCl added to usual concentration of NaCl). Whether this may account for the difference is not known. Also not known is why the calcium dependence of the different components of contraction of visceral smooth muscle differ from the calcium dependence of components in vascular smooth muscle.

SUMMARY

The theme of the foregoing review has been the central role played by calcium in the activation of smooth muscle contractile proteins. Evidence has been provided that smooth muscle stimulants mobilise calcium from several different functional pools either by altering the electrical gradient across the muscle membrane or by a non-electrical 'pharmaco-

mechanical' process. Smooth muscle relaxants, likewise, may decrease mobilisation of calcium by electrical or non-electrical means. Several models for the arrangement of the various calcium pools have been proposed and the series/parallel arrangement appears to be currently acceptable. There is some evidence that mechanical manifestation of activation of contractile protein can be resolved into several components and these in turn can be related to the different mechanisms or functional pools of calcium that eventually contribute to muscle contraction.

STATEMENT OF PROBLEM

It is generally agreed that calcium is the most important factor in coupling excitation of smooth muscle membrane to its eventual contraction. Multiple pools (sources) of calcium have been proposed and as a result of several lines of evidence it appears that different types of stimuli utilise different pools of calcium for initiating contraction. Several ions, drugs and procedures alter the responsiveness of smooth muscle to stimulants. It is conceivable that many of these may be due to changes in the mode of utilisation of the various calcium pools. The purpose of this study is to develop a method for assessing the contribution of any particular calcium pool to contraction by analysing the mechanical activity of the muscle. The cat splenic capsular smooth muscle was found suitable for this purpose.

The isometric contractile response of the capsular smooth muscle to noradrenaline consists of an initial rapid phase which is followed by a further slow increase in tension. These two phases have been found to rely on a tightly and a loosely bound pool of calcium respectively. With this system an attempt was made to study the effect of a variety of agents which inhibit noradrenaline responses. These included an alphaadrenoceptor blocking agent as well as several others which are better known as nonspecific smooth muscle relaxants.

It was hoped that some agents might have differential effects on the two phases of contraction which in turn would suggest differential effects on the two calcium pools.

Radioactive calcium movement across the resting or stimulated

splenic capsular muscle was also studied to attempt to characterise the various pools of calcium.

The effect of pre-existing membrane potential on the contractile response to stimulating agents was studied by employing procedures that depolarise or hyperpolarise muscles.

Many agents or procedures are known to cause supersensitivity of smooth muscle to agonists. These, in the case of sympathomimetic agents, include cocaine, reserpine and denervation of sympathetic nerves. From the recent findings of several workers the phenomenon of supersensitivity appears to be mediated at least partly by alterations in the mode of utilisation of calcium. Hence the effects of various drugs on the two phases of contraction were studied to elucidate the mechanism of supersensitivity.

The investigation is mainly directed towards the development of a procedure for studying utilisation of different calcium pools by analysis of a single isometric contraction of the capsular smooth muscle and to the use of this method to study the interrelationships of the different calcium pools and the effect of drugs which alter muscle reactivity on these pools.

The cat spleen was chosen for this study because the two phases of contraction due to noradrenaline are very distinct. The preparation is quiescent. A considerable amount of work has previously been done in our laboratory on this tissue, which responds to a variety of agonists such as catecholamines, acetylcholine, histamine, angiotensin and 5-hydroxytryptamine. Previous work has also characterised the effect of a variety of procedures which interfere with the mechanism of storage

and release of noradrenaline in the sympathetic nerve endings of the spleen and cause supersensitivity (Davidson, 1970; Mailhot, 1970). Preliminary work showed that it was easy to prepare a slice of the spleen containing mainly the capsular smooth muscle.

METHODS

I. PREPARATION OF THE CAT ISOLATED SPLEEN STRIP

Cats (1.5 - 2 kg) of either sex were stunned by a blow on the head and bled to death by cutting the heart. The spleen was quickly removed and placed in Krebs-Henseleit solution at room temperature (23°C). A thin (0.5 mm) slice was cut from the surface opposite the hilum with a Stadie-Rigg microtome. Strips (15 mm x 2 mm) were cut from this slice in the longitudinal, oblique or transverse directions with a device made of five razor blades mounted parallel to one another 2 mm apart. In some initial experiments the conventional preparations obtained by cutting along the edges of the spleen were utilised for comparison.

Histological sections showed that the slice included mainly the capsular smooth muscle and contained much less pulp and blood vessels than the thicker preparations obtained by cutting along the edges of the spleen. The main reason for using a thinner preparation was to reduce the problems of diffusion of drugs and ions and of heterogeneity of muscular and other tissue elements.

The spleen strips were mounted in individual 10 ml jacketed organ baths, bathed in Krebs-Henseleit solution (pH 7.4; temperature 37°C) and equilibrated with 95% O_2:5% CO_2. Changes in isometric tension were recorded with a Grass FT-03D force displacement transducer on a Grass Polygraph. The preparations were initially stretched to exert a steady resting tension of 1 g. Before the experiment began the tissues were stimulated with noradrenaline, usually at intervals of 15 min until the responses became stable. Further procedures will be described in the 'RESULTS' section.

II. BATHING SOLUTIONS

The following bathing solutions were made with glass distilled water.

a) Krebs-Henseleit solution: NaCl 118 mM; KCl 4.7 mM; $CaCl_2$ 2.5 mM; KH_2PO_4 1.4 mM; $MgSO_4$ 1.2 mM; $NaHCO_3$ 25 mM and glucose 11 mM. In the preparation of this solution calcium chloride and magnesium sulphate were added only after all the other ingredients had been dissolved and the solution equilibrated with the O_2-CO_2 mixture for fifteen minutes.

b) Modified Krebs-Henseleit solutions

i) Calcium-free Krebs-Henseleit solution

The composition was similar to that of Krebs-Henseleit solution with the omission of calcium chloride. Solutions to contain various low concentrations of calcium were obtained by addition of appropriate volumes of a concentrated stock solution of calcium chloride to the calcium-free solution. No substitution was made to compensate for the decrease in osmolarity of the bathing medium caused by reduction of the amount of calcium chloride. In several experiments the free calcium concentration was reduced by adding the chelator ethylene glycol bis (2-aminoethylether) tetraacetic acid (EGTA) to the standard Krebs-Henseleit solution. Specific details will be provided in the 'RESULTS' section.

ii) Potassium-free Krebs-Henseleit solution

This was made by omission of potassium chloride from the Krebs-Henseleit solution and substitution of equimolar

concentrations of sodium dihydrogen phosphate for potassium dihydrogen phosphate.

iii) Low chloride solution

All the ingredients were similar to those used for making Krebs-Henseleit solution with the exception of sodium chloride which was replaced by an equimolar concentration sodium isethionate. The solution still contained 7.2 mM of chloride.

iv) High potassium depolarising solutions

These were prepared by substituting different amounts of potassium bicarbonate and potassium chloride for equimolar concentrations of sodium bicarbonate and sodium chloride.

III. EXPERIMENTAL PROCEDURES

a) Treatment with reserpine

Noradrenaline stores in spleen were depleted by intraperitoneal injection of 1 mg/kg reserpine twenty four hours prior to experiments. Tissues were tested for depletion of noradrenaline stores by chemical assay using the method of Bertler et al. (1958) and Euler and Lishajko (1959) and by the failure to elicit contraction with tyramine (33×10^{-5} g/ml) at the beginning of the experiment.

b) Sympathetic denervation with 6-hydroxydopamine

This was done according to the method recommended by Mailhot (1970). A dose of 35 mg/kg was administered intravenously. The drug was dissolved immediately before use in 0.5 ml of 0.1 N hydrochloric acid. The spleen was taken out

for experiments twenty four hours later. This procedure is known to produce an impairment of noradrenaline uptake and a specific supersensitivity to noradrenaline and adrenaline but not to isoprenaline or histamine (Mailhot, 1970).

c) Ionic analyses

i) Estimation of inulin space

Inulin was used as an extracellular marker since it is generally believed not to penetrate the cell. In each determination of the effect of a treatment on the ionic content of spleen slices, inulin-methoxy-^{3}H was added to the incubating medium thirty minutes before the slices were removed for analysis. When the incubation period was over some of the slices were dipped in non-radioactive incubation medium for four seconds, blotted gently, weighed on a torsion balance, homogenised in 5% trichloracetic acid (TCA) and centrifuged for ten minutes at 3000 rpm. Aliquots (0.3 ml) from the supernate were added to vials containing 10 ml of scintillation fluid and the samples were counted in a Phillips liquid scintillation spectrometer. The radioactive incubation medium was diluted 50 fold and TCA in amounts similar to that existing in the samples from the tissue, was added to this. An aliquot (0.3 ml) from this was mixed with 10 ml of scintillation fluid and counted.

Another set of tissues was identically tested. Wet weight was determined after gentle blotting and dry weight after dessication in vacuum at 80°C for twelve hours.

Inulin space (fraction of total tissue water) was determined as follows:

$$\text{Inulin space} = \frac{a\left[2 + (d-e)\right]}{(b)\ (c)\ (d-e)}$$

where a = counts/min in aliquot from homogenate

b = counts/min in aliquot from medium

c = dilution of medium

d = wet weight of tissue

e = dry weight of tissue

ii) Determination of intracellular sodium and potassium content

After appropriate pre-incubation, the spleen slices were removed from the bathing media, blotted gently, weighed and put into polyethylene tubes containing concentrated nitric acid (0.2 ml) and deionised distilled water (0.2 ml). The ions were extracted for two hours at 37°C. Additional distilled water was added to make up a volume of 3.0 ml and extraction continued for another eighteen hours. The aqueous extracts of ions were analysed for sodium and potassium in a Perkin Elmer Atomic Absorption Spectrophotometer. An amount of nitric acid was added to the sodium and potassium standard solutions to equal the concentration of the acid in tissue extracts. The potassium standards in addition to containing potassium chloride and nitric acid also contained 0.15 M of sodium chloride.

Concentrations of the ions were expressed in terms of mEq/l intracellular water and were calculated by correcting the total tissue ion concentration (mEq/kg wet weight) for the sodium and potassium concentration in the extracellular (inulin

space). The concentration of ions in the extracellular space was considered to be the same as that in the bathing medium.

Concentration of ion (mEq/l) intracellular water) =

$$\frac{A - B \times C}{1 - [(\text{dry weight/wet weight}) + B]}$$

Where A = total tissue ion concentration (mEq/kg wet weight)

B = extracellular space (fraction of total tissue water)

C = concentration of ion (mEq/l) in external medium

iii) Determination of total tissue calcium

Tissue samples after appropriate treatments were blotted gently and put in weighed platinum crucibles. After being weighed again, the crucibles were put in a vacuum dessicator at 80°C for twelve hours, reweighed and finally ashed at 600°C for twelve hours in a muffle furnace. This left a white powdery residue which was then dissolved in 1.0 ml of a solution of 1% lanthanum chloride in 5% hydrochloric acid. Lanthanum chloride was used to prevent interference by tissue phosphates in the calcium determination. The tissue calcium dissolved in the lanthanum-hydrochloric acid solution was estimated by atomic absorption spectrophotometry. The instrument was initially adjusted to indicate a null reading with a blank solution (1% lanthanum chloride in 5% hydrochloric acid). Standard solutions of calcium chloride were prepared in the lanthanum chloride-hydrochloric acid solution. Tissue concentrations of calcium were expressed in mM/kg wet weight.

d) Tracer radioactive calcium efflux

Spleen slices were tied to thin glass rods with terylene threads and preincubated in Krebs-Henseleit solution at 37°C for 1 hour. In some experiments they were exposed to 20 ml of calcium-free Krebs-Henseleit solution containing ethylene glycol bis (2-amino-ethylether) tetraacetic acid (EGTA) (6 mM) for 15 min, followed by incubation in 20 ml of calcium-free Krebs-Henseleit solution for 15 min. Subsequently the slices were placed for 60 min in 5 ml of calcium-free Krebs-Henseleit solution containing 2 μCi of $^{45}CaCl_2$. The total radioactive calcium concentration of this medium was 2.7 μg/ml. Loading under the above condition (low total external calcium concentration) ensured a large uptake of ^{45}Ca. The tissues were then dipped successively for one second into each of four test tubes containing Ca-free Krebs-Henseleit solution in order to remove radioactivity from the muscle surface. The tissues were then passed through a series of tubes containing normal Krebs-Henseleit solution or calcium-free Krebs-Henseleit solution (2 ml) to allow efflux of ^{45}Ca. The incubation time in each tube was usually 5 min but varied from 15 sec to 30 min in some experiments. The agents whose effect on ^{45}Ca had to be studied were added to appropriate tubes and a similar volume of calcium-free or normal Krebs-Henseleit solution (depending on the particular case) was added to the remaining tubes. After passage through the entire series of tubes in any given experiment, the tissues were quickly removed from the glass rods,

blotted gently and ashed at 600°C. The ash was dissolved in 1% lanthanum chloride in 5% hydrochloric acid (2 ml). Aliquots (0.3 ml) from the dissolved ash as well as the media in the efflux tubes were added to 10 ml of scintillation fluid and counted in a Phillips Liquid Scintillation spectrometer for 4 minutes. The tissue counts, after correcting for wet weight were added to the cumulative sum of all counts in the efflux media to determine the total tissue radioactivity at the inception of washout. The efflux was depicted either as the residual radioactivity of the tissue, plotted on a semi-log scale as a function of time (desaturation curve) or plotted as the rate of ^{45}Ca emergence into the efflux medium as a function of time. Further details of these procedures will be described at appropriate places in the 'RESULTS' section. An eye line of best fit was applied to the data points and the curve analysed for its component exponentials by the graphical method of Riggs (1963).

e) Tracer radioactive calcium influx

Spleen strips 2 cm x 0.3 cm x 0.05 cm were tied with terylene thread to hooks placed 2.5 cm apart on thin glass rods. The strips were then equilibrated in Krebs-Henseleit solution (gassed with O_2-CO_2 mixture) at 37°C for one hour. During this period they were challenged twice with noradrenaline (10^{-6} g/ml). After the preincubation period of one hour pairs of tissues were subjected to one of two procedures:

i) One strip of the pair was incubated in Krebs-

Henseleit solution containing 5×10^{-6} g/ml of noradrenaline for 1 minute and then transferred to a beaker containing 10^{-6} g/ml noradrenaline and 4 μCi $^{45}CaCl_2$ in Krebs-Henseleit solution for 1 minute. The other tissue was kept only in Krebs-Henseleit solution containing 4 μCi $^{45}CaCl_2$ for 4 minutes.

ii) One muscle of the pair was incubated in Krebs-Henseleit solution containing 4 μCi $^{45}CaCl_2$ for 1 minute and then kept in the same solution with noradrenaline added to make a concentration of 5×10^{-6} g/ml for 3 minutes. The other muscle of the pair was kept in Krebs-Henseleit solution containing 4 μCi $^{45}CaCl_2$ for 3 minutes.

After the necessary incubations, the muscles were cut away from the threads which secured them to the rods. After gentle blotting the muscles were transferred to weighed platinum crucibles, weighed again, dessicated in vacuum at 80°C for twelve hours and ashed for another twelve hours at 600°C. Ashed samples were dissolved in 1 ml of a 1% solution of lanthanum chloride in 5% hydrochloric acid.

Aliquots (0.3 ml) obtained from the solution of the ashed tissue as well as from a 50 fold diluted radioactive incubation medium were counted by the liquid scintillation technique after mixing with 10 ml of the scintillating fluid. The radioactivity/gm tissue wet weight was obtained by multiplying counts/min by 3.3 and dividing the product by the tissue wet weight (g). Similarly the counts/min/ml of the incubation

medium was also calculated. ^{45}Ca space in the tissue was obtained by dividing the tissue counts/min/gm by the medium counts/min/ml.

f) Procedure for increasing intracellular concentration of sodium of spleen strips

Spleen strips were incubated in Krebs-Henseleit solution for one hour at 37°C and then in four changes of potassium-free solution (10 ml/strip). The strips stayed for a total of two hours in the potassium-free solution at 37°C before being finally placed in 400 ml of potassium-free solution at 4°C for twenty four hours. Subsequently the strips were utilised for measurement of tension or ionic changes produced by several different treatments that will be described in greater detail in the 'RESULTS' section.

g) Histology of spleen strips

i) Light microscopy

Spleen strips cut along the longitudinal axis of the spleen were tied to glass rods and then incubated in Krebs-Henseleit solution for 30 min. The strips were fixed in a 10% solution of formaldehyde in normal saline for 7 days, embedded in paraffin and sectioned for light microscopy. Sections were stained with haematoxylin-eosin or Verhoff's connective tissue stain before examination.

ii) Electron microscopy

After incubation in Krebs-Henseleit solution for 30 min the strips were initially fixed in 2% glutaraldehyde in

Millonig's phosphate buffer (pH 7.1) for 30 min at 4°C, washed with the buffer for one hour, post-fixed with osmic acid and stained with 2% uranyl acetate. The tissues were embedded in Epon and the ultra thin sections cut from the blocks were stained with Reynold's lead citrate before electron microscopic examination.

IV. DRUGS AND OTHER EXPERIMENTAL MATERIALS

a) Scintillation fluid consisted of diphenyloxazole (PPO) 11.6 g; p-bis-2 (5-phenyloxazolyl) benzene (POPOP) 0.22 g; toluene 2 l and ethyleneglycol monomethyl ether 1 l.

b) Method of preparation of solutions of drugs. Stock solutions of l-noradrenaline bitartrate, l-isoprenaline bitartrate, phenylephrine hydrochloride and histamine diphosphate were prepared in 0.1 N HCl and frozen. Dilutions were freshly made in 0.1N HCl. The stock solutions were made in concentrations of 1 and 10 mg free base/ml.

Phenoxybenzamine hydrochloride (1 mg/ml) was prepared in propylene glycol acidified with 0.05 ml of 12 N HCl per 10 ml of solution. Appropriate dilutions were prepared with distilled water immediately before use.

Solution of reserpine (5 mg/ml) was made by dissolving reserpine (50 mg) in propylene glycol (5 ml), glacial acetic acid (2.5 ml) and ethanol (2.5 ml).

6-hydroxydopamine was dissolved in 0.1 N HCl immediately before use.

All other drugs and inorganic substances were

dissolved in distilled water and the pH brought as close to 7.3 as possible by titrating with NaOH.

Stock solutions of organic substances except EGTA were stored in the frozen state while the EGTA and the inorganic substances were stored at room temperature.

c) List of drugs and chemicals used

aminophyllin (B.D.H.)

barium chloride (B.D.H.)

$^{45}CaCl_2$ (Amersham/Searle)

calcium chloride (Fisher)

chlorpromazine (May and Baker)

clonidine hydrochloride (Boehringer Ingelheim)

cocaine hydrochloride (B.D.H.)

diazoxide (Schering)

ethylene glycol bis (beta-aminoethyl ether) N, N-tetraacetic acid (EGTA) (Fisher)

histamine diphosphate (Nutr. Biochem. Co.)

inulin-methoxy-^{3}H (New England Nuclear)

l-isoprenaline bitartrate dihydrate (Sterling Winthrop)

lanthanum chloride (Fisher)

lithium chloride (B.D.H.)

manganese chloride (Fisher)

l-noradrenaline bitartrate (Calbiochem)

ouabain (Nutr. Biochem. Co.)

papaverine (S.K. and F.)

phenoxybenzamine hydrochloride (S.K. and F.)

l-phenylephrine hydrochloride (Sterling Winthrop)

procaine hydrochloride (B.D.H.)

sodium cyanide (B.D.H.)

sodium isethionate (Eastman Kodak)

sodium pyruvate (Fisher)

sucrose (Fisher)

sucrose-^{14}C (New England Nuclear)

zinc chloride (Fisher)

V. STATISTICAL EVALUATION OF RESULTS

The effect of any treatment or procedure on a response was compared with control values by the Student's t test. Comparisons involving more than two data points were made using the Duncan's multiple range test. (new).

RESULTS

I. Effect of noradrenaline, isoprenaline, histamine and potassium chloride on the capsular smooth muscle of the cat spleen

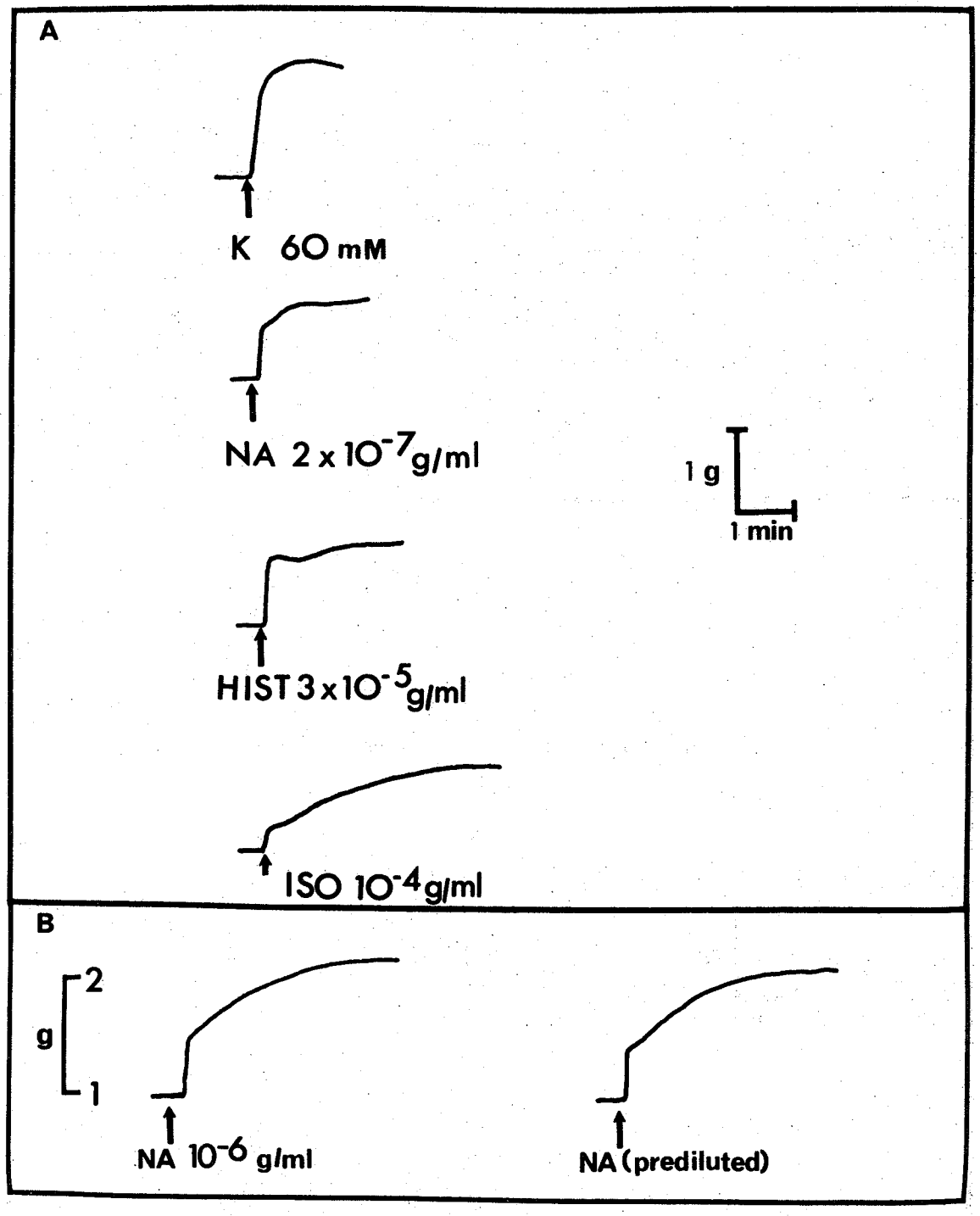
Strips cut along the longitudinal axis of the spleen were incubated in Krebs-Henseleit solution at 37°C. For studying the responses to noradrenaline, isoprenaline and histamine, the spleens were obtained from normal cats. The response to high concentrations of potassium chloride were studied in spleens depleted of catecholamines (< 0.05 g/gm) by pretreatment of the cats with reserpine. This was done to avoid the complication of release of endogenous catecholamines by potassium which would have interfered with the direct depolarising effect of potassium on the muscle. Strips depleted of catecholamines failed to respond to tyramine, 10^{-5} g/ml. This was tested routinely in the beginning of every experiment involving a reserpine-treated strip. The results are shown in Fig. 1A. Equivalent responses to all three amines consisted of an initial fast increase in tension followed by a second slower increase. These will subsequently be referred to as the 'fast' and 'slow' phases of contraction. The effect of potassium was studied by replacing the standard Krebs-Henseleit solution with one in which 90 mM potassium chloride was present and an appropriate amount of sodium chloride removed. The contractile response to this procedure consisted of a single phase. An unexpected observation was that isoprenaline which acts on the same alpha adrenergic receptors for producing contraction as noradrenaline (Davidson, 1970) produced a much smaller fast phase compared to that in an equivalent contraction produced by noradrenaline.

To investigate whether the two phases of contraction produced by the drugs could be due to uneven mixing in the bathing medium, spleen

Fig. 1. Variation in the ratio of slow to fast phases of contraction with different agonists. Lack of effect of preliminary dilution of the agonist.

A. Contractions due to noradrenaline (NA), histamine (HIST) and isoprenaline (ISO) of spleen capsular smooth muscle from normal cats, and to KCl (K) in spleen capsular smooth muscle from cats treated with reserpine, 1 mg/kg, 24 hours beforehand.

B. Responses to noradrenaline, 10^{-6} g/ml, added as a concentrated solution for the first contraction, and added as a premixed bathing solution after emptying the bath for the second contraction.


A
K 60 mM
NA 2 x 10⁻⁷ g/ml
1 g
1 min
HIST 3 x 10⁻⁵ g/ml
ISO 10⁻⁴ g/ml
B
2
g
1
NA 10⁻⁶ g/ml
NA (prediluted)

strips were challenged with noradrenaline in two different ways (Fig. 1B). A response was obtained with concentrated noradrenaline solution added to the bath to produce a concentration of 10^{-6} g/ml. The drug was washed out and 15 min later the bathing medium was replaced with a 10^{-6} g/ml solution of noradrenaline in Krebs-Henseleit medium gassed with O_2-CO_2 mixture and kept at 37°C in a water bath. The prior dilution of noradrenaline was made to eliminate the delay in attainment of uniform mixing when concentrated drug solutions are added directly to the bath. As can be seen in Fig. 1B the premixed solution of noradrenaline also produced two phases. The two phases of contraction are not artefacts of mixing.

Little is known about the orientation of smooth muscle fibres in the spleen capsule. It is conceivable that the two phases may arise due to muscle fibres being arranged in different directions. If this is true then marked differences in the relative proportions of the two phases should be obtained from strips cut at different angles to the longitudinal axis. To test this spleen strips were obtained by cutting the thin slice from the surface of the spleen either longitudinally, transversely or diagonally. The strips were cut 1.5 cm long and were subjected to a tension of 1 g before stimulation (Fig. 2). Noradrenaline (10^{-6} g/ml) produced nearly similar proportions of the two phases in the preparations cut in three different ways. However, the strip that was cut longitudinally developed the greatest amount of tension. This indicates that a majority of the muscle cells are arranged parallel to the longitudinal axis of the spleen. Histological sections of the capsule show that this is true in most areas examined. It was also noted that the capsular muscle cells were 4-5 layers deep and were surrounded by

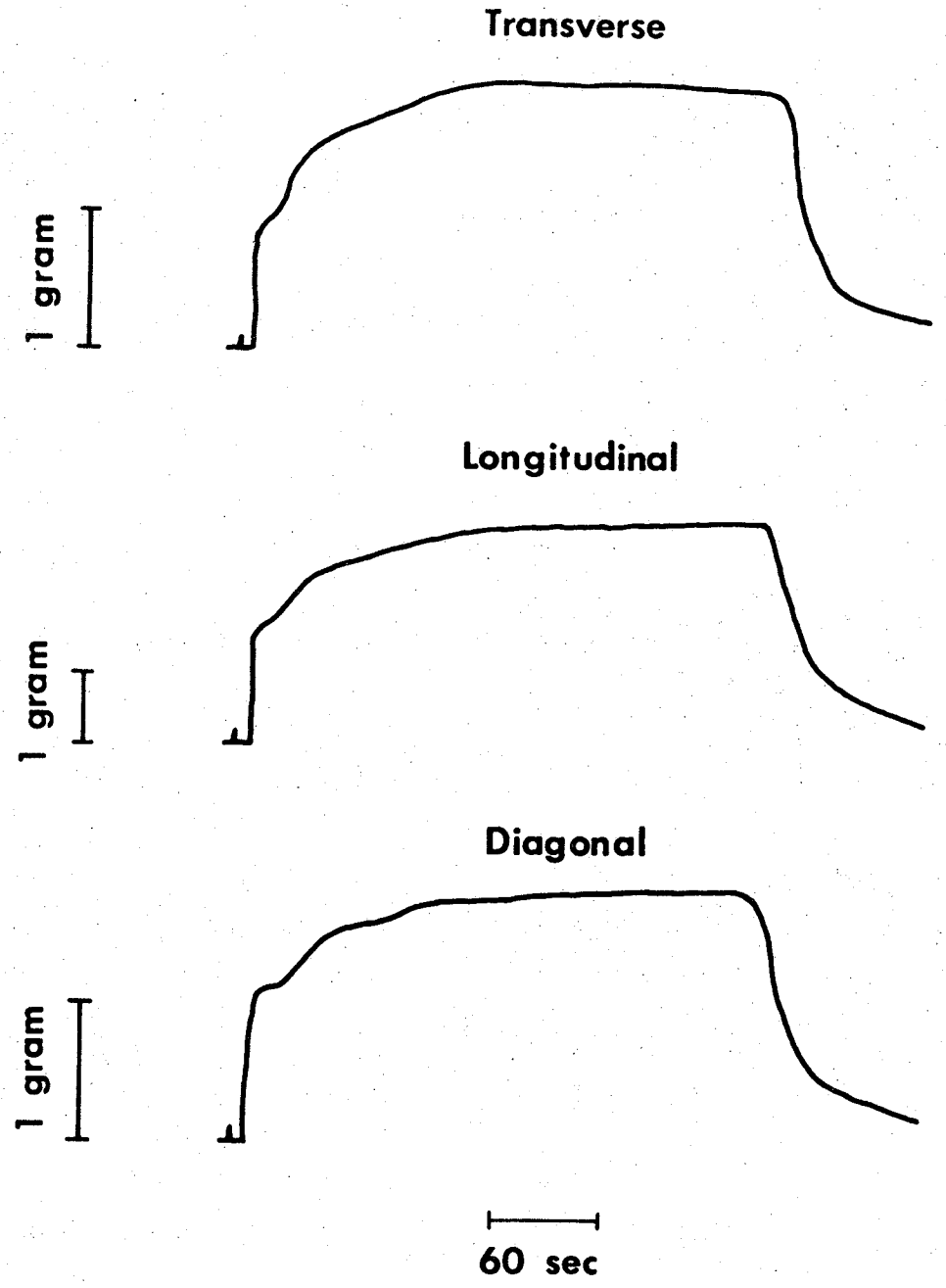


Fig. 2. Effect of different orientation of spleen strips on the two phases of contraction due to noradrenaline, 10^{-6} g/ml.

connective tissue. In all future experiments, the longitudinal strips were employed.

II. Effect of various resting tensions on the response to noradrenaline

Since muscles possess an optimum length at which contraction is largest, preliminary experiments were done to find the effect of various degrees of stretch on the resting tension, length of the preparation and active tension produced in response to a constant submaximal concentration of noradrenaline (10^{-6} g/ml).

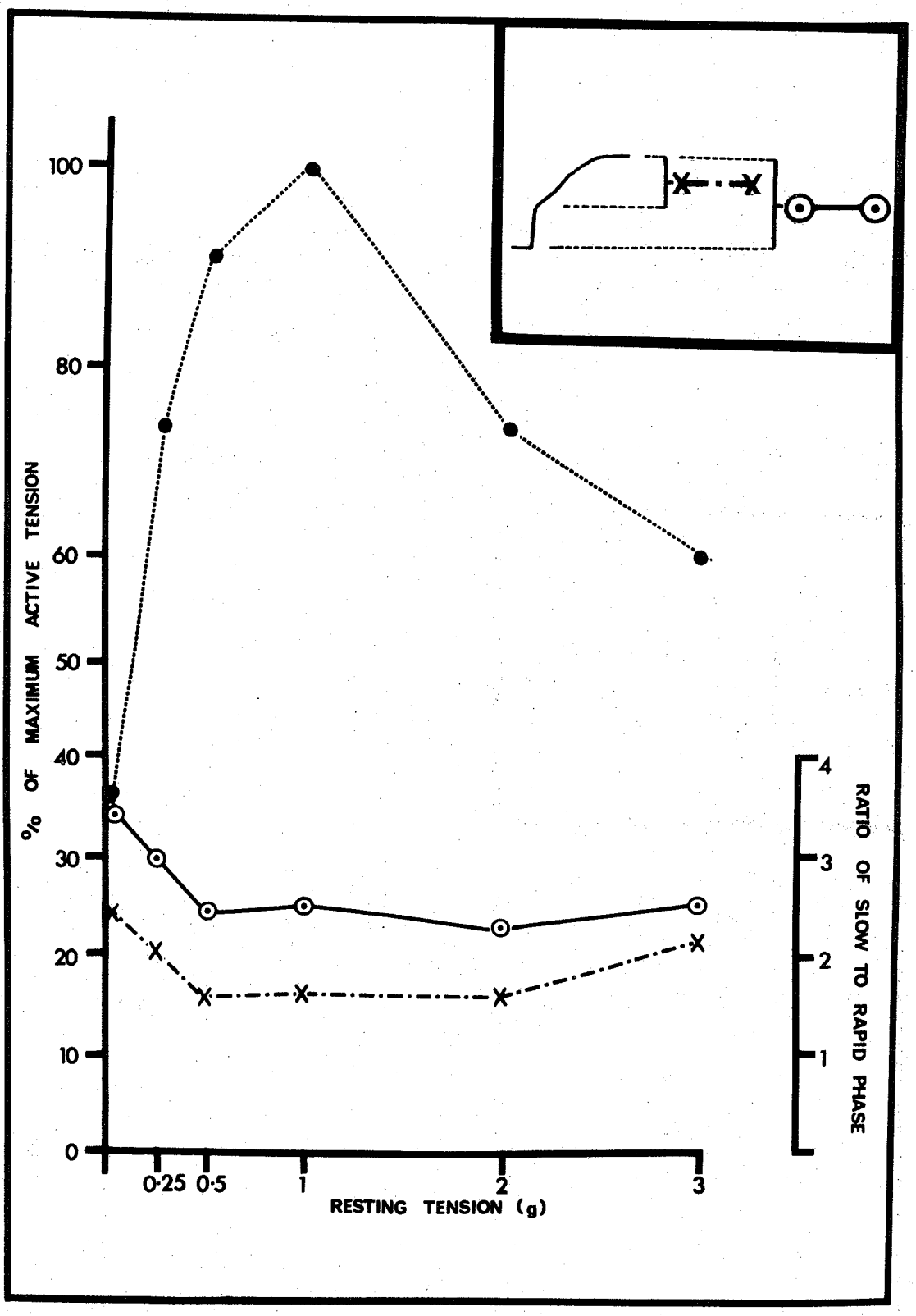
Eight spleen strips were initially stretched to exert a tension of 1 g and repeatedly stimulated with noradrenaline until reproducible responses were obtained. The stretch on the muscles was then completely withdrawn by lowering the transducers. After 10 min the transducers were raised until the muscles were under a tension of no more than 20 mg; 5 min later the muscles were stimulated with noradrenaline and the magnitude of fast and slow phases observed. The slow phase was measured in two different ways. In one method the slow phase was measured from the point at which the initial rapid increase in tension slowed down (resulting in a change in slope of the curve) to the point of maximum tension. In the other method the slow phase was measured from the beginning of the contraction to the point of maximum tension. These two methods were used provisionally. From subsequent analysis of the two phases by techniques designed to dissociate them (to be presented later in this section) it was decided to retain the second method of measurement of slow phase.

After two contractions were elicited 15 min apart, with the muscles bearing a minimum resting tension, the transducer was moved further away until the muscles exerted a steady tension of 0.25 g. Again

two tests were made with noradrenaline. In a similar manner, responses were tested at 0.5, 1, 2 and 3 g initial tensions. The results are shown in Fig. 3. The total active tension produced by noradrenaline increased steadily to a maximum when the resting tension was increased from the minimum level to 1 g. Further increase in resting tension caused a decrease in active tension. Although not shown in this figure, the resting length of the muscles when minimum tension was exerted increased by 40.3% when the tension was increased to 1 g. Increase in tension to 2 and 3 g caused proportionately smaller increases in length of 45.0% and 47.4% of the minimally stretched length respectively. When the changes in active tension and ratios of the slow to fast phases as a function of resting tension were compared, the change in active tension was greater than the change in the ratio of slow to fast phase, irrespective of how the slow phase was measured. For example, at minimum resting tension the mean active tension due to noradrenaline was only 35.5% of the maximum increase in active tension observed at a resting tension of 1 g. After the resting tension was increased from the minimal level to 0.25 g the contraction induced by noradrenaline was 75% of the maximum. This response was significantly ($p < 0.01$) larger than the previous one. The ratio of slow to fast phase decreased from a mean value of 3.48 to 3.05 when the slow phase was measured as the total increase in tension above the resting tension. This was not significant at the 0.05 level. However, since the terylene thread that was used for attaching the muscle to the transducer was far from being an ideal non-compliant system necessary for such studies, the responses at minimal resting tensions were not given any importance. Further increase in resting tension from

Fig. 3. Effect of resting tension on the active tension produced by noradrenaline (10^{-6} g/ml).

(●) active tension; (⊙) ratio of slow to fast phase with the slow phase considered equal to the total active tension; (X) ratio of slow to fast phase with the slow phase was measured as the difference between the total active tension and fast phase (see inset).


100
80
60
50
40
30
20
10
0
% OF MAXIMUM ACTIVE TENSION
0·25
0·5
1
2
3
RESTING TENSION (g)
4
3
2
1
RATIO OF SLOW TO RAPID PHASE

0.25 g to 0.5 g produced a response that was 90.7% of the maximum. This response also was significantly larger ($p<0.01$) than the previous response. However, the change in ratio of the two phases from 3.05 to 2.49 was also not found to be statistically significant. At 1 g resting tension the active tension was maximum but was not larger than the previous response in a statistically significant manner. Further increase in resting tension to 2 g produced a statistically significant ($p<0.01$) decrease in the magnitude of active tension without producing a significant change in the ratio of the two phases (2.56 to 2.26). A further significant decrease in tension occurred when the resting tension was increased to 3 g. The ratio slightly increased from 2.26 to 2.51 but was not significantly different. Essentially similar results were obtained when the slow phase was measured as the difference between total active tension and fast phase (Fig. 3).

These results show that between 0.5 g and 3 g the changes in active tension due to changes in initial resting tension affect both phases in a nearly similar fashion. Since the change in active tension resulting from increased resting tension is due to the change in the position of the actin and myosin filaments in relation to each other (Huxley and Peachey, 1961; Gordon et al., 1966) the lack of change of the relative magnitude of fast and slow phases suggests that the mechanisms involved in their production are earlier than the step in which activation of contractile proteins occurs. A resting tension of 1 g was selected for subsequent experiments as this appeared to be optimal for development of active tension.

III. The role of calcium in the two phases of contraction due to noradrenaline

As it is well known that smooth muscle contraction depends upon the availability of calcium to the contractile proteins and several lines of evidence suggest that there may be multiple sources for this 'activator' calcium, the effect of altering external calcium on the two phases of noradrenaline contraction were studied. A total of 8 spleen strips were obtained from 4 cats and incubated in Krebs-Henseleit solution. The muscles were repeatedly challenged at 15 min intervals with noradrenaline (10^{-6} g/ml) and isometric tension changes were recorded. After the responses of the muscle to noradrenaline became constant, the calcium content of the Krebs-Henseleit bathing solution was reduced to 2 mM for 10 min and the muscles tested again with noradrenaline. In a similar stepwise fashion the calcium concentration of the medium was reduced to 1, 0.8, 0.4, 0.2 and 0.1 mM and response to noradrenaline obtained at each concentration. Finally the muscles were incubated in a calcium-free medium for 2 hr. The effect of these procedures on the two phases are shown in Fig. 4. The slow phase was measured as the total increase in tension from the baseline. Reduction of external calcium from 2.5 mM to 0.8 mM had no effect on the fast phase of contraction. Reduction to 0.4 mM decreased the fast phase by 7.5% which was not statistically significant ($p < 0.05$) (Fig. 5). Further decrease in calcium concentration to 0.2 and 0.1 mM reduced the fast phase by 10.6% and 19.2% respectively. Both these changes were statistically significant at the 0.05 level. Compared to the fast phase, the slow phase was much more sensitive to variations in the extracellular calcium concentration. Reduction of the

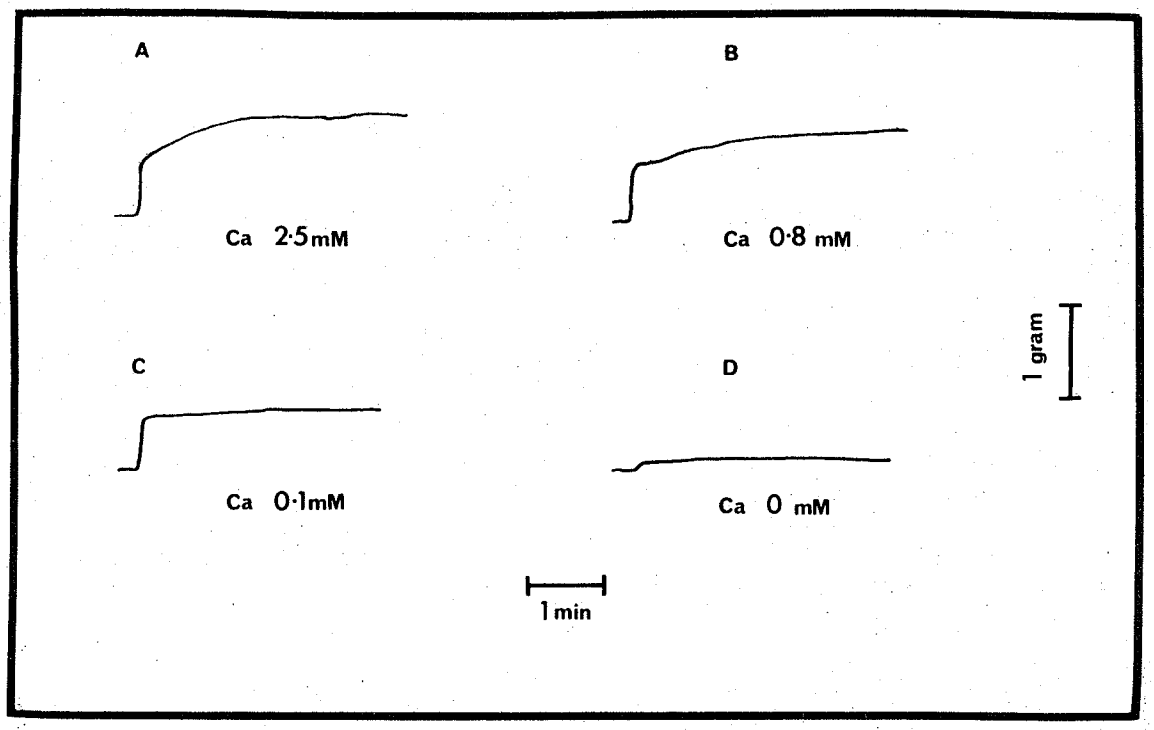


Fig. 4. Effect of reduction of external calcium concentration on the two phases of contraction due to noradrenaline (10^{-6} g/ml).

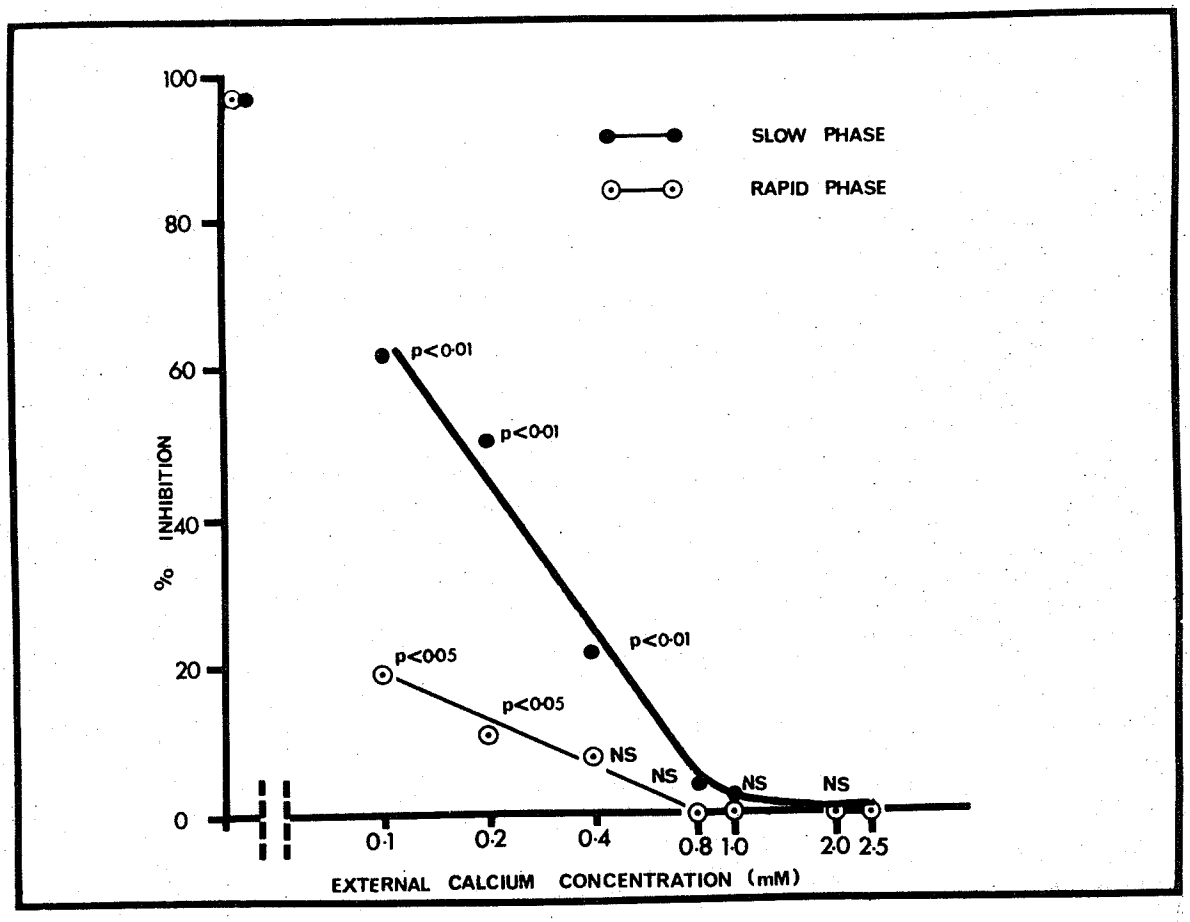


Fig. 5. Effect of external calcium concentration on the magnitude of slow and fast phases.

Inhibition of each phase is expressed as a percentage of the control value of the corresponding phase in the presence of 2.5 mM external calcium. Each point on the curves was compared with the adjacent point obtained with a higher concentration of external calcium. Note the preferential effect of all levels of calcium depletion on the slow phase except that of prolonged depletion in a calcium-free medium.

external calcium concentrations down to 0.8 mM did not cause a statistically significant decrease in the magnitude of slow phase. However, concentrations of 0.4, 0.2 and 0.1 mM reduced the slow phase by 22.5%, 50.5% and 62.4% respectively. All these changes were significantly ($p<0.01$) different from each other as well as from the slight (4.7%) inhibition at 0.8 mM external calcium. Prolonged incubation for 2 hr in the calcium-free medium resulted in nearly complete abolition of both phases (98%). Thus the slow phase is more sensitive than the fast phase to reduction in extracellular calcium concentration. It is reasonable to think that the fast phase relies upon a source of calcium that is more tightly bound than the source which contributes to the slow phase.

IV. Effect of reduced external calcium on the calcium content of spleen strips

In the previous experiment with the external calcium concentration lowered to 0.1 mM, the slow phase was reduced by 62.4% while the fast phase was reduced only 19.2%. In the following experiment the time course of calcium depletion of spleen strips incubated in a medium containing 0.1 mM calcium chloride was studied. 48 spleen strips were suspended in Krebs-Henseleit solution, 6 for recording tension and the remainder for analysis of calcium content. The muscles were stimulated with noradrenaline (10^{-6} g/ml) every 15 min until constant responses were obtained. 6 strips were then removed for analysis of the calcium content. The calcium content after this treatment was regarded as the normal value. All the muscles were then exposed to a medium containing 0.1 mM calcium chloride and the responses to noradrenaline were obtained after 5, 10, 15, 30 and 45 min in two strips after each of the first three

time periods and in all the strips after the remaining two time periods. 6 strips were removed after each time period for analysis of their calcium content. The bathing medium was changed every minute during the first 10 min, every 5 min during the next 20 min and twice in every 15 min thereafter.

The effect of reduction of external calcium on the slow phase of contraction was compared with the reduction in the total tissue calcium. After 5 min, the slow phase decreased by an average of 48.2% and by 10 min it decreased by 61%. The fast phase decreased by only 18% at this stage. During the same periods the total calcium concentration of the muscles had decreased from a control value of 1.75 mM/kg to 1.4 mM/kg and 0.8 mM/kg respectively (Fig. 6). These values were significantly different from one another. Between 10 min and 45 min the calcium concentration decreased very little, from 0.8 mM/kg to 0.67 mM/kg. This decrease was not statistically significant. The muscles were then placed in a calcium-free medium. After exposure to this solution for 55 min the muscle responded very feebly to noradrenaline. The calcium concentration decreased by another 0.22 mM/kg and this was significant at the 0.05 level.

The decrease in calcium concentration of the spleen strips after 10 min exposure to the medium containing 0.1 mM calcium chloride amounted to 0.95 mM/kg. The spleen has an extracellular space of 22% (determined with inulin). If the effect of decreasing the calcium concentration in the medium was only on the extracellular calcium but not on the cellular depot of calcium then the decrease in the total calcium concentration of the tissue should be only 0.22 times the decrease in calcium concentration of the bathing medium (2.5 mM reduced to

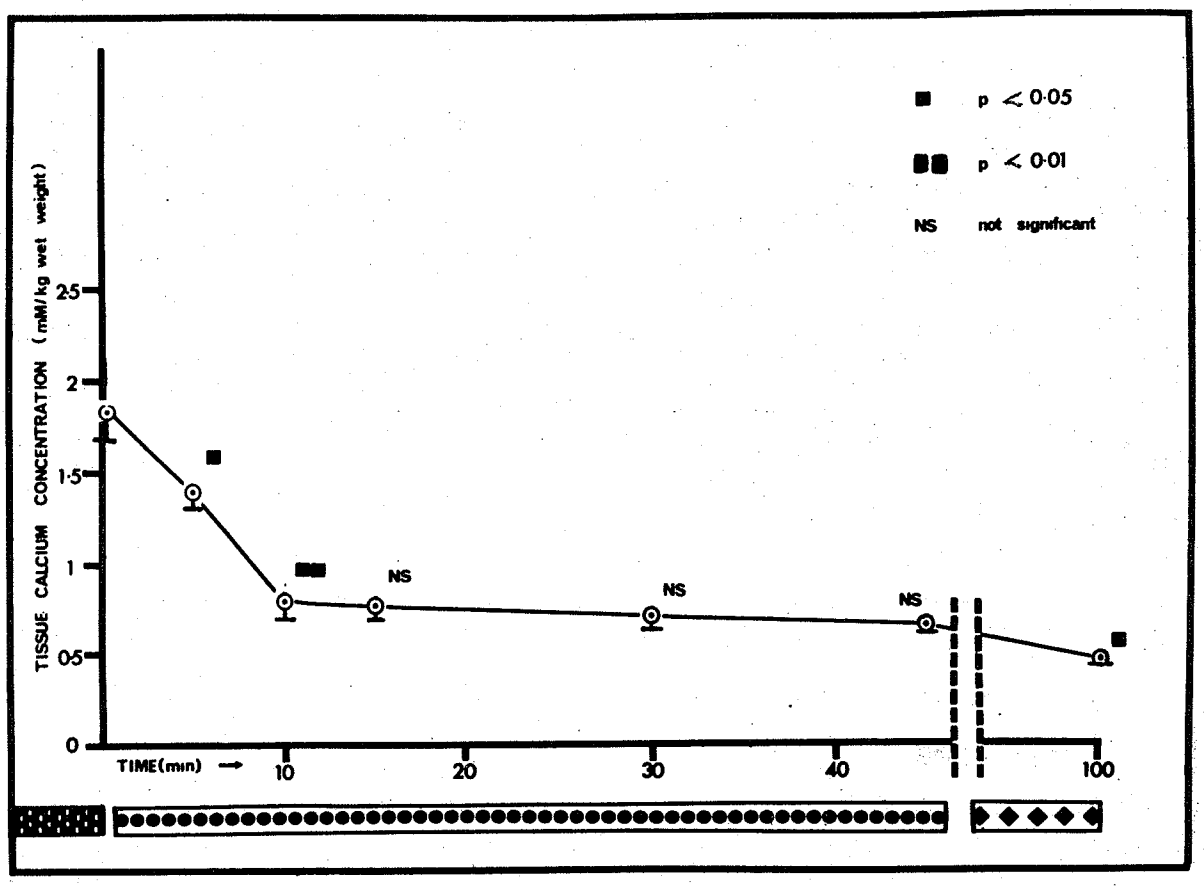


Fig. 6. Time course of loss of tissue calcium in low calcium medium. A rapid initial decrease in tissue calcium content occurred when the bathing medium was changed from Krebs-Henseleit solution (▬▬) to one containing only 0.1 mM calcium (⊙). After 45 min the bathing medium contained no calcium (◆). A small further decrease in tissue calcium is seen.

0.1 mM = 2.4 mM), i.e. only 0.53 mM. The observed decrease of 0.95 mM/kg in total tissue calcium content is 0.42 mM/kg more than calculated above. This appears to be the amount of calcium lost from the cellular stores. This was associated with a preferential decrease of 61% of the slow phase and only 18% of the quick phase. In the total absence of external calcium, the additional loss of tissue calcium (0.22 mM/kg) exceeded the decrease accountable by the reduction in the calcium concentration of extracellular space (0.022 mM) by 0.20 mM/kg. This was associated with a decrease in the fast phase by 82% and in the slow phase by 35%.

V. Effect of change in external calcium concentration on the response to high external potassium concentration

These studies were done on strips from reserpine treated cats to avoid complications due to release of endogenous catecholamines. These strips were exposed to a medium in which the potassium concentration was raised to 90 mM by substitution of potassium for appropriate amounts of sodium chloride. A prompt increase in tension consisting of a single steep phase occurred. Control responses were obtained in 8 strips bathed in Krebs-Henseleit solution. The external calcium chloride concentration was successively reduced to 1.5 mM, 0.75 mM, 0.5 mM and 0 mM every 15 min. After the muscles had remained in each of the above concentrations of calcium chloride for 10 min, they were challenged with the high potassium solution containing an identical concentration of calcium chloride. The response always consisted of a single phase. When the percentage inhibition of the response to high potassium was expressed as a function of external calcium chloride (Fig. 7) a steep relationship was obtained between reduction in external calcium levels and contractile force. This

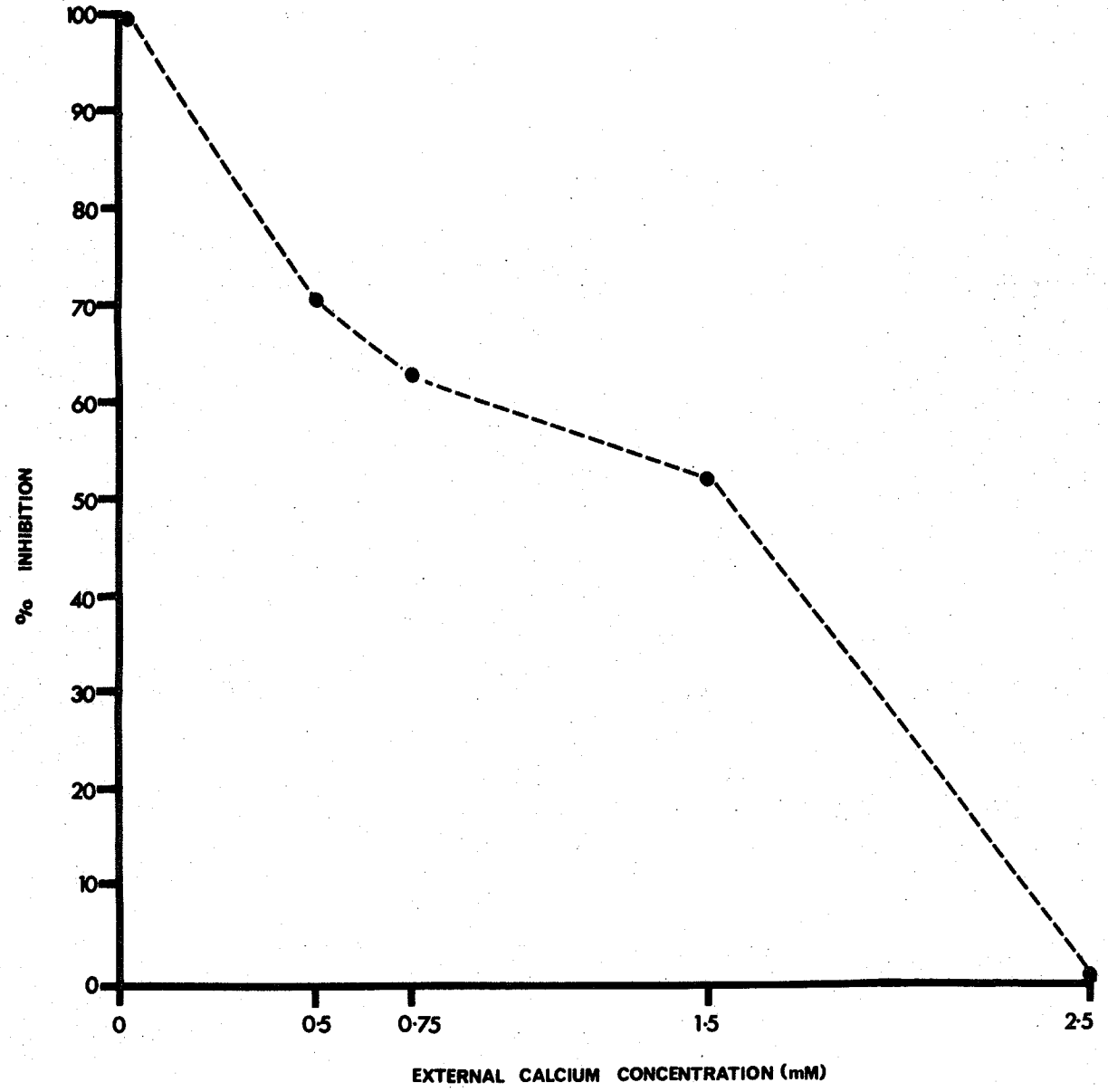


Fig. 7. Effect of reduction of external calcium on the contractions of spleen strips due to a high concentration of potassium (90 mM). Spleen strips were taken from cats treated with reserpine, 1 mg/kg, 24 hours before the experiment.

relationship was qualitatively similar to the relationship between decrease in extracellular calcium and inhibition of the slow phase of the splenic response to noradrenaline. One important difference between the responses to the two agonists was the marked sensitivity of the responses to potassium to reduction of calcium concentrations from 2.5 mM to 1.5 mM in contrast to the responses to noradrenaline, where inhibition of the slow phase was seen only when the external calcium concentration was reduced to 0.8 mM or less. Nevertheless it appears that potassium contractions and the slow phase of response to noradrenaline utilise a similar source or mechanism of utilisation of calcium.

VI. Dissociation of two phases of contraction produced by noradrenaline by a calcium chelator, EGTA

In the previous experiment reduction of external calcium concentration from 0.8 mM to 0.1 mM preferentially decreased the slow phase. The remaining contraction was fast and the tension was maintained. Krejci and Daniel (1970b) suggested that in a calcium-free medium internal tissue calcium may be released into a restricted biophase from which it can be taken back by an uptake mechanism that is saturable at low external calcium concentrations. Presence of a calcium chelating agent in the external medium will prevent the reuptake of calcium if this is indeed the case. Whether this would interfere with contraction could then be seen.

In six experiments, spleen strips were stimulated with noradrenaline (10^{-6} g/ml) until consistent responses were obtained. The second response (Fig. 8A) was obtained 15 sec after adding a calcium chelator, EGTA (2.5 mM). An undiminished fast phase followed by a slow

and small further increase in tension were seen. Addition of 2.5 mM calcium chloride (a concentration normally present in Krebs-Henseleit solution) to the bath resulted in a further increase in tension which approached control levels obtained in the first contraction. The latency of the contraction due to calcium was longer than that due to noradrenaline. On the other hand the response appeared to reach a plateau level faster than in the control response due to noradrenaline. Another difference between the response in the presence of EGTA and the response in Krebs-Henseleit solution is that in the latter solution the slow phase appeared to begin as soon as the fast phase reached a maximum. However, in the presence of EGTA, there was a delay between the attainment of the maximum of the fast phase and the commencement of a small component of the slow phase. This was even more accentuated in the next two responses which were obtained with noradrenaline 15 sec after addition of EGTA, 6 mM and 15 mM. In the presence of 6 mM EGTA the fast phase was potentiated in 3 out of the 6 strips tested. A small depression of tension was seen between the fast phase and slow phase. Increase in the concentration of EGTA was associated with a progressive decrease in the slow phase. In the presence of 15 mM EGTA, the fast phase was still not different from the one in the control response. The tension was not maintained and there was no secondary increase in tension (slow phase).

The strips were then depleted of calcium by incubation in a calcium-free medium containing EGTA (12 mM) for 30 min followed by a further period of incubation only in calcium-free medium. The muscles failed to respond to noradrenaline. However, without washing noradrenaline out, when calcium chloride (2.5 mM) was added to the bath 1 min later,

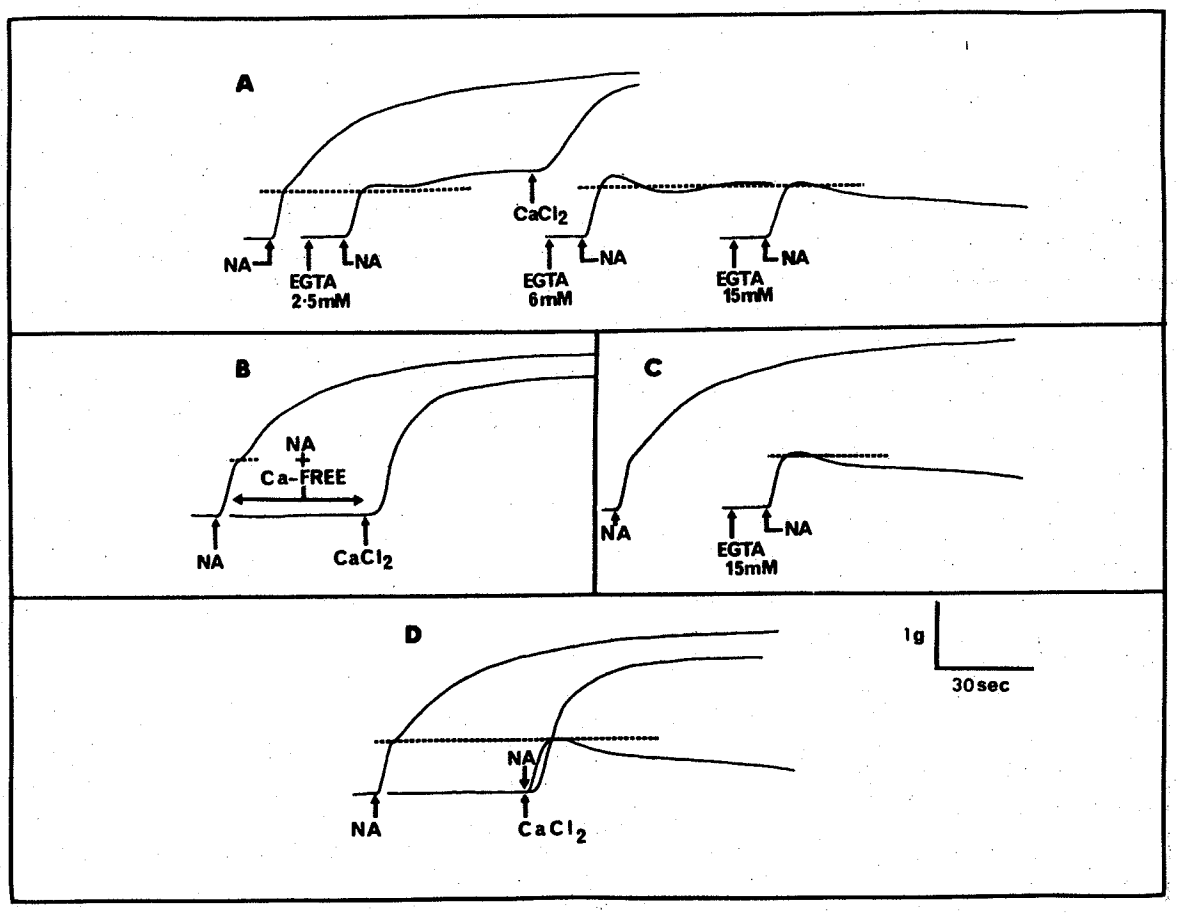


Fig. 8. Dissociation of fast and slow phases of splenic responses to noradrenaline (NA).

A. Responses to noradrenaline, 10^{-6} g/ml: contractions in Krebs-Henseleit solution, with EGTA and calcium chloride added as indicated. Note decrease of slow phase and slight potentiation of fast phase after EGTA.

B. Responses after depletion of calcium by exposure for 30 min to a calcium-free medium and 12 mM EGTA and 15 min to calcium-free medium. NA added now did not result in contraction; with NA still present, $CaCl_2$ 2.5 mM added 2 min later caused a single phase contraction with a latency greater than the latency in C.

C. Responses to NA in standard Krebs-Henseleit solution; first contraction before, second contraction after 15 sec exposure to EGTA, 15 mM. Note fast phase is followed by a progressive decrease in tension.

D. Contractions of B and C are superimposed to indicate relative sizes of the phases, onsets and times to peak tension.

The tension increased in an 'S' shaped fashion and the curve could not be resolved into two components (Fig. 8B). In Fig. 8D the larger contraction produced by the addition of calcium in the presence of noradrenaline has been superimposed on the smaller response (fast phase) which was produced by noradrenaline in the same strip (Fig. 8C), in the presence of EGTA (15 mM) in Krebs-Henseleit medium. The delay preceding the larger response, which was produced by utilisation of extracellular calcium, was greater than the delay preceding the smaller response (fast phase) produced presumably from a tightly bound source of cellular calcium. The response of the calcium depleted tissues to calcium, in the presence of noradrenaline, reached a maximum much faster than the response of a normal tissue to noradrenaline. It is not known whether the time taken by a response to noradrenaline to attain a plateau level is limited by the rate of attainment of an equilibrium of the amine in the vicinity of the alpha adrenoceptors or if the rate limiting step is one of the subsequent events in the sequence of changes which result in the eventual shortening of the contractile proteins. The response to noradrenaline in the control tissues attained a peak level in 134 ± 6 sec. However, if the calcium depleted tissue was first kept in contact with noradrenaline for 2 min the subsequent response to calcium reached its peak in only 57.6 ± 3.2 sec. Thus it seems that noradrenaline takes longer to equilibrate in the vicinity of the receptors than the time taken for extracellular calcium to enter the tissue to activate the contractile process. The mobilisation of the tightly bound calcium (evidenced by the fast phase) is an even quicker process taking an average of 4.5 ± 0.4 sec to reach a maximum. Another important finding was that the sum of the peak of the

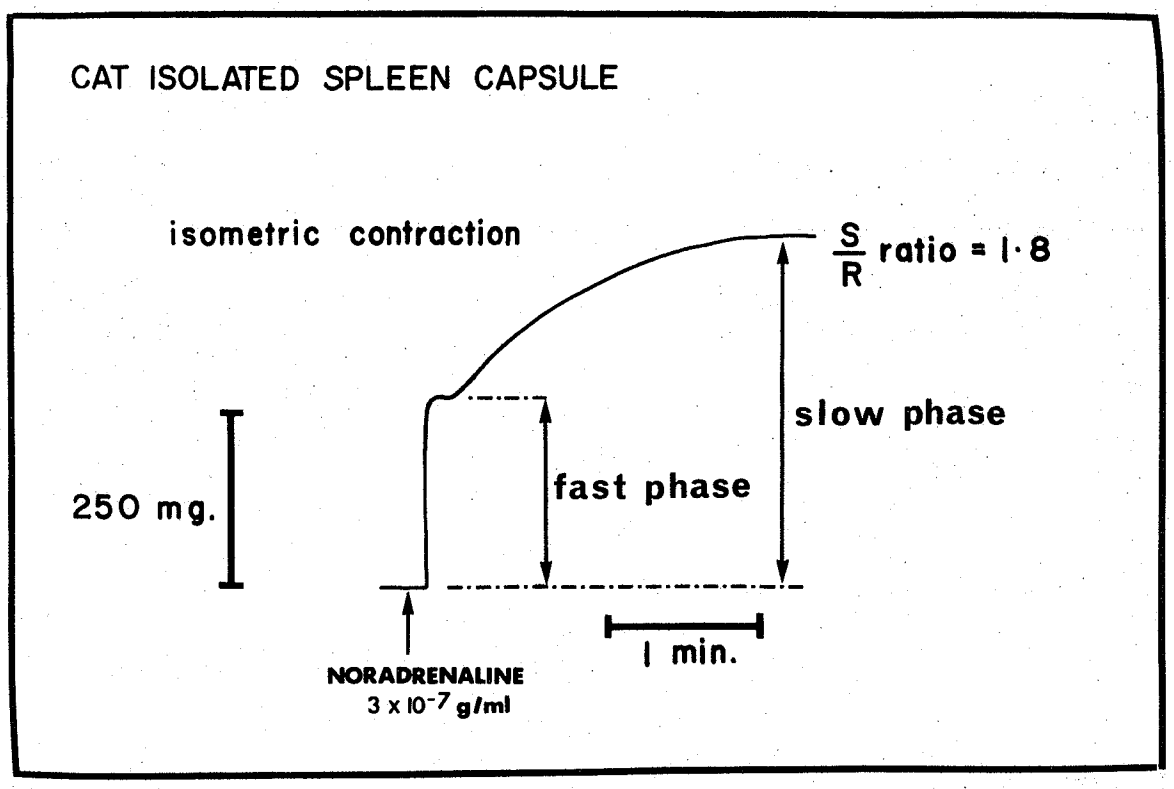


Fig. 9. Method of measuring slow and fast phases of response to noradrenaline (NA).

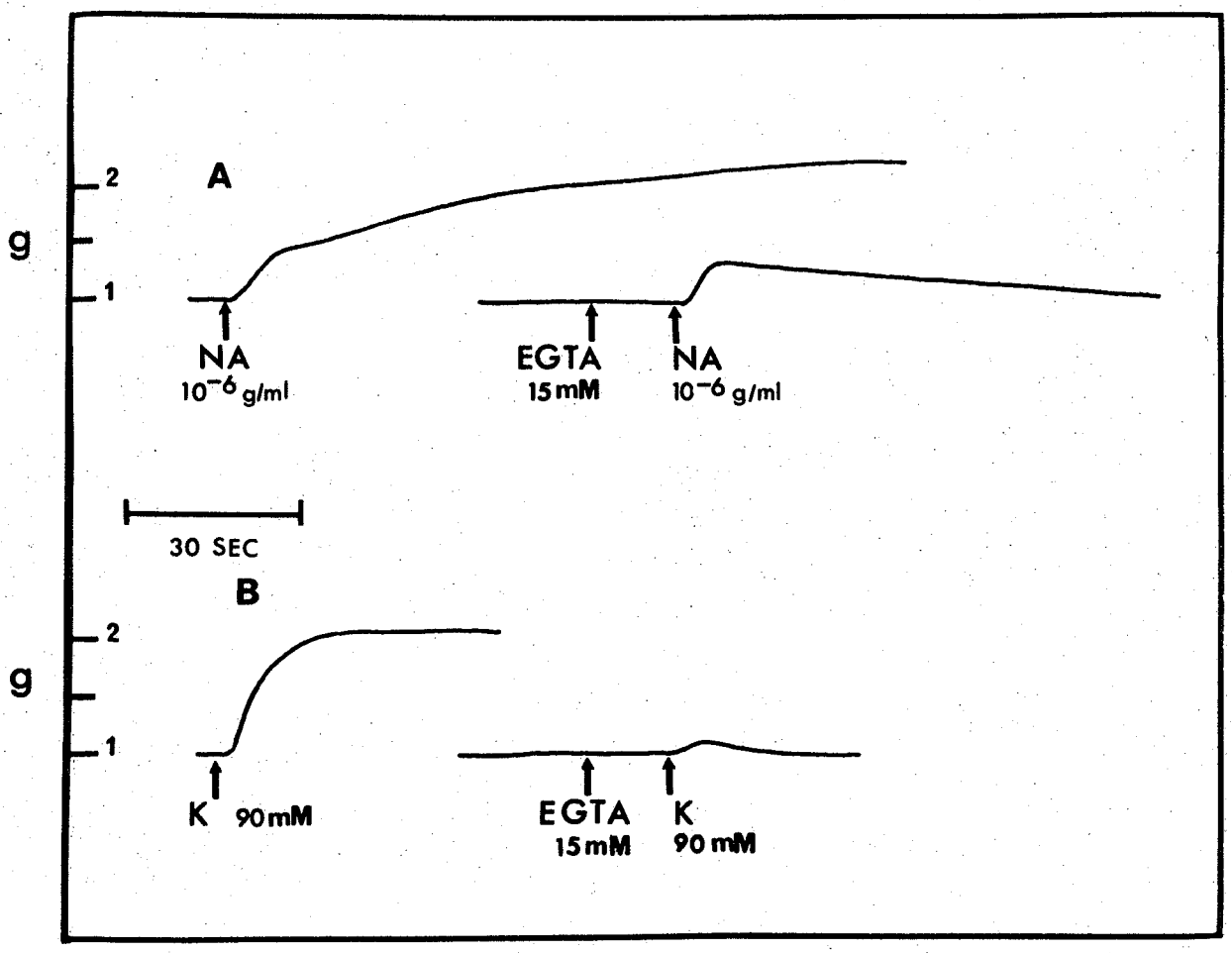


Fig. 10. Effect of EGTA on the responses of spleen to noradrenaline (NA) or potassium (K).

A. Response of normal spleen strips to NA, 10^{-6} g/ml before and 15 sec after EGTA.

B. Response of spleen strip from cat treated with reserpine, 1 mg/kg, 24 hours prior to K, 90 mM before and 15 sec after EGTA, 15 mM.

fast phase (the response in Fig. 8C) and the phase due to restoration of extracellular calcium (Fig. 8B) exceeded the total contraction in the normal tissue (Fig. 8 B or C, lst contraction) by 38%. The slow phase of the normal response to noradrenaline is dependent upon extracellular calcium. Hence if the contraction produced by calcium in the calcium depleted tissue in the presence of noradrenaline is analogous to the slow phase, then it is likely that the contribution of the fast phase to the total tension induced by noradrenaline is less than the peak tension of the fast phase seen early in the contraction. If one postulates that the fast phase is a transient process then the sum of a smaller fast phase (at the later time when the muscle has developed peak tension) and the calcium contraction (analogous to a true slow phase) will be closer to the observed total tension in the normal tissue. The transient nature of the fast phase in the presence of EGTA supports this assumption. Hence in all succeeding experiments, the slow phase was measured from the peak tension to the baseline tension rather than to the peak of the fast phase. The measurements have generally been expressed as a ratio of slow/fast phase (Fig. 9).

In another 4 tissues obtained from a reserpine treated cat, responses were obtained with a high potassium (90 mM) Krebs-Henseleit solution (Fig. 10B). The contractions consisted of a single phase. EGTA (15 mM) was added to the Krebs-Henseleit medium 15 sec before changing to the high potassium solution, which also contained the same amount of EGTA. A very small response was seen compared to that produced by noradrenaline under identical conditions (Fig. 10A). This shows a smaller dependence of potassium-induced contraction on tightly bound calcium.

VII. Preferential modifications of individual phases of contraction

The results to this point show that any change in external

calcium first affects the component of contractile response dependent upon a loosely bound calcium store. The tightly bound pool, which is presumably intracellular, is affected later. This favours a model in which the loosely bound store is in 'series' with the intracellular tightly bound calcium store on one side and the extracellular calcium on the other side. Our results do not at this stage allow any differentiation between extracellular and loosely bound stores. An alternate possibility is that the loosely bound and tightly bound stores of calcium, instead of communicating with each other and with the extracellular depot of calcium in a series arrangement, may have independent access to the extracellular store. In other words the two stores may be arranged 'parallel' to each other. If the rates of loss of calcium from the two pools into a calcium deficient medium are different, then the phases of contraction dependent on these two stores will be inhibited with different time courses as seen in the experiments reported so far. Further experiments were done to find out if it was possible to modify either phase of contraction due to noradrenaline selectively or preferentially. Such evidence if obtainable would dictate against the 'series' model of calcium stores.

Modification of slow phase

Several divalent cations are known to interfere with the calcium dependent action potentials in the barnacle muscle fibres, manganese chloride being one of them (Hagiwara and Nakajima, 1966). The effect of manganese chloride on the two phases of contraction of noradrenaline was therefore studied.

Eight strips of spleen capsule bathed in Krebs-Henseleit solution were stimulated with noradrenaline (10^{-6} g/ml) until consistently equal

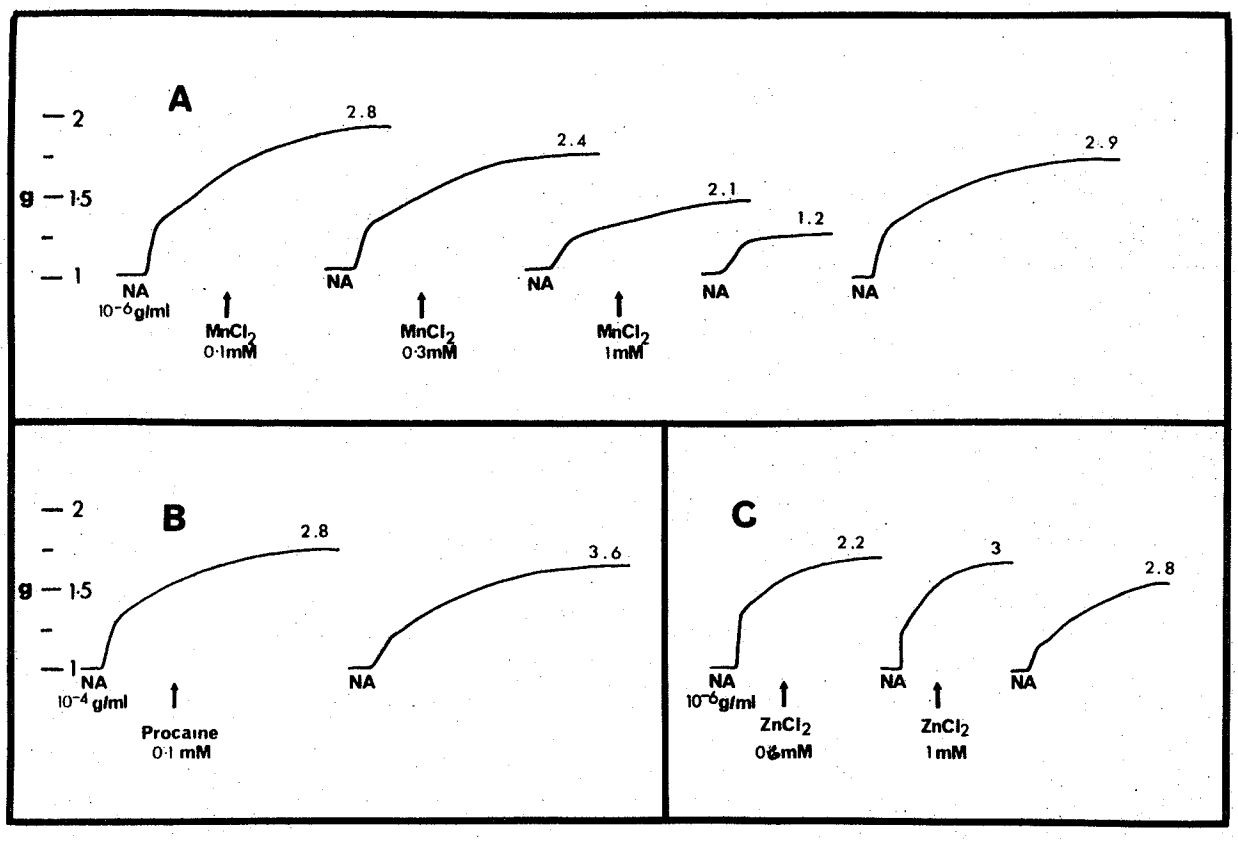


Fig. 11. Effect of $MnCl_2$, procaine and zinc on the phases of contraction due to noradrenaline (NA), 10^{-6} g/ml.

A. Effect of $MnCl_2$, 0.1, 0.3 and 1 mM;

B. Effect of procaine, 0.1 mM;

C. Effect of $ZnCl_2$, 0.6 and 1 mM.

Numbers above each contraction indicate the proportion of slow to fast phases.

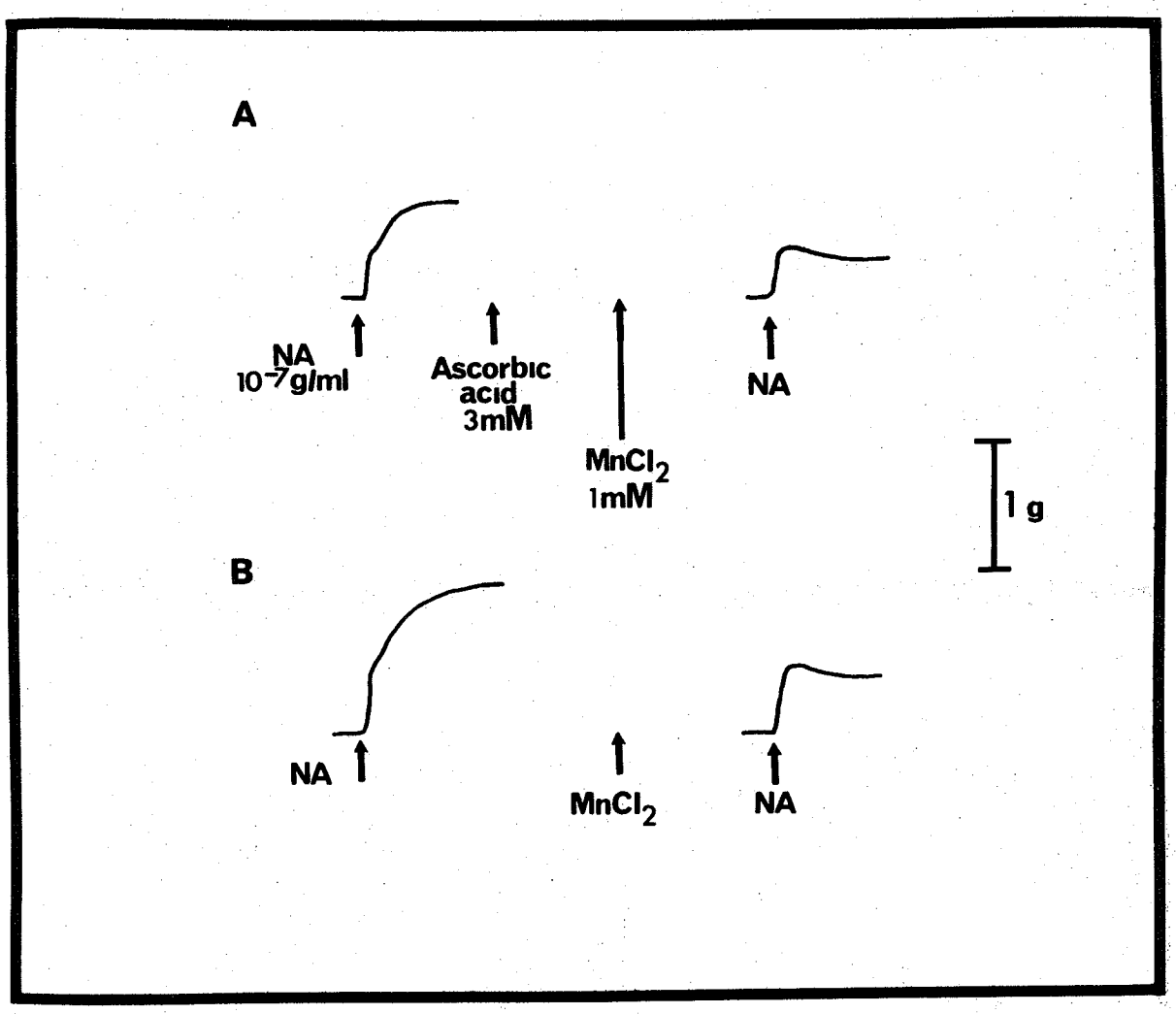


Fig. 12. Effect of ascorbic acid on the inhibition of noradrenaline (NA) induced response in the spleen.

A. $MnCl_2$, 1 mM added in the presence of ascorbic acid, 3 mM.

B. $MnCl_2$, added alone.

Numbers above each contraction denote the proportion of slow to fast phases. Note the lack of protective effect of ascorbic acid against inhibition of responses to noradrenaline by $MnCl_2$.

responses were obtained. Manganese chloride (0.1 mM) was added to the bathing medium and 15 min later the preparations were challenged with noradrenaline. After the response, the strip was washed and the concentration of manganese chloride increased first to 0.3 mM and then to 1 mM and the strip stimulated each time. Finally the strip was washed free of manganese and tested with noradrenaline at 15 min intervals for evidence of recovery. A typical experiment is depicted in Fig. 11A. Manganese chloride reversibly decreased mainly the slow phase of contraction. This was indicated by a decrease in the ratio of slow to fast phase. The total contractions decreased 17%, 48% and 69% with 0.1 mM, 0.3 mM and 1 mM of manganese chloride respectively.

Löffelholz and Scholz (1970) cautioned that inhibition by manganese ion of responses of a variety of tissues to adrenaline need not exclusively be due to an effect on calcium utilisation. In fact they showed that manganese (0.1 mM) during an observation period of 20 min increased the auto oxidation of adrenaline approximately 3 fold. This effect of manganese was markedly inhibited by ascorbic acid in concentrations of $1 - 5 \times 10^{-5}$ g/ml. To test whether manganese inhibited the splenic capsular contraction to noradrenaline by causing an increased oxidation of the drug the following experiment was done. Three strips were stimulated with noradrenaline, (10^{-6} g/ml) until the responses became reproducible. Ascorbic acid (3 mM) was added to one bath, manganese chloride (1 mM) and ascorbic acid (3 mM) into the second and manganese chloride (1 mM) alone to the third bath. The response to noradrenaline was tested again 15 min later. Ascorbic acid did not alter the response to noradrenaline. Manganese chloride decreased the slow phase markedly,

Fig. 12B. The presence of ascorbic acid in a concentration that was greater than that used in the study cited earlier, failed to decrease the effect of manganese chloride (Fig. 12A). It seems very unlikely that in the short duration (3 min) during which a response to noradrenaline is studied, manganese ions can promote destruction of the agonist sufficiently to affect the response.

Modification of fast phase

Local anaesthetics have been postulated to act on smooth muscle as membrane stabilisers (Feinstein, 1966). It was therefore considered desirable to investigate the action of procaine on the response to noradrenaline. The experimental design was similar to that in the previous experiment. Procaine in a concentration of 0.1 mM preferentially decreased the fast phase of the response to noradrenaline and consequently increased the ratio of slow to fast phase (Fig. 13B). Higher concentrations of 0.2 mM, 0.3 mM and 1 mM of procaine did not further decrease the fast phase but reduced the slow phase (Fig. 14). With high doses, 1 mM and 2 mM, procaine induced a tonic increase in tension superimposed on rhythmic contractions. Procaine effects were reversible.

Zinc chloride in concentrations of 0.6 mM to 2 mM had effects on the fast phase similar to those of low concentrations of procaine (Figs. 12C, 14). The inhibitory effect of zinc was more resistant than the inhibition due to either manganese or procaine to washout of the substance from the bath. Recovery of contractility after washout of the inhibitory substances from the bath was less complete with zinc than with either manganese or procaine.

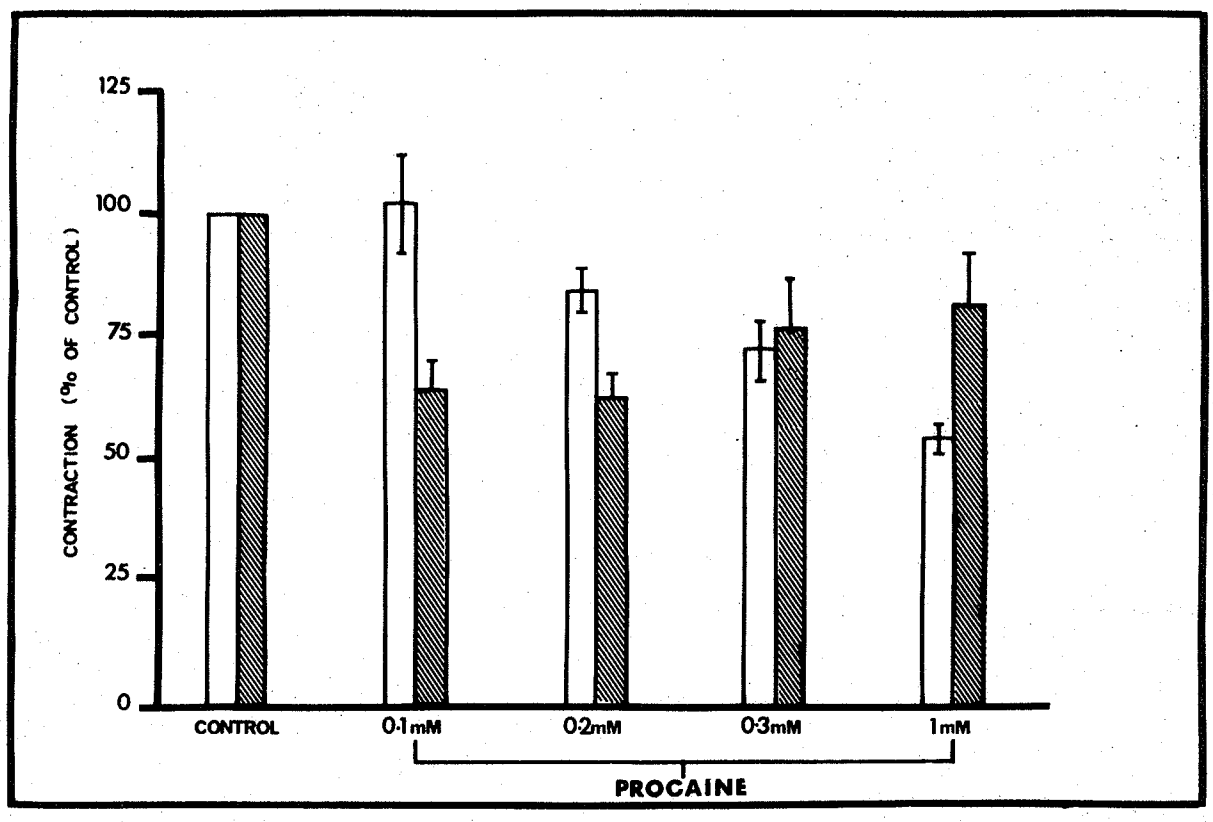


Fig. 13. Effect of procaine on the two phases of contraction produced by noradrenaline, 10^{-6} g/ml.

Magnitude of slow phase (open bars) and fast phase (hatched bars) after various concentrations of procaine have been expressed as a percentage of the control responses.

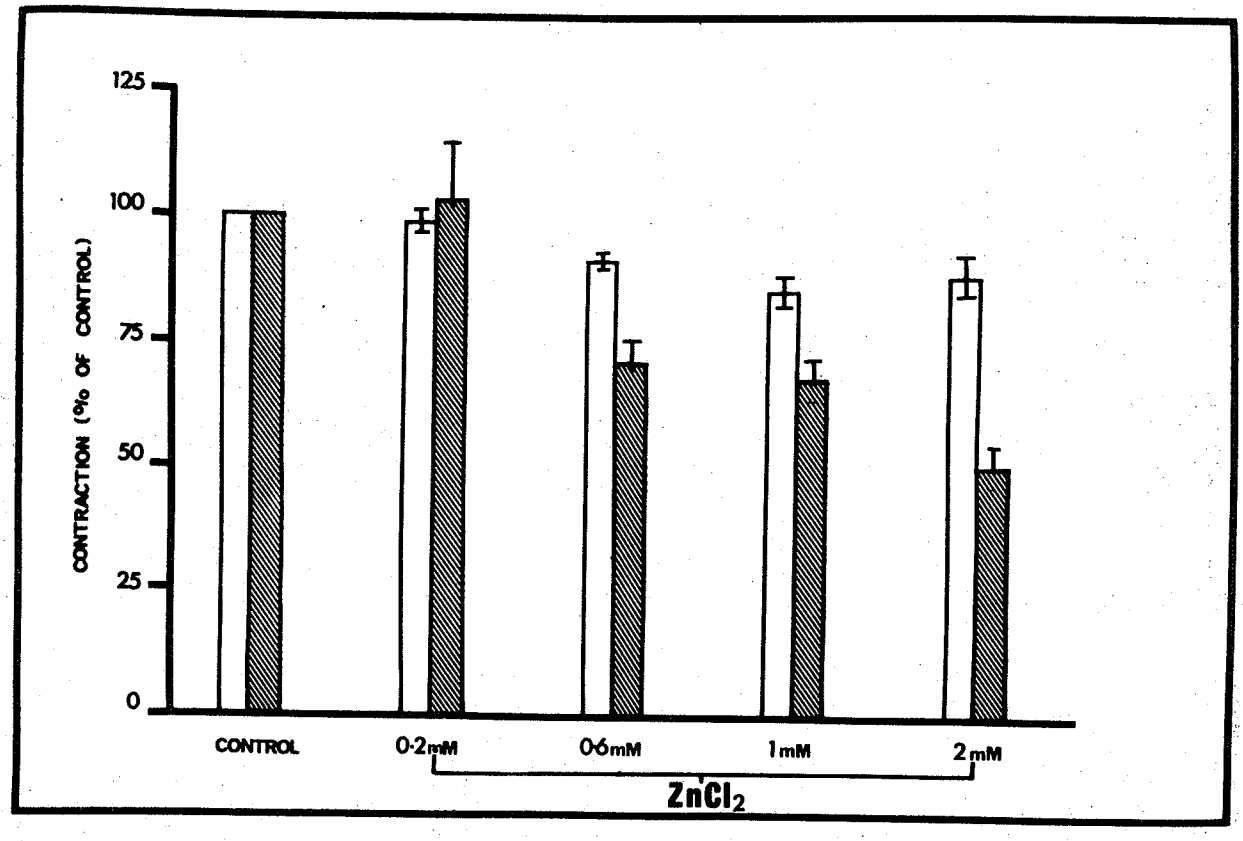


Fig. 14. Effect of $ZnCl_2$ on the two phases of contraction produced by noradrenaline, 10^{-6} g/ml.

Magnitude of slow phase (open bars) and fast phase (hatched bars) after various concentrations of $ZnCl_2$ have been expressed as a percentage of the control responses.

VIII. Effect of noradrenaline on ^{45}Ca efflux in spleen capsular smooth muscle

The previous experiments have provided indirect evidence that the two phases of contraction due to noradrenaline depend upon two different stores or mechanisms of utilisation of calcium. It was also noticed that pharmacological agents could preferentially decrease one or the other phase of contraction. To gather more direct information about the above phenomenon, experiments were done to find out whether noradrenaline affected radiocalcium release from muscles loaded with the isotope or affected the uptake of the isotope by the muscles from the bathing medium. Further, if any of these changes did take place, it was planned to investigate the effect of agents which modify the phases of contraction on these alterations in radiocalcium movements.

a) Effect of noradrenaline on ^{45}Ca and sucrose-^{14}C efflux

Spleen strips were equilibrated in Krebs-Henseleit solution at 37°C for 1 hr. They were then depleted of calcium by incubation in calcium-free Krebs-Henseleit solution for 30 min. During the first 15 min of this incubation, EGTA (6 mM) was also present in the bath. After this step, the muscles were soaked for 60 min in a calcium-free Krebs-Henseleit solution containing 2 μci of $^{45}CaCl_2$. Hudgins and Weiss (1969) have earlier shown that the uptake of radioactive calcium by aortic smooth muscle was enhanced in the absence of external non-radioactive calcium. The spleen strips, after incubation in the radioactive medium were dipped in non-radioactive calcium-free Krebs-Henseleit solution for 4 sec and then passed through a series of tubes containing 2 ml of either calcium-free or standard Krebs-Henseleit solution. The muscles were kept in each

tube for 5 min before being moved to the next one. Radioactivity remaining in the tissue at the end of the experiment and the radioactivity in the individual tubes were determined. The rate of ^{45}Ca efflux into each tube was obtained by correcting for the weight of the tissue and the period of incubation in that tube. The rate of efflux at each interval has been depicted as a percentage of the initial rate of efflux during the first 5 min (Fig. 15C). Efflux in the Ca-free and Ca-containing media have also been shown as the rate of desaturation of tissue radioactivity as a function of time (Fig. 15A,B). For this, the tissue radioactivity was added to the radioactivity in each of the efflux tubes in a cumulative manner. The total radioactivity thus accumulated denotes the radioactivity in the tissue at the beginning of the experiment. The level of tissue radioactivity at the end of each 5 min incubation in an efflux tube has been expressed as a percentage of the initial radioactivity in the muscle and a desaturation curve plotted.

When the efflux was determined in a calcium-containing medium (2.5 mM), neither the rate of efflux nor the tissue radioactivity due to ^{45}Ca, plotted semilogarithmically as a function of time, decreased as single exponentials. The percentage of ^{45}Ca remaining in the muscle decreased fast initially but gradually the rate of decrease became less (Fig. 15B). The points on the curve after 35 - 40 min appear to be in a straight line, indicating an exponential decrease (Fig. 16B2). The intercept of this line with the abscissa gives the percentage of total ^{45}Ca in the tissue that contributes to this slow efflux. The difference between the percentage of ^{45}Ca remaining in the tissue at various times between 0 to 35 or 40 min and the extension of the slow efflux component

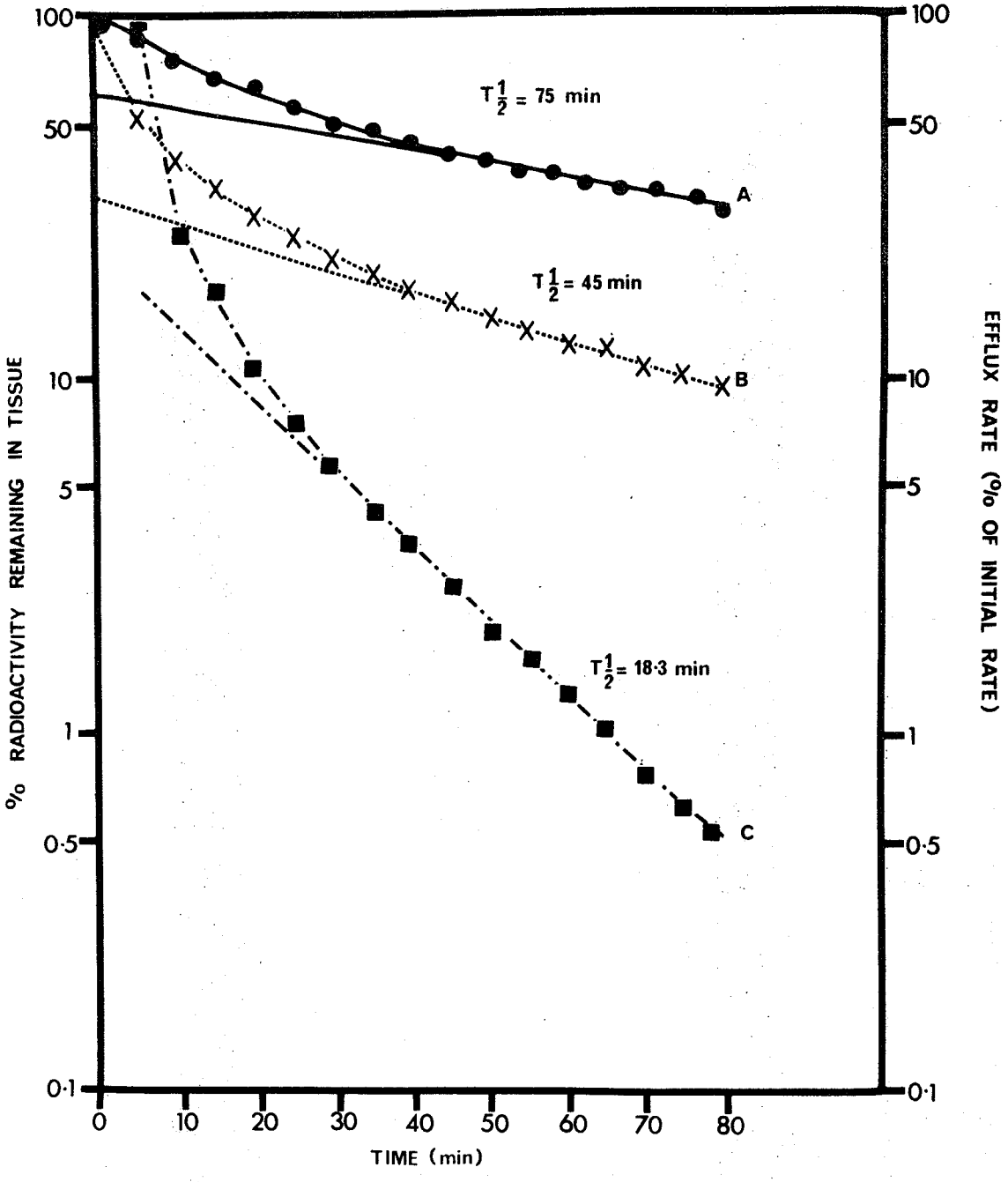
Fig. 15. Loss of ^{45}Ca from spleen strip in ^{40}Ca-free or Krebs-Henseleit solutions.

Strips were loaded with ^{45}Ca in a ^{40}Ca-free medium. Efflux of ^{45}Ca was studied under conditions indicated as follows:

A. Efflux occurred in a ^{40}Ca-free medium. ^{45}Ca contents of the muscle at different times were plotted as percentages of the ^{45}Ca content at the beginning of efflux.

B. Efflux occurred in Krebs-Henseleit solution. Similar plot as in A.

C. Efflux occurred in Krebs-Henseleit solution. Rates of efflux at different times have been plotted as percentages of the rate of efflux during the first 5 min. $T\frac{1}{2}$ values are half times of decay of the late exponential portions of each curve.


100
50
10
5
1
0·5
0·1
$T\frac{1}{2}$ = 75 min
$T\frac{1}{2}$ = 45 min
$T\frac{1}{2}$ = 18·3 min
A
B
C
0
10
20
30
40
50
60
70
80
TIME (min)
% RADIOACTIVITY REMAINING IN TISSUE
EFFLUX RATE (% OF INITIAL RATE)

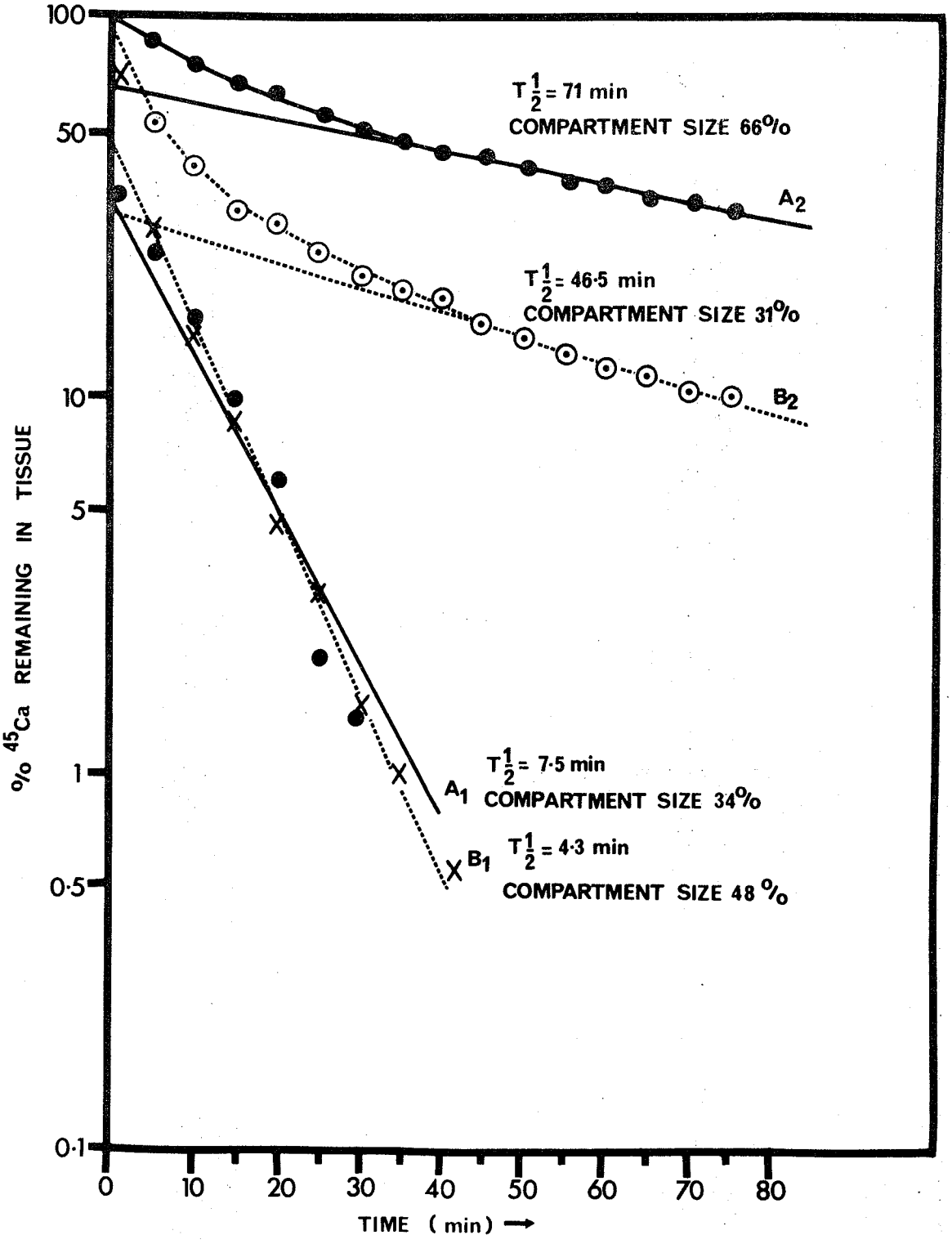
to the left when plotted produced a straight line fit (Fig. 16B1). Again, the intercept of this line on the abscissa gives the percentage of total tissue ^{45}Ca that contributes to this fast efflux component. In about 70% of the experiments the sum of these two components amounted to 100% of the total tissue ^{45}Ca. In the remaining experiments the sum of the two components was less than 100%. The remaining ^{45}Ca, in these experiments, appeared to be coming out as a very fast component that could not be resolved clearly because the sampling interval (5 min) was not short enough. Obviously, if such a component does occur, it would be largely over during the first 5 min of the efflux. A quantitatively different result was obtained when the rate of emergence of radioactivity instead of the percentage of total ^{45}Ca remaining in the tissue was plotted in relation to time. The counts/min coming out into the medium during the first 5 min of efflux were considered 100%. All the subsequent radioactivity in counts/min coming out into the medium at 5 min intervals were expressed as a percentage of the initial rate. The resulting curve again was not a single exponential function. However, like the earlier curve showing 'desaturation' of tissue ^{45}Ca, the rate of efflux also decreased less steeply and in an exponential fashion after 40 min. The slope of this slower component was not parallel but steeper than the slope of the slow component of the desaturation curve.

When desaturation of tissue ^{45}Ca was measured in a calcium-free medium, the rate of loss of radioactive calcium was markedly decreased and an appreciably greater fraction of tissue became available to the slow component (Fig. 16A1,A2).

In a total of 10 tissues each, in calcium-containing or calcium-

Fig. 16. Efflux of ^{45}Ca from spleen strip in the presence or absence of ^{40}Ca in the medium.

Spleen strips were loaded with ^{45}Ca in a ^{40}Ca-free medium. Efflux was then conducted in a ^{40}Ca-free medium (●) or in Krebs-Henseleit solution (⊙). The radioactivity remaining in the strips (expressed as a percentage of the initial radioactivity in the strips) is plotted as a function of time. The last parts of the two curves (A_2 and B_2) are exponentials with half times of 71 and 46.5 min respectively and they met the ordinate at the 66% and 31% marks. Subtracting the difference between the points on these straight lines from the points on the earlier parts of the initial curves gave 2 sets of points (• and x) which also fell on 2 straight lines (A_1 and B_1) having half times of 7.5 and 4.3 min respectively. A_1 and B_1 met the ordinate at the 34% and 48% marks respectively.


100
50
10
5
1
0·5
0·1
% 45Ca REMAINING IN TISSUE
0
10
20
30
40
50
60
70
80
TIME (min) →
T½ = 71 min
COMPARTMENT SIZE 66%
A2
T½ = 46·5 min
COMPARTMENT SIZE 31%
B2
T½ = 7·5 min
A1 COMPARTMENT SIZE 34%
B1 T½ = 4·3 min
COMPARTMENT SIZE 48 %

free medium the following measurements were obtained.

In a calcium-free medium:

half time of decrease in slow component of desaturation =

74.5 ± 8.6 min

half time of decrease in fast component of desaturation =

7.2 ± 0.6 min

compartment size of slow component = $67.8 \pm 6.1\%$ and

compartment size of fast component = $32.2 \pm 4.7\%$

In a calcium-containing medium:

half time of decrease in slow component of desaturation =

44 ± 4.8 min

half time of decrease in fast component of desaturation =

6 ± 0.4 min

compartment size of slow component = $32.3 \pm 5.8\%$ and

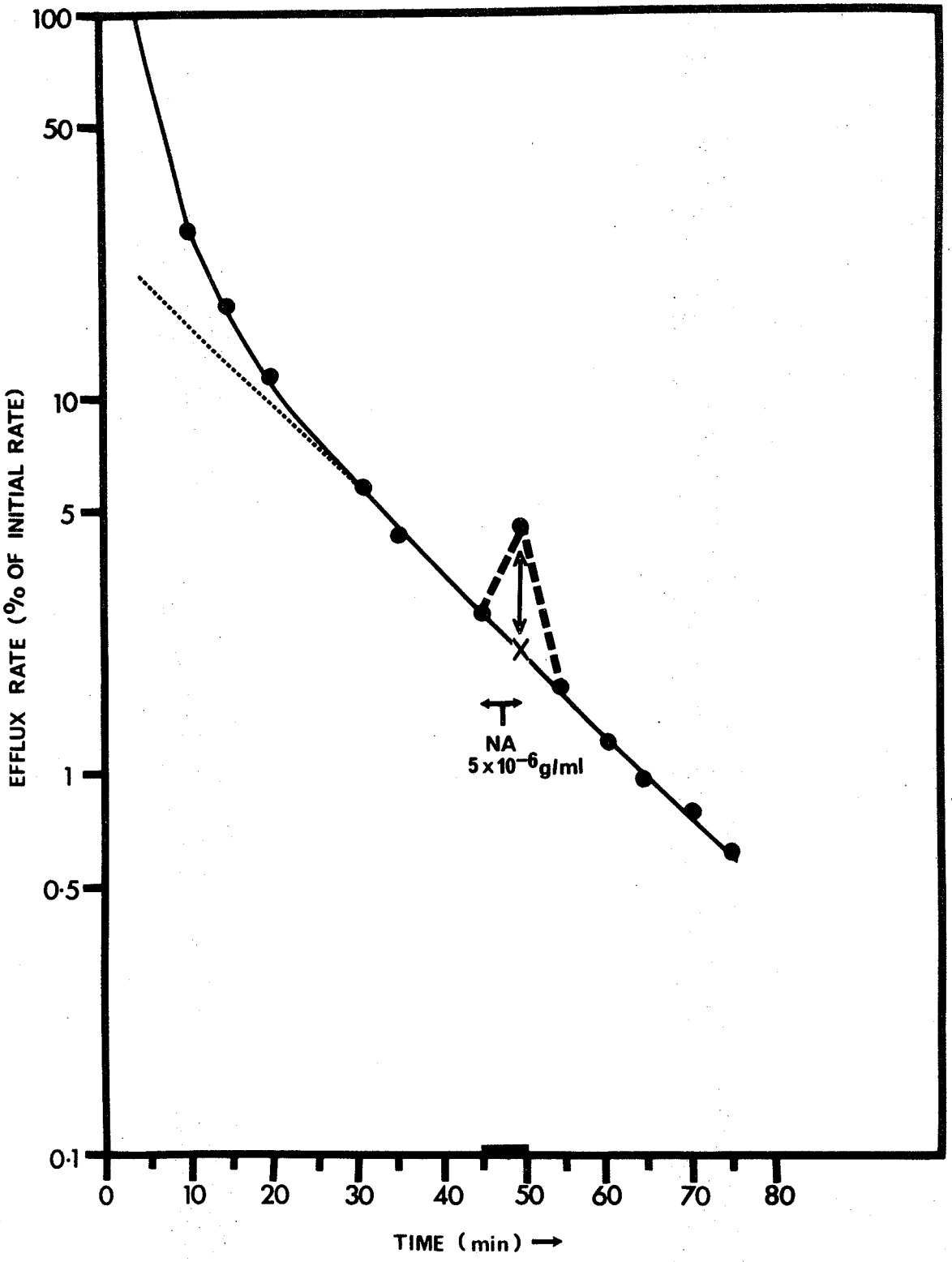
compartment size of fast component = $49.7 \pm 6.2\%$

The sum of the fast and slow compartment sizes was 82%. The remaining 18% of the total tissue radioactivity appeared to come out very early during the efflux. It could not be satisfactorily resolved due to the sampling intervals not being less than 5 min apart.

Thus it can be seen that an appreciable fraction of radioactive calcium that is bound to the muscle in a calcium-free medium is released in the presence of non-radioactive calcium.

To study the effect of noradrenaline on the efflux of ^{45}Ca, the tissues were loaded with radioactive calcium in the same manner as described before. The efflux was carried out for 5 min each for a total of 75 min in a series of tubes containing 2 ml of Krebs-Henseleit solution. Noradrenaline was present in the tube in which efflux occurred between

Fig. 17. Effect of noradrenaline on ^{45}Ca efflux from spleen strips. Strips were loaded with ^{45}Ca in a ^{40}Ca-free medium and efflux conducted in Krebs-Henseleit solution. Every 5 min the strips were moved to fresh medium. Radioactivity coming out into each sample was expressed as a percentage of the radioactivity measured during the first 5 min of ^{45}Ca-efflux and plotted as a function of time. Noradrenaline (NA) was present from the 50th to 55th min and resulted in an increase in efflux. The efflux rate in the absence of NA (x) was obtained by calculating a linear regression of all the data points between 35 and 80 min excluding those obtained between 50 and 60 min.


100
50
10
5
1
0·5
0·1
EFFLUX RATE (% OF INITIAL RATE)
0
10
20
30
40
50
60
70
80
TIME (min) →
NA
5 x 10⁻⁶g/ml

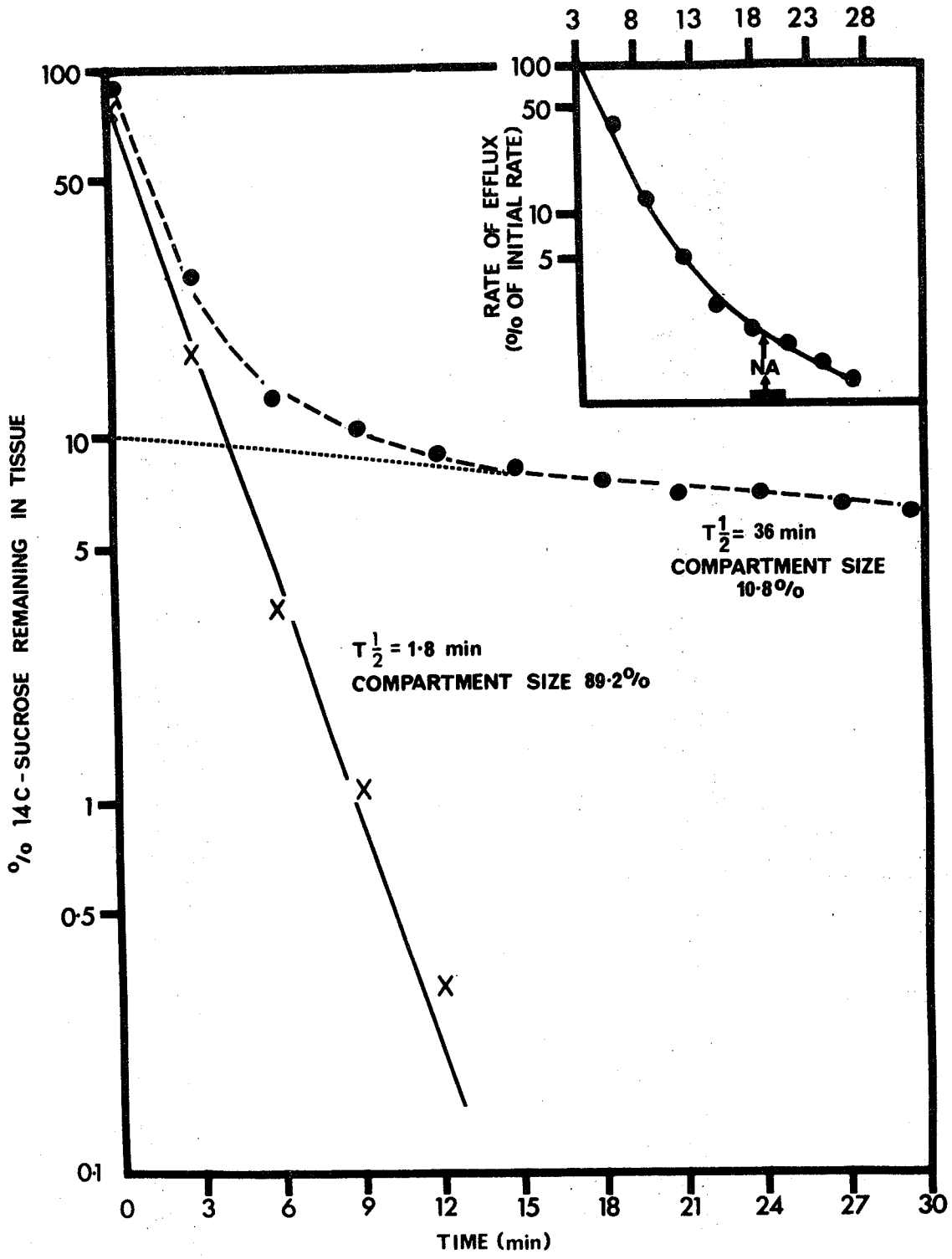
the 45th and 50th min. The rates of efflux were converted to a percentage of the rate during the first 5 min of efflux. Since the last 35 min of the efflux curve was linear on a semilogarithmic plot, a straight line was drawn to fit all the data points between 40 and 75 min inclusive except the ones between 45 and 55 min. The expected efflux rate at the 50th minute was compared with the actually observed rate. The latter was increased by 130.2 ± 9.8% by noradrenaline compared to the expected rate of efflux (Fig. 17). This difference was highly significant statistically ($p < 0.01$).

In another control experiment, four spleen strips were equilibrated in Krebs-Henseleit solution for 60 min and then in 10 ml of Krebs-Henseleit solution containing 1 g of sucrose-^{14}C for 30 min. Sucrose is believed to be largely distributed in the extracellular space. The strips were then dipped for 4 sec in a non-radioactive medium and efflux for 3 min each was performed in a series of 10 tubes containing Krebs-Henseleit solution. The percentage of the initial radioactive sucrose present in the tissue when plotted semilogarithmically against time decreased non-linearly except during the last 15 min (Fig. 18). This slow efflux occurred from a compartment that was only 10.8% of the total sucrose-^{14}C content of the tissue. The remaining 89.2% of the isotope washed out very rapidly with a half time of 1.8 min compared to a half time of 36 min for the slow component. The sucrose coming out in the fast component of efflux is most likely that coming from the extracellular space.

To test whether the observed increase in ^{45}Ca efflux in the presence of noradrenaline is due to a change in extracellular volume with

Fig. 18. Efflux of ^{14}C-sucrose and effect of noradrenaline. Strips incubated in Krebs-Henseleit medium were loaded with ^{14}C-sucrose and then washed in non-radioactive medium for 30 min. The bathing medium was changed every 3 min. The radioactivity remaining in the tissue (expressed as a percentage of the initial radioactivity in counts/min) was plotted as a function of time (●). The resulting curve was a sum of two exponential functions. The slower one consisted of 10.8% of the total tissue radioactivity and decreased with a half time of 36 min, whereas the faster one (x) consisted of 89.2% of the total radioactivity and had a half time of 1.8 min.

The inset shows the result of a similar experiment. The rate of efflux (counts/min during any given 3 min period of efflux) is expressed as a percentage of the rate of efflux during the first 3 min. Noradrenaline (NA), 5×10^{-6} g/ml, was present between 21 and 24 min. Unlike ^{45}Ca efflux, no increase in efflux of ^{14}C-sucrose was seen after noradrenaline.


3 8 13 18 23 28
100
50
10
5
RATE OF EFFLUX (% OF INITIAL RATE)
NA
100
50
10
5
1
0·5
0·1
% 14C-SUCROSE REMAINING IN TISSUE
$T\frac{1}{2}$ = 36 min
COMPARTMENT SIZE 10·8%
$T\frac{1}{2}$ = 1·8 min
COMPARTMENT SIZE 89·2%
0 3 6 9 12 15 18 21 24 27 30
TIME (min)

a consequent displacement of extracellularly present radiocalcium into the bathing medium, three muscle strips were loaded with sucrose-^{14}C and efflux begun as in the previous experiment. Noradrenaline was present in one of each set of tubes between the 15th and 18th min. No significant increase in radioactivity in the medium was observed (Fig. 18 inset).

b) Modification of noradrenaline induced increase in ^{45}Ca efflux

The increase in ^{45}Ca efflux due to noradrenaline appears to be due to a true mobilisation of intracellular calcium, especially since most of the rapidly emerging calcium had already left the tissue before noradrenaline was added. In the next series of experiments it was tested whether the increase in efflux was due to blockade of alpha-adrenoceptors or due to some unspecific phenomenon. As seen in the previous experiment, the increase in efflux of ^{45}Ca with noradrenaline (5×10^{-6} g/ml) was $130.2 \pm 5.8\%$. In another 10 tissues the effect of a smaller dose of noradrenaline (5×10^{-7} g/ml) was studied. A third group of 5 tissues was pretreated with the alpha-adrenoceptor blocking agent phentolamine (5×10^{-6} g/ml) during the first 45 min of efflux and the tissues were then exposed to a similar concentration of phentolamine and also noradrenaline (5×10^{-6} g/ml) between the 45th and 50th minute. Percentage increases in efflux were determined as described earlier and compared with those due to the two concentrations of noradrenaline in the absence of the blocking agent. As can be seen in Fig. 19, the increase in efflux of ^{45}Ca with a smaller dose of noradrenaline was smaller ($67.4 \pm 7.3\%$) compared to the response with the higher concentration ($130.2 \pm 5.8\%$). This difference was statistically significant at the 0.01 level. Phentolamine reduced the response of the higher concentration of noradrenaline

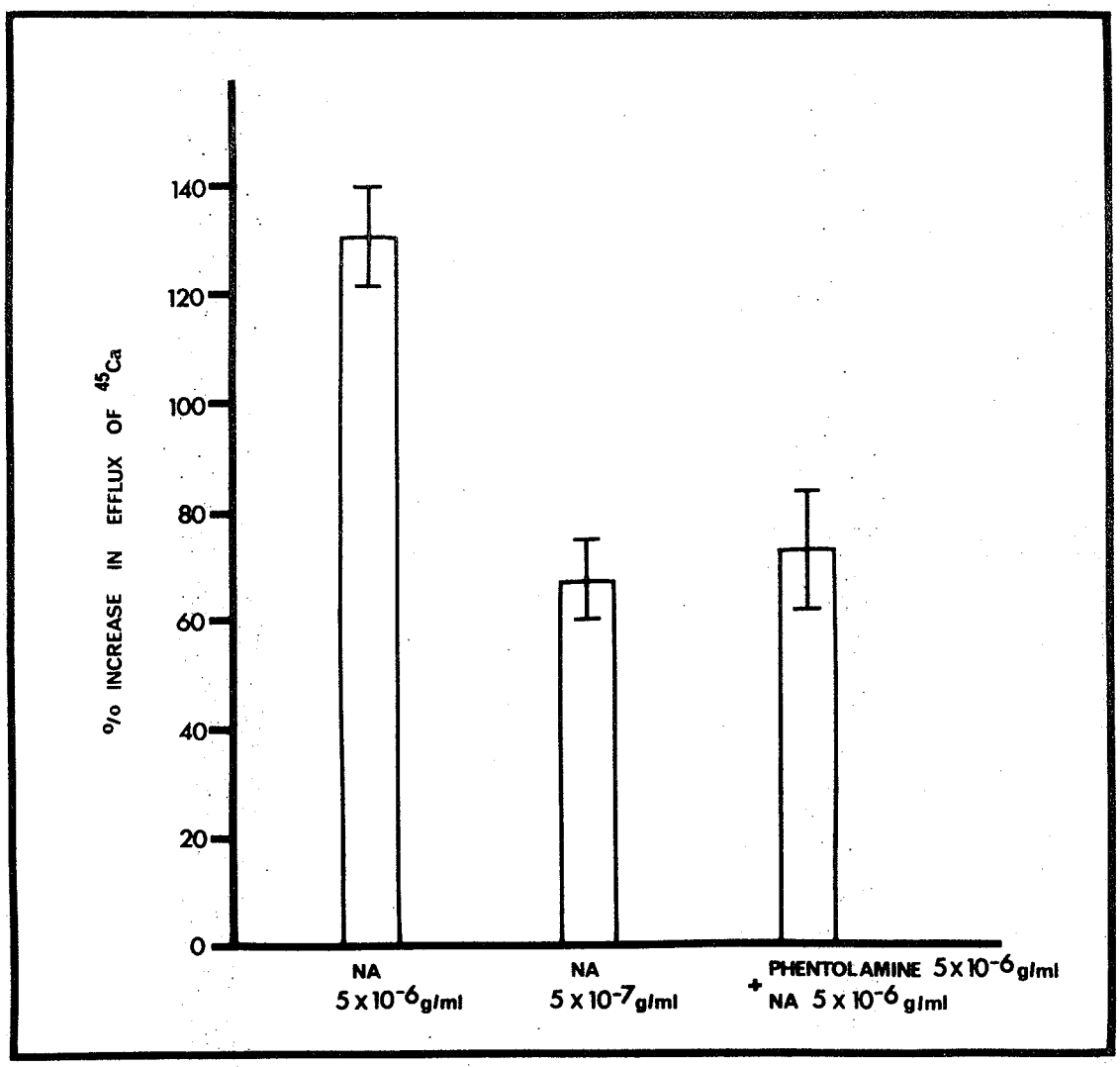


Fig. 19. Effect of phentolamine on the increase in ^{45}Ca efflux from spleen strips caused by noradrenaline (NA). Spleen strips loaded with ^{45}Ca in a ^{40}Ca-free medium were effluxed in Krebs-Henseleit solution and moved to fresh medium every 5 min. Radioactivity (counts/min) was measured in each sample of medium. Presence of NA (5 x 10-6 g/ml) in the medium between 50 and 55 min increased efflux more than NA (5 x 10-7 g/ml). In the presence of phentolamine (5 x 10-6 g/ml) the increase in efflux with the higher concentration of NA was reduced to equal that due to the smaller concentration of NA in the absence of phentolamine.

to a smaller value of 72.2 ± 10.5%. This was significantly different ($p < 0.01$) from the control response. There was no statistically significant difference between the response obtained with noradrenaline (5×10^{-7} g/ml) and that due to the higher concentration (5×10^{-6} g/ml) of the same in the presence of phentolamine.

In the previous studies on the pharmacological modifications of the two phases of contraction due to noradrenaline it was found possible to preferentially block the loosely bound calcium dependent slow phase with manganese chloride and the tightly bound calcium dependent fast phase with zinc chloride or procaine. It was therefore considered of interest to study the effect of these agents on the increase in ^{45}Ca efflux caused by noradrenaline.

Three groups of 5 tissues were loaded with ^{45}Ca as in the previous experiments. They were then washed out in a series of tubes containing 2 ml Krebs-Henseleit solution and manganese chloride (1 mM) or zinc chloride (4 mM) or procaine (0.5 mM). Noradrenaline (5×10^{-6} g/ml) was present in addition in the 10th tube between the 45th and 50th min of washout. Percentage increases in efflux due to noradrenaline were calculated and are shown in Fig. 20. Manganese chloride reduced the noradrenaline response to 114 ± 13.3% which was statistically not significantly different from the control at the 0.05 level. On the other hand procaine and zinc chloride caused the noradrenaline response to decrease to 70.66 ± 12.14% ($p < 0.05$) and 41.4 ± 12.32 ($p < 0.01$) respectively. Thus there appears to be a correlation between the ability of agents which inhibit the fast phase of the contractile response of spleen capsular smooth muscle and their ability to decrease the effect of noradrenaline

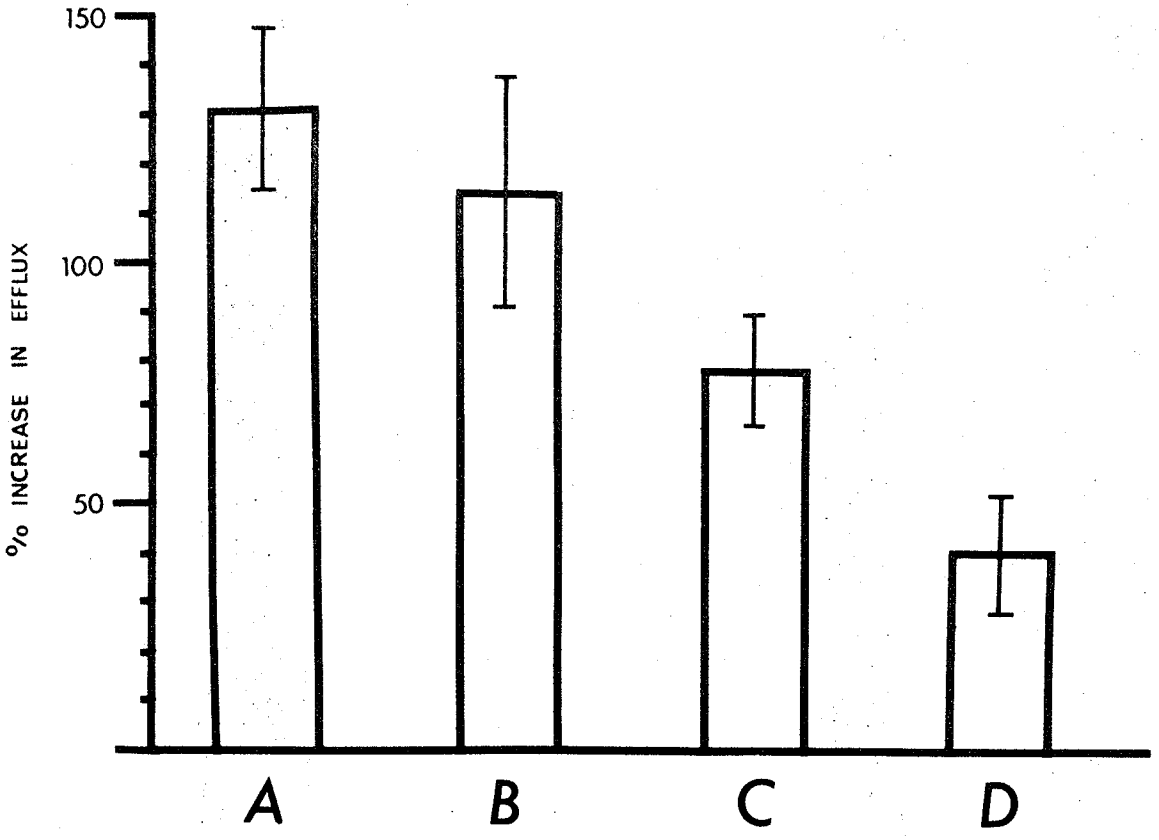


Fig. 20. Effect of $MnCl_2$, procaine and $ZnCl_2$ on the increase in ^{45}Ca efflux from spleen produced by noradrenaline.

Spleen strips loaded with ^{45}Ca in a ^{40}Ca-free medium were effluxed in Krebs-Henseleit solution alone or in Krebs-Henseleit solution containing $MnCl_2$ (1 mM) or procaine (0.5 mM) or $ZnCl_2$ (4 mM). Noradrenaline (5×10^{-6} g/ml) was present in the media between the 50th and 55th min.

A. Percentage increase in rate of ^{45}Ca efflux in control strips due to noradrenaline.

B. Same in the presence of $MnCl_2$.

C. In the presence of procaine.

D. In the presence of $ZnCl_2$.

in releasing calcium from a slowly emerging compartment of radioactive calcium.

c) Effect of manganese chloride and procaine on the increase in ^{45}Ca efflux promoted by ^{40}Ca

In these experiments 3 groups consisting of 10 tissues each were loaded with ^{45}Ca in the same manner as in the previous experiments. Five tissues from the first group were incubated in a calcium-free Krebs-Henseleit medium and the non-radioactive medium was changed every 5 min for 80 min. The remaining 5 tissues were incubated in a calcium-free medium for 50 min and in normal calcium Krebs-Henseleit medium from the 50 min to the 80 min. The incubation medium was changed every 5 min. At the end of the experiment the ^{45}Ca contents of the tissues were measured and the cumulative sum of efflux of ^{45}Ca into the incubation medium at the end of each 5 min period was determined. The ^{45}Ca remaining in the tissue after different times were plotted semilogarithmically. In a typical experiment shown in Fig. 21, the radioactivity in the tissue declined at the same rate in both subgroups of tissues. In the subgroup that was incubated throughout in calcium-free medium, the decline in tissue radioactivity became an exponential function after 30 min. In the other subgroup of tissues, in the presence of external calcium (2.5 mM), a greater decrease in the tissue ^{45}Ca was observed. The residual ^{45}Ca in both groups of tissue at the end of 80 min was measured and the further decrease in tissue ^{45}Ca due to addition of ^{40}Ca was expressed as a percentage of the ^{45}Ca remaining in tissues not exposed to calcium. After 2.5 mM of ^{40}Ca, the tissues contained only $45.3 \pm 2.35\%$ of the ^{45}Ca that was present at that time in untreated tissues.

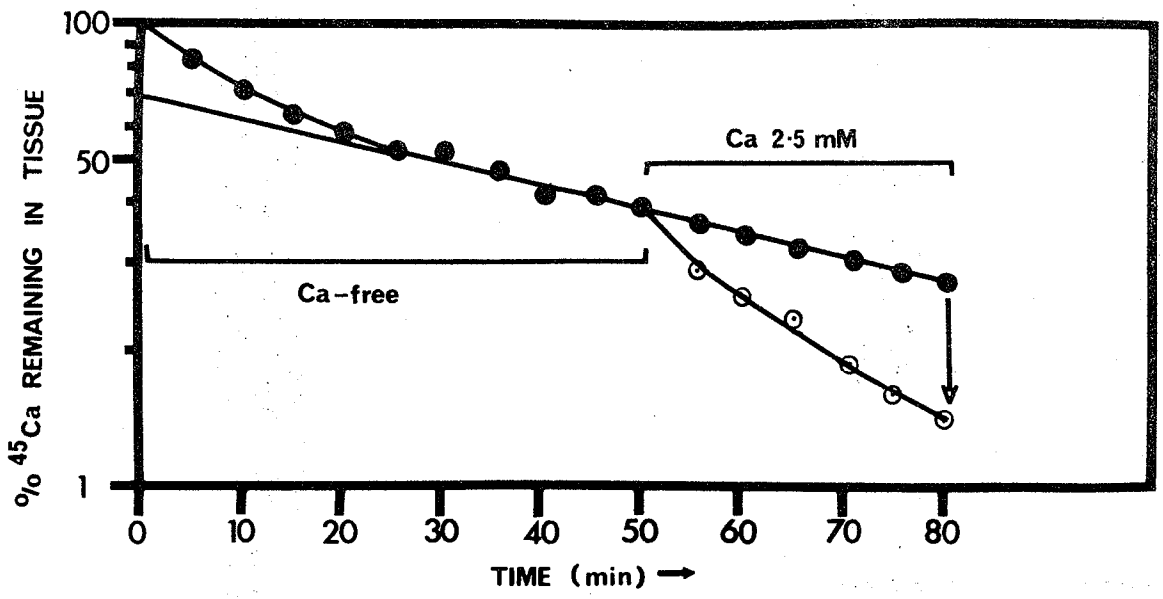


Fig. 21. Effect of ^{40}Ca on the efflux of ^{45}Ca from spleen strips. Two sets of strips loaded with ^{45}Ca in a ^{40}Ca-free medium were incubated in a ^{40}Ca-free medium and changed to a new medium every 5 min for 50 min (●). One group then continued in ^{40}Ca-free medium (●) and the other was transferred every 5 min to a new tube containing Krebs-Henseleit (⊙) solution. Radioactivity remaining in the tissue (expressed as a percentage of the initial radioactivity in the tissue, counts/min) was plotted as a function of time. The increased loss of ^{45}Ca in the presence of ^{40}Ca is noticeable.

Similar experiments were done in which procaine (0.5 mM) or manganese chloride (1 mM) were added to each of the tubes in which efflux occurred. Addition of ^{40}Ca (2.5 mM) resulted in virtually no change in the effect of ^{40}Ca on the residual ^{45}Ca content (48.1 ± 1.72 $p>0.05$) in manganese chloride treated tissues. In procaine treated tissues there was a slight though non-significant decrease in the effect of ^{40}Ca. The decrease in ^{45}Ca level was 41.3 ± 2.49% of the residual level in the absence of external ^{40}Ca.

These results show that the fraction of ^{45}Ca which exchanges with ^{40}Ca does not behave in the same way towards procaine or zinc chloride as the fraction of ^{45}Ca which is released by noradrenaline.

IX. Effect of noradrenaline on calcium influx in spleen capsular smooth muscle

The slow phase of contraction due to noradrenaline was found to be more sensitive than the fast phase to changes in external calcium concentration. It was therefore considered necessary to find out if an influx of extracellular calcium occurred when the capsular smooth muscle was stimulated with noradrenaline.

a) Effect of noradrenaline on total calcium concentration

Twelve spleen strips were incubated in Krebs-Henseleit solution for 1 hr. 6 tissues were stimulated with noradrenaline (5×10^{-6} g/ml) for 5 min, after which all the muscles were blotted gently. Wet and dry weights and total tissue calcium were determined. The untreated strips had a calcium content of 1.91 ± 0.266 mM/kg wet weight and a tissue water content of 80.33 ± 0.23%. The tissues made to contract with noradrenaline showed little change in these values, having a calcium concentration of

2.14 ± 0.33 mM/kg wet weight and a water content of 79.42 ± 0.28%. These changes were not statistically significant.

b) Effect of noradrenaline on ^{45}Ca influx

Ten spleen strips were incubated for 1 hr in Krebs-Henseleit solution. They were then divided into two batches and transferred to two beakers each containing 10 ml of Krebs-Henseleit solution and 4 μCi of $^{45}CaCl_2$. After 1 min noradrenaline (5×10^{-6} g/ml) was added to one beaker and a similar volume of Krebs-Henseleit solution added to the second beaker. 3 min later the strips were taken out, blotted, weighed and ashed at 600°C in a muffle furnace. The ash, dissolved in a 1% solution of lanthanum chloride in 5% hydrochloric acid was counted and the counts/min/g wet weight of tissue was calculated. Similarly the counts/min/ml of the radioactive incubation medium were also determined. The radioactive calcium space in the tissue was obtained by dividing the counts in the tissue by the counts in the medium.

The ^{45}Ca space in the unstimulated tissue was 0.95 ± .09 whereas the space in the tissue stimulated with noradrenaline was 0.96 ± 0.10. The difference between the spaces in the two groups of tissues was not significant at the 0.05 level.

In another experiment 10 tissues were first incubated in Krebs-Henseleit solution for 60 min. Five tissues were first made to contract with noradrenaline (5×10^{-6} g/ml) for 1 min and then 4 μCi of $^{45}CaCl_2$ was added to the bathing medium (volume 10 ml). The second group of 5 tissues was exposed to a similar radioactive bathing medium but not to noradrenaline. After 4 min, both sets of tissues and media were analysed for radioactivity. The ^{45}Ca space in the tissues stimulated with nor-

adrenaline before and during the presence of radioactive calcium had a space of 0.91 ± 0.15 while the unstimulated control had a ^{45}Ca space of 0.96 ± 0.11. Again the difference between the two spaces was not significant.

Since no measureable effect of noradrenaline on total tissue calcium or ^{45}Ca influx was observed, further studies with agents such as manganese chloride which affect the slow phase of contraction due to noradrenaline were not carried out.

X Study of functional interrelationship between multiple calcium stores

The results so far show that preferential modification of either phase of splenic smooth muscle response to noradrenaline is possible. This will suggest that the two different calcium stores or mechanisms involved in their utilisation can function independently and can therefore be considered to be arranged in a 'parallel' fashion. However, it was seen during the previous experiments that when high concentrations of the smooth muscle inhibitors were employed, both phases of contraction were diminished even though one phase may have been affected more. This suggests that either the chemical agents employed are somewhat unspecific as regards their site of inhibitory action or that the two stores of calcium or the different mechanisms of utilisation of calcium from these stores are not completely independent. The following experiments were done to find out whether an alternative arrangement in which the calcium stores besides providing calcium to the contractile machinery by independent mechanisms or pathways also interacted in series, e.g. it is possible that the tightly bound calcium pool may derive its calcium from the loosely bound pool and the contractile machinery its calcium from the two pools independently.

Such an arrangement could be considered as a 'series-parallel' arrangement.

a) Effect of manganese chloride on noradrenaline induced response of normal spleen strips and on restoration of responses to noradrenaline by calcium chloride in calcium depleted strips

In a previous section it has been shown that manganese chloride preferentially decreases the slow phase of contraction of spleen induced by noradrenaline, this being suggestive of an effect on the loosely bound store of calcium. In this series of experiments, the above decrease was measured quantitatively. Further, it was seen that after the strips were depleted of calcium both phases of contractile responses to noradrenaline were abolished, suggesting depletion of both the tightly and the loosely bound stores of calcium. The responses were restored fully on repletion of the stores with calcium chloride. If the tightly bound pool is replenished via the loosely bound pool then manganese should be able to reduce the size of the tightly bound store after the above procedure. Consequently replenished tissues should have a smaller fast phase in the presence of manganese chloride than normal tissues. On the other hand if the tightly bound store refills by an independent route then there should be no differences in the magnitude of the fast phases in the two situations.

In each of five experiments, four spleen strips, incubated in Krebs-Henseleit solution, were tested with noradrenaline (10^{-6} g/ml) until the magnitude of the fast and slow responses became reproducible. Two of the strips were then depleted of calcium by exposure to calcium-free Krebs-Henseleit solution containing EGTA (15 mM) for 20 min and then only to calcium-free Krebs-Henseleit solution for an additional 10 min. Noradrenaline (10^{-6} g/ml) failed to produce contraction in the majority of strips

tested. The residual contraction in some strips disappeared after an additional 15 min exposure to the calcium-free solution. The other two strips in Krebs-Henseleit solution were stimulated with noradrenaline every 15 min. Manganese chloride (1 mM) was added to one strip immersed in Krebs-Henseleit solution and to the other strip in calcium-free solution. Five minutes later calcium chloride (2.5 mM) was added to the two baths containing calcium-free solution. Fifteen minutes after addition of manganese chloride, all four strips were challenged with noradrenaline. The response of the tissue that was in Krebs-Henseleit solution and had not been treated with manganese chloride remained unchanged. The tissue that had been depleted of calcium but was not given manganese chloride recovered very nearly to the level seen at the beginning of the experiment. In Fig. 22, it can be seen that the tissue which was given manganese chloride while bathing in Krebs-Henseleit solution showed a preferential decrease of the slow (53.9 ± 5.6%) compared to the fast phase (29.5 ± 5.2%). This difference was highly significant ($p < 0.01$). Noradrenaline response in the tissue depleted of calcium but which was refilled with calcium chloride in the presence of manganese chloride also improved but was different from the similar tissue not treated with manganese chloride. The manganese chloride treated tissue after being refilled with calcium chloride showed similar inhibition of slow and fast phases (56.2 ± 6.8% and 56.25 ± 6.7% respectively). The inhibition by manganese chloride of fast phase in the 'refilled' tissue was significantly ($p < 0.01$) greater than in the tissue in which the calcium stores were already filled. The difference in inhibition of slow phase in the two sets of tissues, however, was not significant at the 0.05 level.

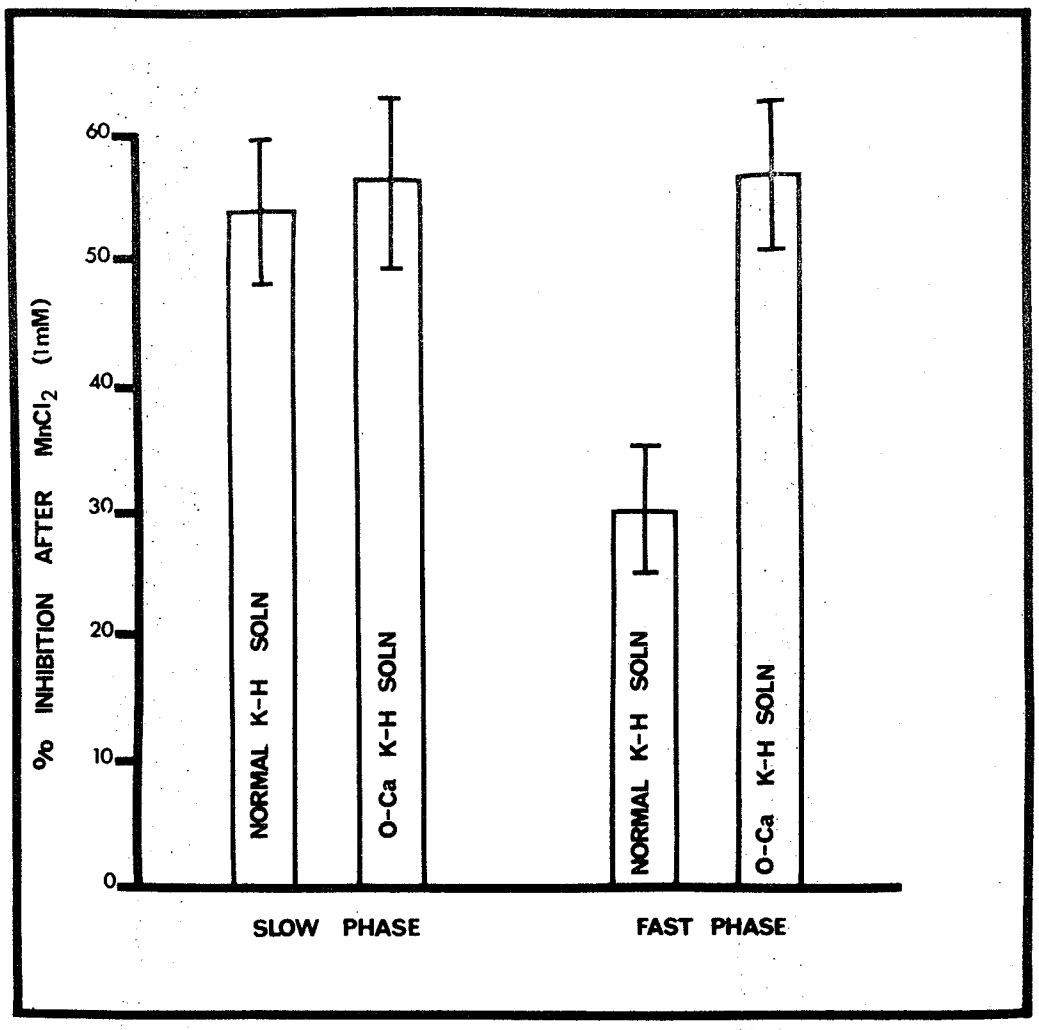


Fig. 22. Effect of $MnCl_2$ on normal spleen strips and on the repletion of calcium stores in depleted strips.

The first and third bars denote the percentage inhibition of slow and fast phases of response of normal spleen strips by $MnCl_2$ (1 mM).

Another set of spleen strips were first depleted of calcium in a Ca-free medium containing EGTA, 15 mM for 20 min and then in a Ca-free medium alone for 10 min. The strips did not respond to noradrenaline, 10^{-6} g/ml. $CaCl_2$ (2.5 mM) was then added in the presence of $MnCl_2$ and responses to noradrenaline obtained. The slow and fast phases of responses to noradrenaline after this procedure expressed as a percentage of the slow and fast phase in the same strips before depletion of calcium are shown in the second and fourth bars. The differences between the first and third and the third and fourth bars are highly significant ($p < 0.01$).

These results indicate that manganese affects the utilisation of tightly bound calcium from intact stores less than loosely bound calcium. However, when both stores are depleted, it also affects the refilling of the tightly bound pool or its subsequent availability for producing the fast phase.

b) Effect of substitution of strontium chloride for calcium chloride on the responses to potassium chloride and noradrenaline

Hudgins (1969) reported that after responses of rabbit aortic strips to noradrenaline and potassium had been nearly abolished by incubating the muscle in a calcium-free medium, substitution with barium or strontium chloride restored the responses to potassium more and for a longer time than those due to noradrenaline. Similar results for barium have been reported by Hinke (1965). The mechanism of this unequal restorative effect of barium and strontium on contractions produced by noradrenaline and potassium chloride is not clear but this evidence has been considered by Daniel (1965) to indicate that the calcium pools, differentially mobilised by noradrenaline or high potassium levels, are in parallel.

Experiments were done to find out first of all if there was a differential effect of strontium chloride in restoring the responses of the spleen strip to noradrenaline and potassium which had been abolished by calcium depletion. Barium could not be used in this study because it causes marked contracture of the spleen. Strontium on the other hand has very feeble effects which develop far too slowly, compared to the required duration of observation of responses in this study. In six experiments pairs of spleen strips obtained from reserpine treated cats were first stimulated with the two agonists in Krebs-Henseleit solution. Noradrenaline

(10^{-6} g/ml) was used for one strip in each experiment and high potassium Krebs-Henseleit solution (KCl 90 mM) for the other. Noradrenaline responses had a fast and a slow phase whereas only one phase could be observed in the response to high potassium. After reproducible responses were obtained with the two agonists, both tissues were repeatedly washed with a calcium-free Krebs-Henseleit solution for 45 min. The strips now failed to respond to high potassium (Ca-free) medium (Fig. 23aB) but noradrenaline still produced a small response. Fig. 23C,B shows that the slow phase of the response to noradrenaline after calcium depletion was only 5.49% of the control slow phase whereas the fast phase was 12.07% of the control. The muscles were washed free of agonists and strontium chloride (3 mM) was added to the calcium-free bathing media. Responses to high potassium were obtained with high potassium (Ca-free) bathing medium to which strontium chloride (3 mM) had been added. Noradrenaline was also tested in the Ca-free Krebs-Henseleit medium in the presence of strontium chloride.

As can be seen in Fig. 23 and 24A, the response to high potassium was restored by strontium chloride appreciably. The response to high potassium now showed two distinct phases and resembled the responses normally obtained due to noradrenaline. Repeated stimulation at 15 min intervals with high potassium produced a small preferential decrease in the fast phase after five to six responses. Exposing the tissue to a calcium-free solution, containing EGTA (15 mM) for ten minutes followed by a 5 min period during which EGTA was washed out and strontium chloride replaced resulted in a decrease in the response to high potassium (23.23% of control). This new response was significantly different ($p<0.01$) from the response to high potassium in Krebs-Henseleit solution containing

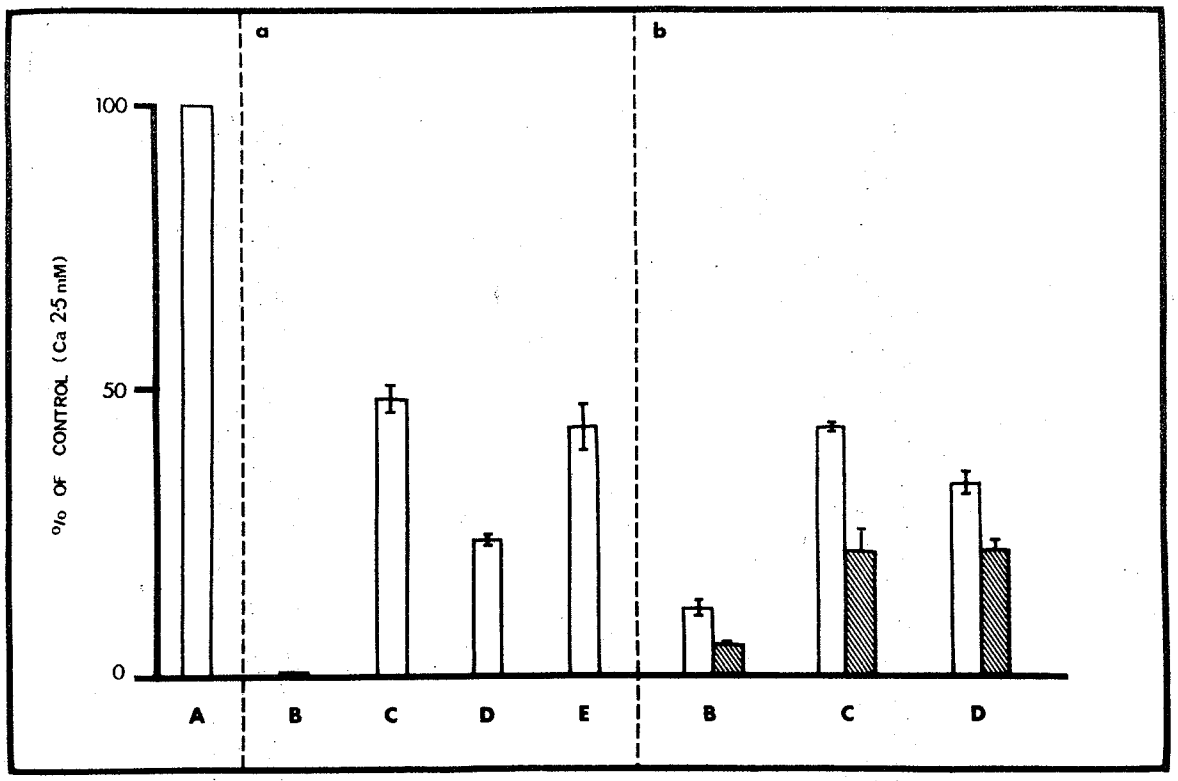


Fig. 23. Effect of $SrCl_2$ on the diminished responses of reserpine treated spleen to KCl and noradrenaline in calcium-free medium.

A. Control response to KCl or slow or fast phase of noradrenaline induced contraction in Krebs-Henseleit medium.

(a) Responses to potassium chloride

B. Response to KCl (90 mM) in Ca-free medium.

C. Response to KCl in Ca-free medium containing $SrCl_2$ (3 mM) .

D. Response to KCl in Ca-free medium containing $SrCl_2$ (3 mM) after brief exposure to EGTA (15 mM).

E. Response of the tissue to KCl in Ca-free $SrCl_2$ containing medium after brief prior exposure to Ca (2.5 mM) followed by exposure to Ca-free medium until responses were abolished.

(b) Responses to noradrenaline, 10^{-6} g/ml: slow (hatched bars) and fast (open bars) phases

B. In Ca-free medium.

C. In Ca-free medium after addition of $SrCl_2$ to Ca-free medium.

D. Tachyphylaxis after four contractions.

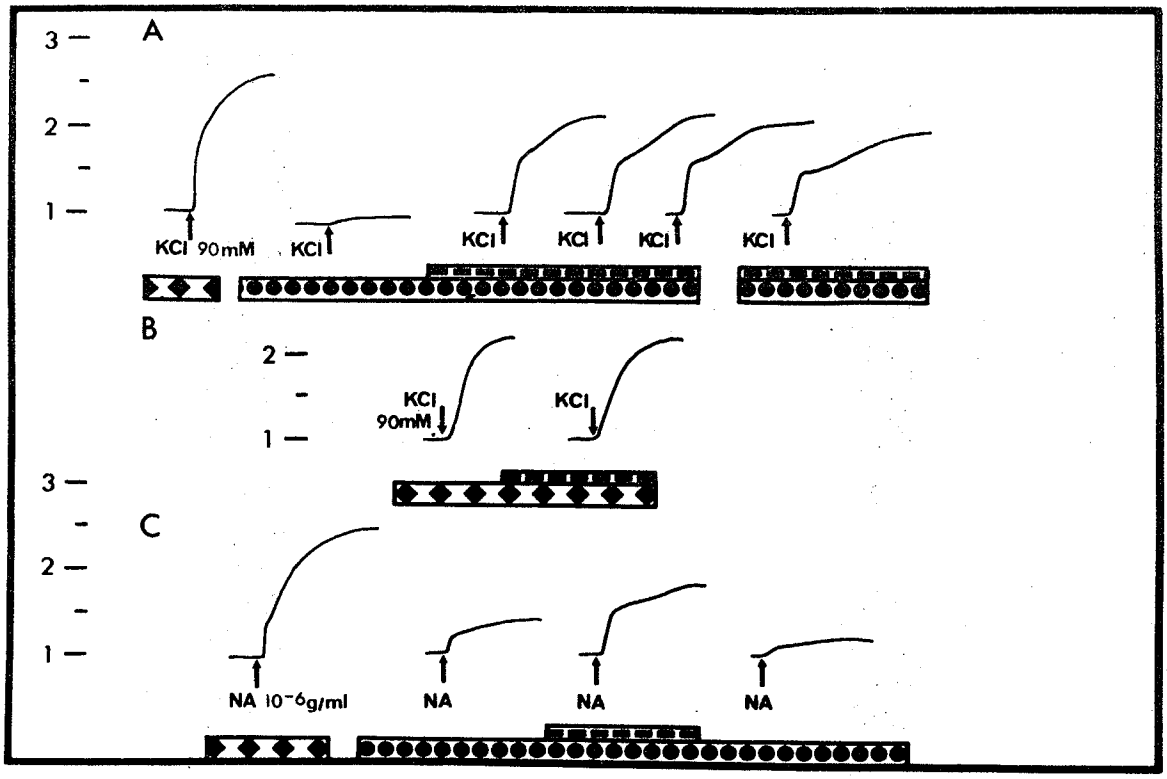


Fig. 24. Substitution of strontium for calcium.

A. Response of reserpine treated spleen to KCl, 90 mM, added as indicated, in Krebs-Henseleit solution (◆) was markedly decreased in calcium-free medium (●). After addition of $SrCl_2$, 3 mM, (▬) the first response showed recovery of amplitude and two phases. The next 2 responses at 15 min intervals showed little change, but the 7th response after 105 min in $SrCl_2$ was diminished. The intervening responses are not shown.

B. Responses to KCl, 90 mM, in Krebs-Henseleit solution. $SrCl_2$, 3 mM, added as indicated, had no effect on the response.

C. Response to noradrenaline, 10^{-6} g/ml, (NA) in Krebs-Henseleit solution, followed by diminished response in Ca-free solution. Addition of $SrCl_2$, 3 mM, resulted in partial recovery which was reversed on removal of $SrCl_2$ from the Ca-free medium.

strontium chloride, before the addition of EGTA. The strip was then exposed for 2 min to calcium chloride (2.5 mM) and the strip again restored to calcium-free solution until the response to high potassium was abolished. Strontium chloride was added to the calcium-free medium and the response to high potassium that was now obtained was significantly greater (43.21%) compared to 23.23% after EGTA ($p < 0.01$).

In another experiment (Fig. 24B) a single phase response to high-potassium in Krebs-Henseleit solution was first obtained. The solution was then washed out and strontium chloride (3 mM) was added to the standard Krebs-Henseleit solution bathing the muscle. After 20 min the muscle was challenged with a high-potassium Krebs-Henseleit solution that contained strontium chloride (3 mM). No difference was seen in the contraction due to this solution and the previous one. Thus the conversion of a single phase high potassium contraction into one with two phases is not an effect of strontium alone but needs the absence of external calcium.

Compared to high potassium the noradrenaline responses which had decreased in the calcium-free medium were restored from 12.07% (fast phase) and 5.49% (slow phase) to only 43.94% (fast phase) and 32.3% (slow phase) of the original response in Krebs-Henseleit solution in the presence of strontium chloride (Fig. 23bC and 24B). Strontium restored the noradrenaline response much less than the high potassium response ($p < 0.01$) and the restored noradrenaline response exhibited tachyphylaxis (Fig. 23bD).

The response of spleen to potassium chloride is more sensitive to reduction of external calcium than the fast phase of noradrenaline response. This was tested in the following manner: Spleen strips incubated in Krebs-Henseleit solution first stimulated with high potassium

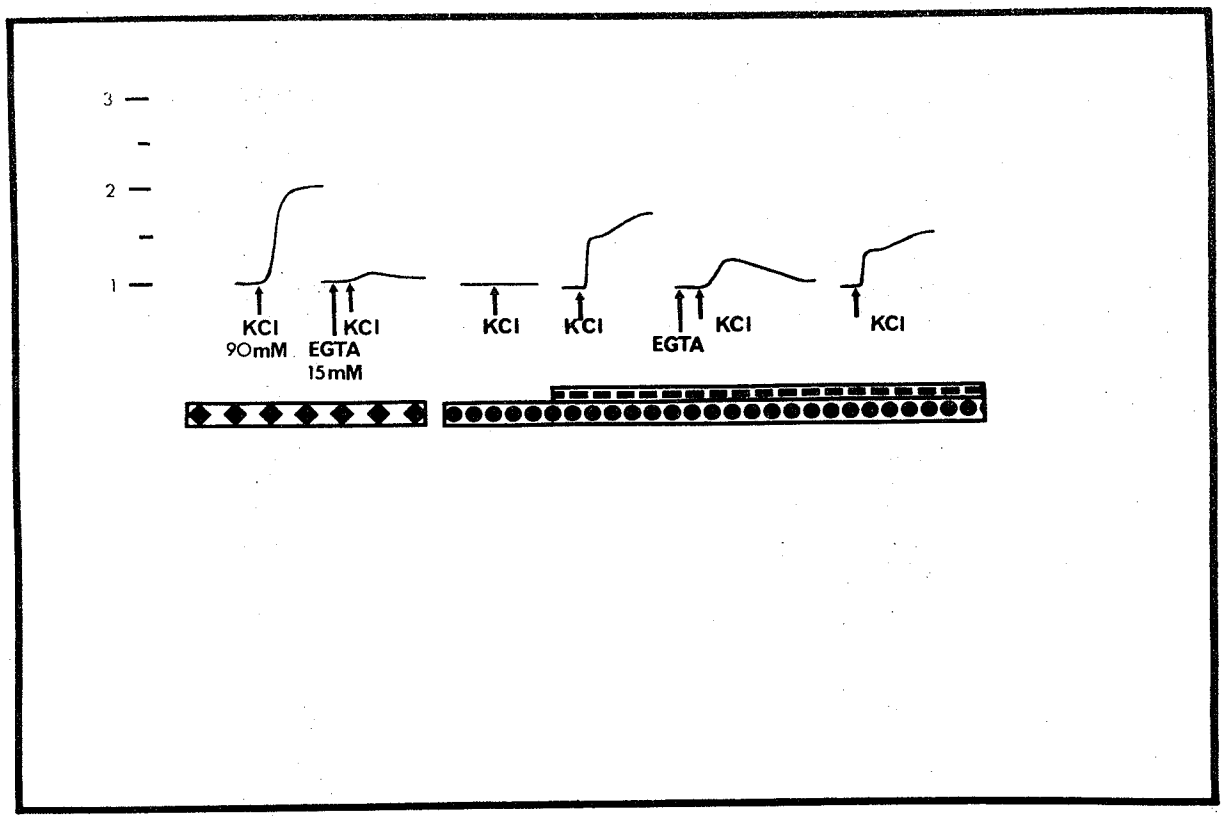


Fig. 25. Effect of strontium on the response of reserpine treated spleen to KCl in a Ca-free medium.

Response due to KCl, 90 mM, (1st response) in Krebs-Henseleit solution (◆) was markedly reduced when EGTA, 15 mM, was added 15 sec before KCl (2nd response). In a Ca-free medium (●) the response to KCl was abolished (3rd response). In the presence of $SrCl_2$, 3 mM, (▬) the KCl response was restored (4th response) and had 2 phases. EGTA reduced the restored response less (5th response) than the response in the presence of calcium (2nd response). Note the progressive decrease in the restored response (6th response).

solution at 15 min intervals to obtain control responses. EGTA (15 mM) was added to the Krebs-Henseleit solution and 15 sec later the strip was tested by replacing the bathing medium with the high potassium Krebs-Henseleit solution containing EGTA (15 mM). As seen in Fig. 25, a marked reduction in the response to high potassium solution occurred. The strips were then bathed for 5 min in Krebs-Henseleit solution and then in calcium-free Krebs-Henseleit solution until a Ca-free high potassium medium did not produce any contraction. Strontium chloride (3 mM) was then added to the Ca-free Krebs-Henseleit medium and a response was obtained with high potassium, Ca-free, medium containing strontium chloride. Fifteen minutes later a similar procedure was done except that EGTA (15 mM) was added to the calcium-free, $SrCl_2$-containing, Krebs-Henseleit solution 15 sec before the test and also added to the high potassium test solutions. The response to potassium was larger (280%, $p<0.05$) in this case than the one in the absence of strontium in the external medium.

These results indicate that unlike normal strips, a part of the response to high potassium which is restored by strontium chloride in calcium-depleted strips, is dependent on a tightly bound pool of calcium. It is also likely that strontium itself can substitute for calcium for activating the contractile mechanism directly. The relationship of extracellular strontium and the tightly bound calcium store seems to be of a 'series-parallel' type.

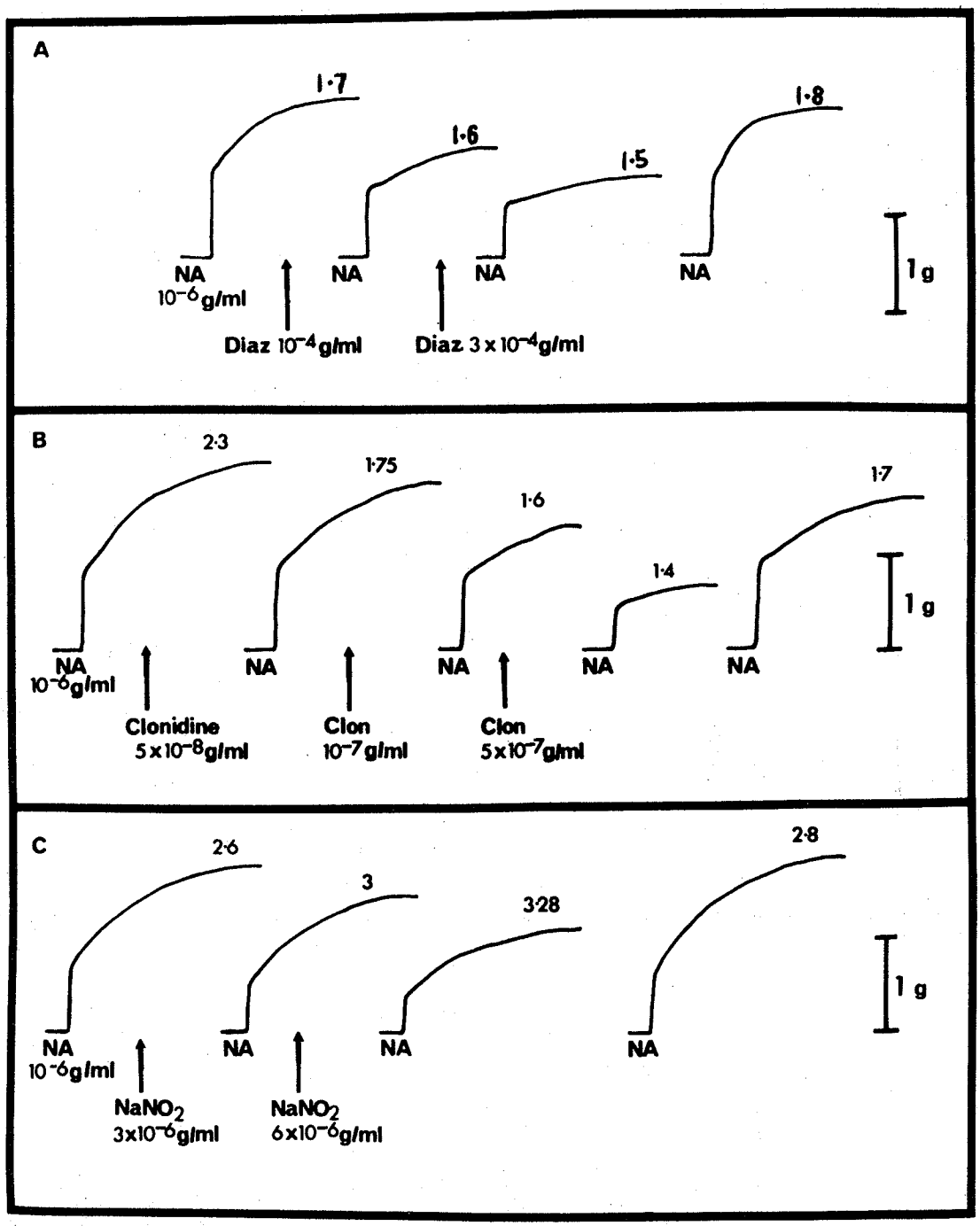
XI. Effect of smooth muscle relaxants on the two phases of contraction due to noradrenaline

Several drugs decrease smooth muscle activity and are considered as smooth muscle relaxants. Their therapeutic applications range from

hypertension and angina pectoris, to spastic conditions of the gastro-intestinal tract. The sites of action also vary widely; for example even among agents acting on vascular smooth muscle some act more on arteriolar muscles while others possess effects on the venous bed as well. The effects of a variety of smooth muscle relaxants on the two phases of contraction produced by noradrenaline were therefore studied to find if qualitative differences existed among these agents insofar as their effect on various stores or mechanisms of utilisation of calcium was concerned. Considerable differences were found between different smooth muscle relaxants in their ability to decrease the two different components of the response of spleen to noradrenaline. The experimental design consisted of initial testing of the spleen strips with noradrenaline (10^{-6} g/ml) at 15 min intervals until the responses became consistent. The smooth muscle relaxant under study was then added to the bathing medium and the muscle stimulated with noradrenaline in the presence of the test substance. If no change occurred, the concentration of the test substance was increased. This was done till a clear change in the response to noradrenaline occurred. The smooth muscle relaxant was then washed away and recovery of the muscle from inhibition was followed. Three to five experiments were done with each substance, and in the following description the figures are typical of the results obtained in the majority of experiments with each substance being tested.

a) <u>Agents preferentially affecting the slow phase of response to noradrenaline</u>

Diazoxide is an antihypertensive agent having a preferential effect on arteriolar smooth muscle in comparison with venous smooth muscle


A
1·7
1·6
1·5
1·8
NA
10⁻⁶g/ml
NA
NA
NA
1 g
Diaz 10⁻⁴g/ml
Diaz 3 x 10⁻⁴g/ml
B
2·3
1·75
1·6
1·4
1·7
NA
10⁻⁶g/ml
NA
NA
NA
NA
1 g
Clonidine
5x10⁻⁸g/ml
Clon
10⁻⁷g/ml
Clon
5x10⁻⁷g/ml
C
2·6
3
3·28
2·8
NA
10⁻⁶g/ml
NA
NA
NA
1 g
NaNO2
3x10⁻⁶g/ml
NaNO2
6x10⁻⁶g/ml

(Rubin et al., 1963). It has been shown to be a competitive antagonist of calcium in the rat aorta (Wohl et al., 1967). In the spleen, as can be seen in Fig. 26A, diazoxide (10^{-7} and 3×10^{-4} g/ml) caused a preferential decrease in the magnitude of the slow phase in response to noradrenaline (10^{-6} g/ml). This was indicated by a decrease in the ratio of slow to fast phases. The inhibitory action of diazoxide could be removed by washing the drug out.

A similar result was obtained using clonidine, 5×10^{-8}, 10^{-7} and 5×10^{-7} g/ml (Fig. 26B). This drug is also a new antihypertensive believed mainly to have a central site of action (Nayler et al., 1968) although there is some evidence of a peripheral site of action too (Larbi and Zaimis, 1970).

b) Agents preferentially acting on the fast phase of response to noradrenaline

Unlike diazoxide or clonidine, addition of sodium nitrite, 3×10^{-6} and 6×10^{-6} g/ml (Fig. 26C), aminophylline, 3×10^{-4} g/ml (Fig. 27a), chlorpromazine, 10^{-9} g/ml (Fig. 27B), papaverine, 3×10^{-6} and 5×10^{-4} g/ml (Fig. 27C) resulted in a preferential decrease of the fast phase of contraction. The effect of sodium nitrite on the vascular system differs from that of diazoxide in that this drug has a greater effect on the venous capacitance bed (Åblad and Mellander, 1963). Chlorpromazine is a smooth muscle depressant under experimental conditions (Godfraind and Kaba, 1969), aminophylline has a generalised smooth muscle relaxing ability and papaverine besides being a smooth muscle relaxant is also an inhibitor of oxidative phosphorylation (Ferrari and Carpenedo, 1968).

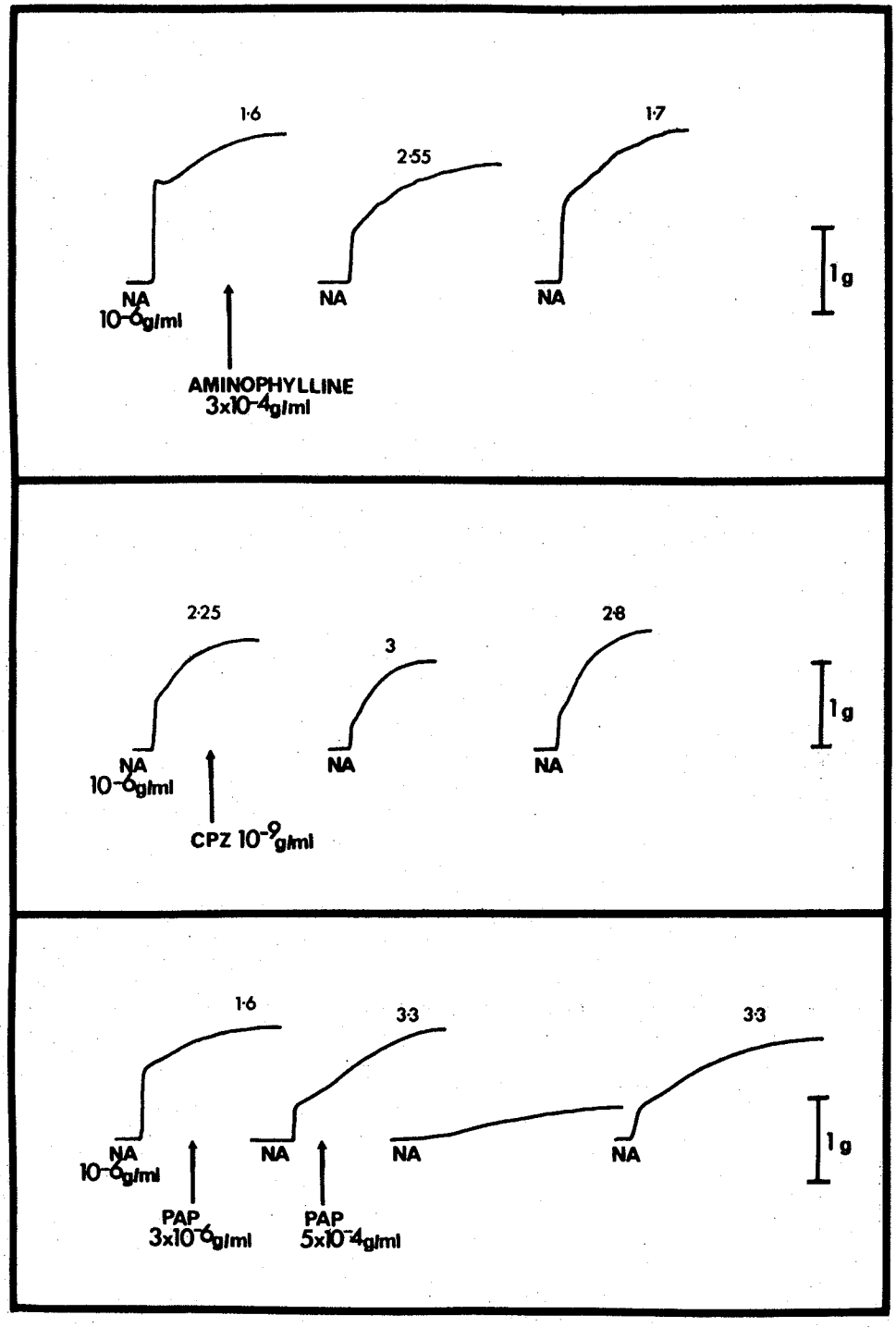
Fig. 27. Effect of aminophylline, chlorpromazine (CPZ) and papaverine (PAP) on the phases of contraction due to noradrenaline (NA), 10^{-6} g/ml.

A. Effect of aminophylline, 3×10^{-4} g/ml;

B. Effect of chlorpromazine, 10^{-9} g/ml;

C. Effect of papaverine, 3×10^{-6} and 5×10^{-4} g/ml.

Numbers above each contraction indicate the proportion of slow to fast phases.


1·6
2·55
1·7
NA
10⁻⁶g/ml
NA
NA
1g
AMINOPHYLLINE
3x10⁻⁴g/ml
2·25
3
2·8
NA
10⁻⁶g/ml
NA
NA
1g
CPZ 10⁻⁹g/ml
1·6
3·3
3·3
NA
10⁻⁶g/ml
NA
NA
NA
1g
PAP
3x10⁻⁶g/ml
PAP
5x10⁻⁴g/ml

XII. Effects of substrate depletion on two phases of contraction of spleen strips

Metabolic energy is required not only for the work performed by the contractile proteins but also for maintaining the integrity of the cell membrane and for driving the various active processes that maintain the homeostasis of cells. Accordingly, the effect of substrate (glucose) depletion on the contractility of the spleen was studied. Spleen strips were first stimulated in Krebs-Henseleit solution with noradrenaline (3×10^{-7} g/ml) every 15 min until the responses became constant. The muscles were then exposed to a Krebs-Henseleit medium from which glucose had been removed. No substitution was made to maintain normal osmolarity of the medium. The muscle continued to respond to noradrenaline for 30 - 60 min without any change in contractility or in the ratio of slow to fast phases. After this or earlier if the muscle was stimulated for 5 min with a high concentration of noradrenaline (10^{-4} g/ml), there was a preferential decrease in the fast phase as well as a decrease in total active tension in 3 out of 5 experiments (Fig. 28A). After prolonged absence of glucose (2 hr or more) both phases were similarly decreased. On returning the strips to a glucose containing medium, the contractility returned to normal.

Since there was considerable scatter in the trend and magnitude of change of the fast and slow phases in the absence of glucose no attempt was made to analyse in detail the effect on the two phases. However, in a further series of experiments spleen strips were first exposed to the substrate free medium until contractility was diminished. After this a series of metabolic intermediates were tested for their ability to restore the splenic responses to noradrenaline. Fig. 28B shows a typical response

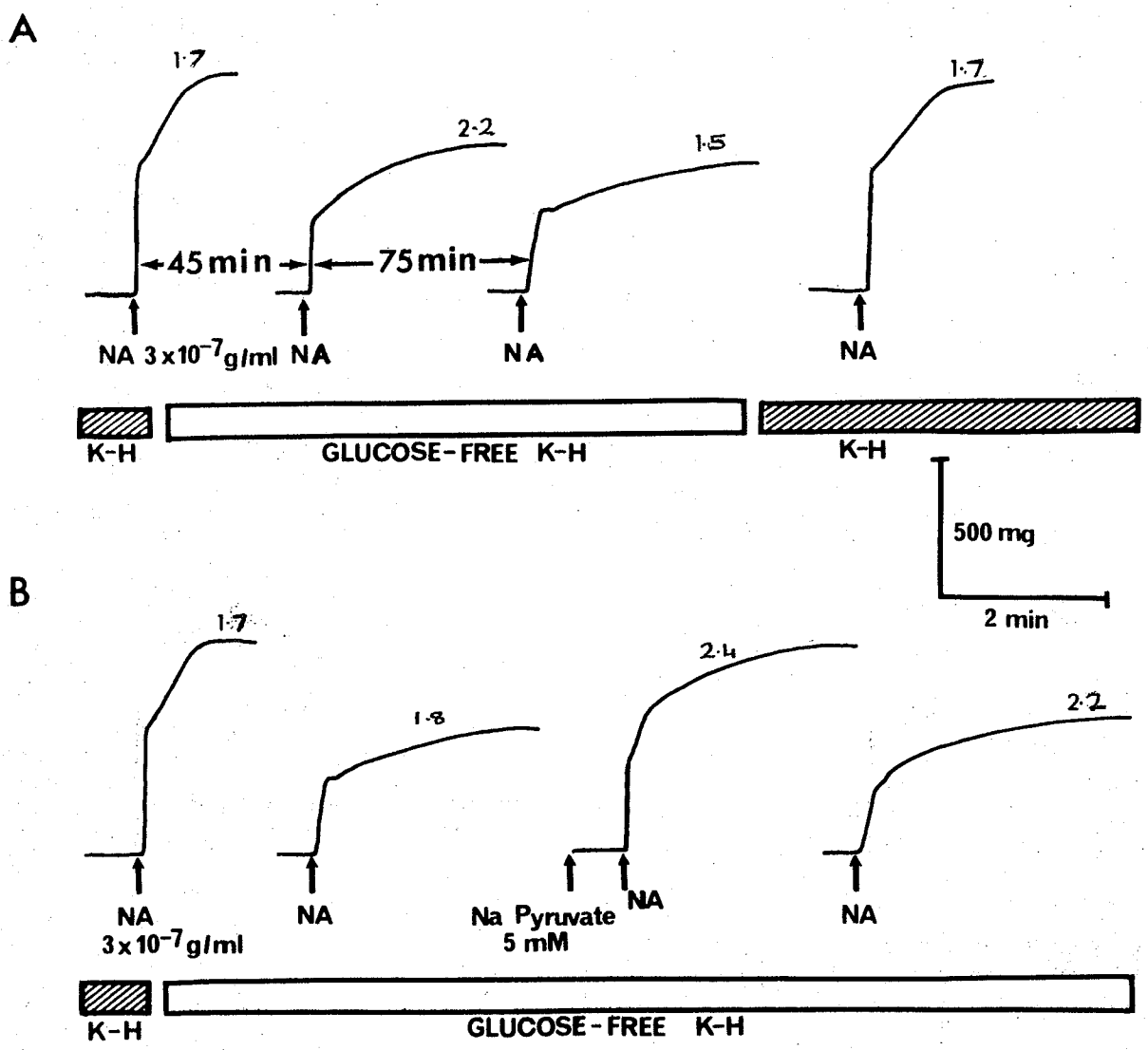


Fig. 28. Effect of substrate depletion on slow and fast phases of contraction due to noradrenaline.

Contractions of spleen strips to noradrenaline, 10^{-6} g/ml, (NA). Numbers above the records indicate the ratio of slow to fast phases.

A. After responding to NA in Krebs-Henseleit solution (KH) the strip was repeatedly stimulated in a glucose-free medium (glucose-free K-H). After 45 min there was a preferential decrease in fast phase but 30 min later both phases were nearly equally affected. Restoration of glucose (KH) reversed the inhibition of tension production.

B. After a response to NA in K-H medium the strip was exposed to glucose-free K-H medium. Decrease in tension and preferential decrease in fast phase was seen. Addition of sodium pyruvate restored the response to NA. Removal of pyruvate resulted in inhibition of active tension production.

to sodium pyruvate (5 mM). It can be seen that the response to noradrenaline recovered to near normal level and was depressed again when pyruvate was withdrawn. This result suggests that the Krebs cycle can provide metabolic energy for contraction of the spleen. Similar restorative effects were seen after oxaloacetate (5 mM) and sodium acetate (5 mM). On the other hand succinate (5 mM) and citrate (5 mM) failed to reverse the inhibitory effect of glucose deprivation.

XIII. Effects of altered external potassium chloride concentration on responses to noradrenaline

Smooth muscle membrane potential is influenced to a large extent by the transmembrane potassium gradient. Elevation of external potassium is expected to depolarise the muscle, while a decrease should produce hyperpolarisation. The magnitude of these changes will, to some extent, be decreased by other effects of external potassium, e.g. on potassium permeability. The aim of these experiments was to alter the external potassium chloride concentrations and study the effects on the two phases of the contraction produced by noradrenaline. If the production of these phases is dependent on electrical or nonelectrical processes, the results will shed some light on the mechanisms involved.

a) Effect of increased concentration of external potassium chloride

In six experiments, strips of spleen capsule obtained from cats which had been administered reserpine (1 mg/kg) 24 hours earlier, were stimulated with noradrenaline (10^{-6} g/ml) until constant responses were obtained. The Krebs-Henseleit bathing medium was then changed to one containing 10 mM potassium chloride and a proportionately smaller concen-

tration of sodium chloride. After 15 min a response to noradrenaline was obtained. The bathing medium was then changed to one containing 30 mM potassium chloride and the response to noradrenaline tested. In a similar fashion the responses were tested in the presence of 45 and 60 mM potassium chloride and finally in a medium containing 140 mM potassium substituting for all the sodium in the medium for producing nearly complete depolarisation. The tissues were then bathed again in Krebs-Henseleit solution and the response to noradrenaline obtained. The results are presented in Figs. 29 and 30A.

Elevation of external potassium chloride concentration from 5.4 mM to 10 mM did not change the magnitude of the fast or slow phases of noradrenaline responses although the slow phase became steeper. In the presence of 30 mM potassium chloride a small contraction occurred and the slow phase of the response to noradrenaline increased to 126% of the control. This small increase, however, was not statistically significant at the 0.05 level. On the other hand, the fast phase was 152% of the control and this potentiation was highly significant ($p < 0.01$). With an external potassium chloride concentration of 60 mM the muscle tension increased from 1 g to 1.6 g. Noradrenaline now produced a fast phase which was 116% of control, while the slow phase was smaller than control (69%). In the presence of the maximally depolarising concentration of 140 mM potassium the muscle tension increased to 1.8 g, the slow phase was decreased further (59% of control, $p < 0.05$) while the rapid phase changed little (122% of control). With 5.4 mM potassium in the external medium the fast phase constituted 30% of the total contraction; in the muscle depolarised by 140 mM potassium it made up nearly 80% of the total contraction. This was

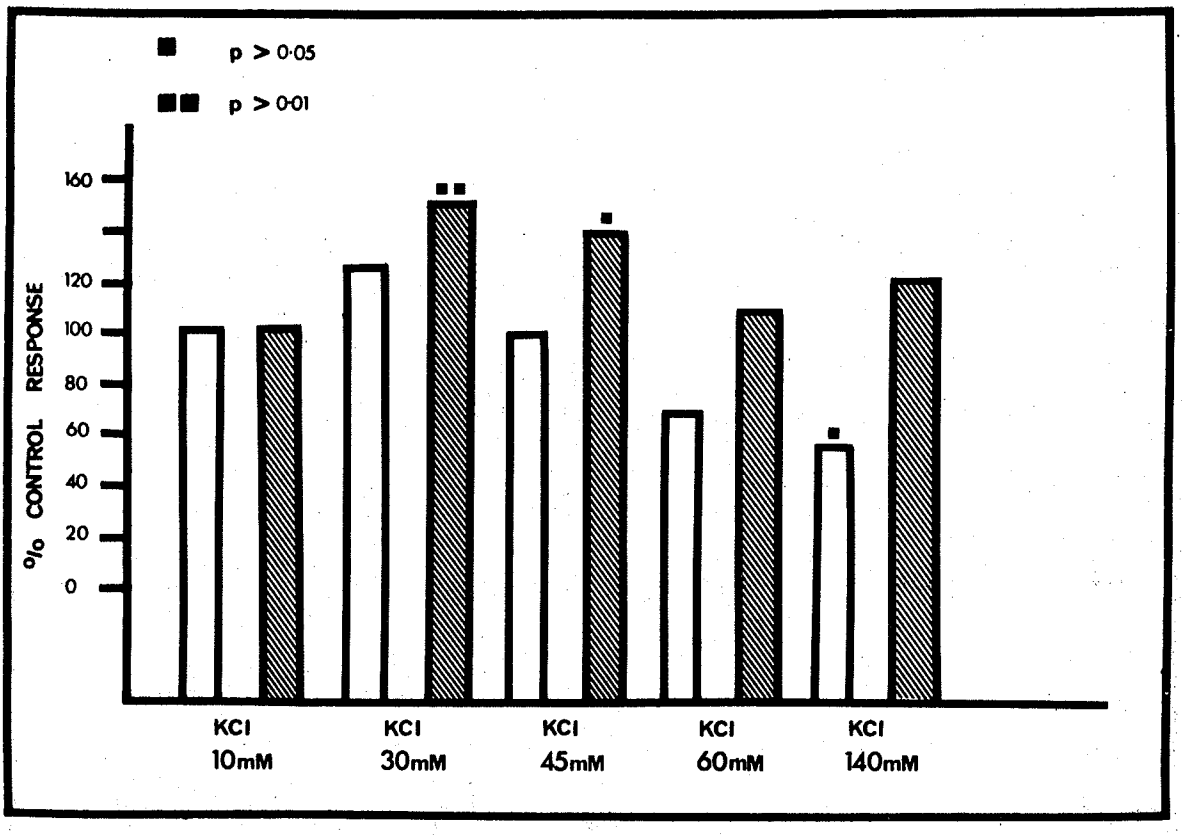


Fig. 29. Effect of potassium on the slow or fast phases of contraction.

Slow (open bars) and fast (hatched bars) phases of contraction due to noradrenaline, 10^{-6} g/ml, in spleen strips from cats treated with reserpine, 1 mg/kg, 24 hours before experiment. Responses in bathing solution containing concentrations of KCl, as indicated, are expressed as a percentage of the corresponding response to noradrenaline in Krebs-Henseleit solution.

due to a decrease in the slow phase.

Earlier it was shown that when 15 mM EGTA was added to a medium containing 2.5 mM calcium chloride 15 sec before noradrenaline, the slow phase was markedly reduced while the fast phase was very little affected. In order to find out if the fast phase of the response to noradrenaline in the medium containing 140 mM potassium was as dependent on tightly bound calcium as the fast phase in Krebs-Henseleit solution the following procedure, recorded in Fig. 30B, was carried out. A control response to noradrenaline (10^{-6} g/ml) was obtained. The fast phase was 0.55 g and constituted 40% of the total response. The response to noradrenaline was then determined in the presence of 140 mM potassium. The fast phase was now 0.56 g, about 69% of the total response. Subsequently the muscle was immersed in 140 mM potassium and EGTA (15 mM) was added, causing a prompt decrease in tension. After 15 sec when noradrenaline was added, a rapid but transient increase in tension occurred. The increase in tension was equal in magnitude to the fast phase of noradrenaline in 140 mM potassium in the absence of EGTA.

It has been shown earlier that the fast phase of noradrenaline response obtained when the muscle is incubated in Krebs-Henseleit solution is also selectively preserved while the slow phase is reduced if EGTA (15 mM) is added 15 sec before the contraction. It can be concluded from these observations that an increase in external concentration of potassium chloride has a dual effect on contractility. Up to a concentration of 30 mM, potassium chloride increases both phases but at higher concentrations the slow phase dependent on extracellular calcium is preferentially reduced. The rapid phase dependent on tightly bound calcium is resistant

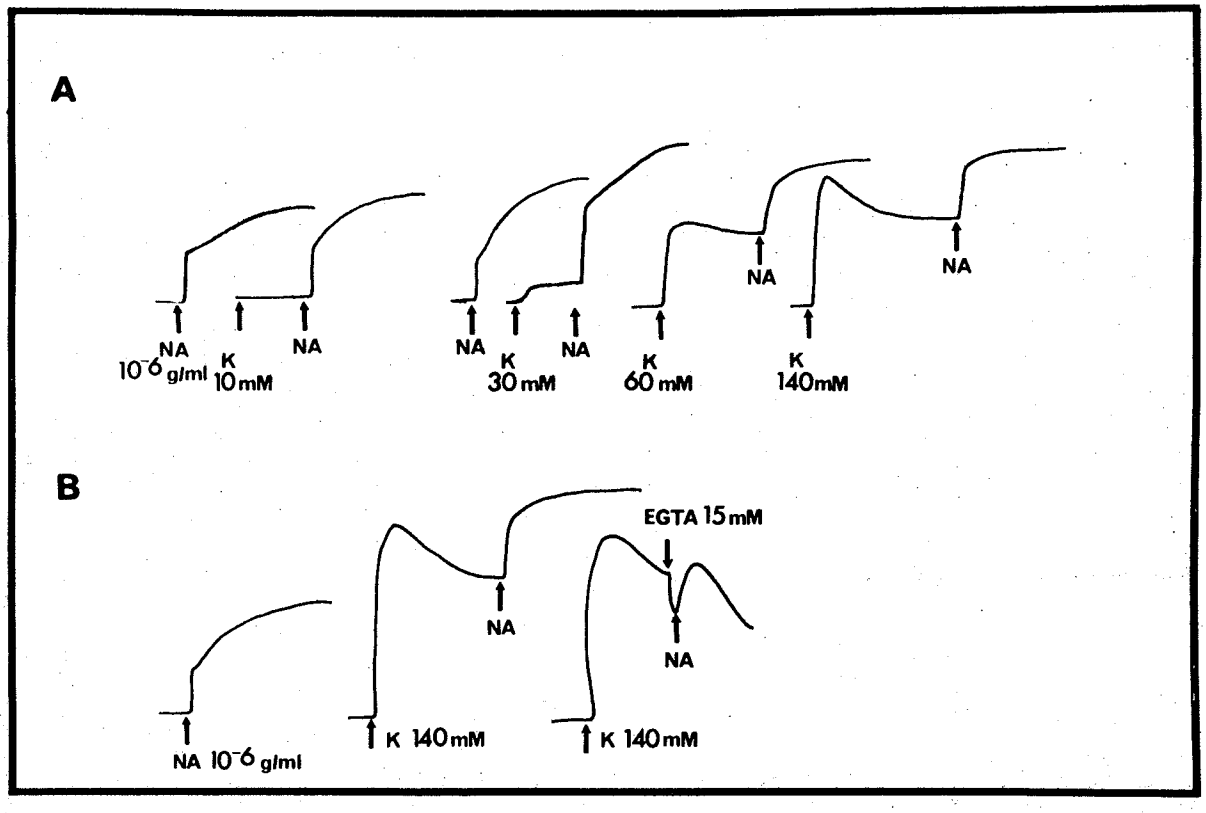


Fig. 30. Effect of increasing concentrations of potassium on the phases of contraction due to noradrenaline.

A. Responses to noradrenaline, 10^{-6} g/ml, (NA) in the presence of 10, 30, 60, and 140 mM potassium.

B. Responses to noradrenaline, 10^{-6} g/ml, (NA); the effect of potassium, 140 mM, and the effect of rapid reduction of available external calcium by EGTA, 15 mM.

to changes in membrane potential due to very high concentrations of potassium chloride.

b) Effect of reduction of potassium chloride on responses to noradrenaline

In six experiments (represented in Fig. 31A), spleen strips in standard Krebs-Henseleit solution were stimulated with noradrenaline (10^{-6} g/ml). After the preparations gave reproducible responses the bathing medium was changed to one which contained no potassium. The medium was at first changed every minute for 5 min and then every 5 min thereafter. Noradrenaline was added to the bath every 15 min. Removal of potassium from the bathing medium did not change the resting tension of the muscle. This is unlike bovine facial arteries, which contract under similar circumstances (Brecht et al., 1969). Responses to noradrenaline did not change during the first 30 - 60 min in potassium-free solution (Fig. 31B). When the muscle was returned to the original potassium-containing Krebs-Henseleit solution no change in tension occurred. When noradrenaline was tested 2 min later, very little change was seen in the fast phase but the slow phase was markedly reduced (Fig. 31C). This inhibition was reversed promptly if the muscle was returned to a potassium-free medium. Addition of potassium chloride (5 mM) to the medium during a steady contraction induced by noradrenaline resulted in a prompt relaxation of the muscle (Fig. 31D).

In another three experiments spleen strips were stimulated with noradrenaline (10^{-6} g/ml) in Krebs-Henseleit solution. After being left in a potassium free medium for thirty minutes without being stimulated they were restored to Krebs-Henseleit solution for 2 min and then challenged

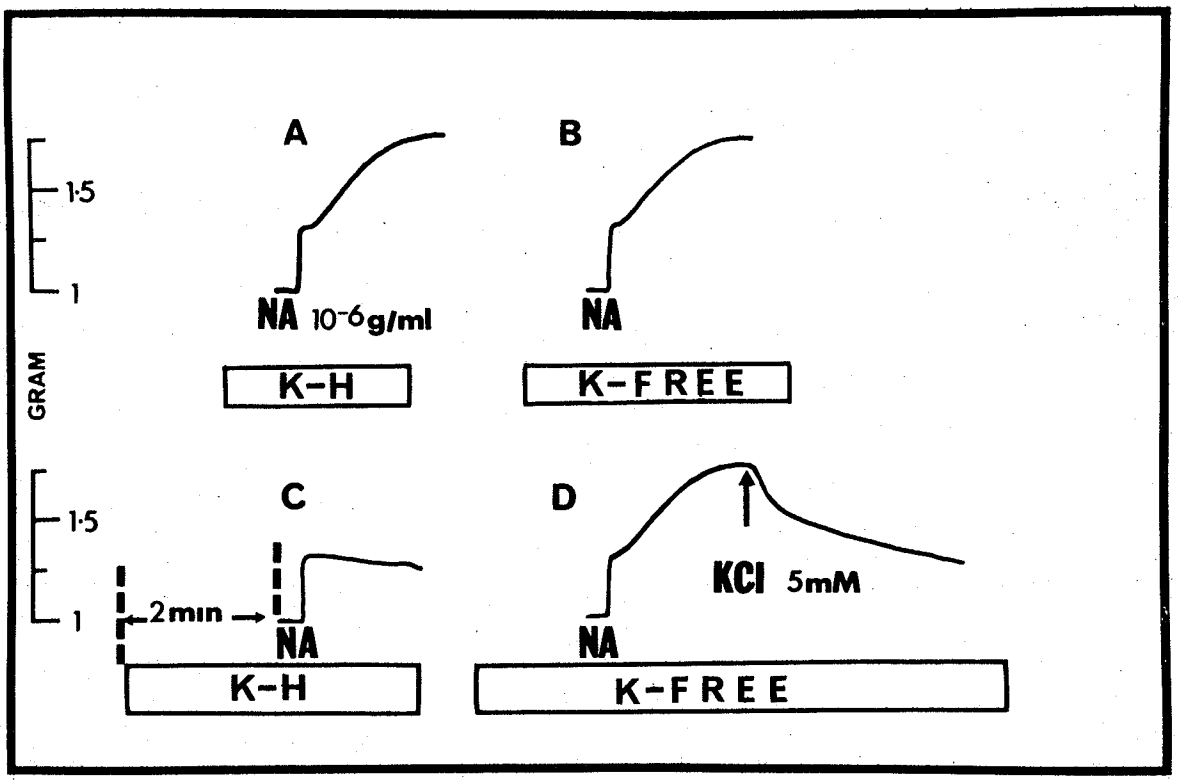


Fig. 31. Effect of exposure to potassium-free medium and subsequent restoration to Krebs-Henseleit medium on the responses of spleen to noradrenaline.

A, B, C, D. Contractions to noradrenaline, (NA), 10^{-6} g/ml.

A, in Krebs-Henseleit medium; B, after change of bathing fluid to K-free solution; C, 2 min after restoration of K to the solution; D, after replacement in K-free solution. KCl added at the peak of contraction resulted in a decrease in tension.

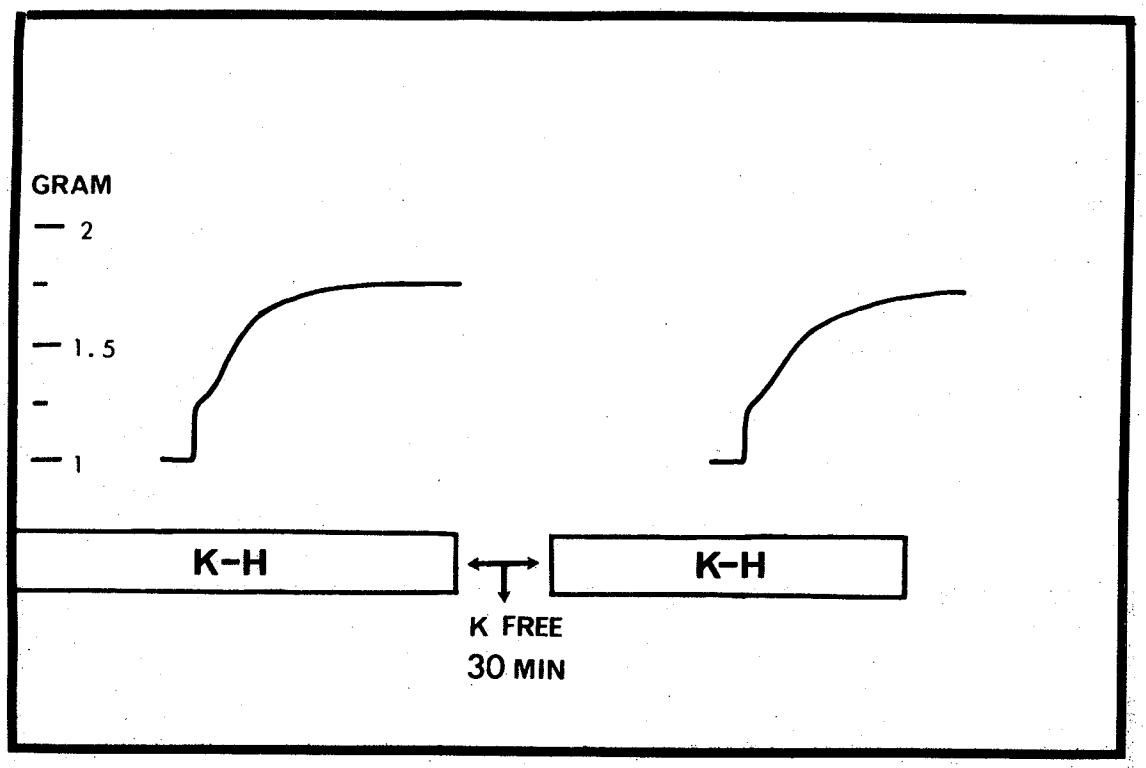


Fig. 32. Lack of effect of simple exposure of spleen to a K-free bathing medium on the subsequent effect of noradrenaline (10^{-6} g/ml) in normal Krebs-Henseleit solution (K-H).

with noradrenaline (Fig. 32). Unlike the previous experiment, in the absence of previous stimulation of the muscle while in a potassium-free environment, subsequent restoration of potassium did not result in inhibition of the slow phase of noradrenaline responses.

These experiments indicate that absence of potassium does not markedly alter the responsiveness of splenic capsular smooth muscle to noradrenaline, but creates a situation in which the muscle is inhibited by subsequent restoration of potassium to the external medium. It is also evident that the depression produced by potassium depends upon some changes occurring during stimulation of the muscle in a potassium-free medium.

In a further series of six experiments the muscles were stimulated in a potassium-free medium and the medium was then replaced with Krebs-Henseleit solution. Responses to noradrenaline decreased markedly when tested 5 min after the replacement and were 63.4% of the control responses in the potassium-free solution. However, with the muscles kept continuously in Krebs-Henseleit solution, the responses recovered, reaching control levels in 45 min (Fig. 33).

Although the inhibition of noradrenaline response by a pretreatment with potassium (5.4 mM) for 5 min before stimulation became less when the muscle was continuously exposed to the same concentration of potassium and tested later, when the inhibition was produced in another way as shown in Fig. 34 a different result was obtained. In the upper panel (Fig. 34A) potassium (5 mM) decreased the response of noradrenaline (10^{-6} g/ml) to 64% of the control response in a potassium-free medium. Noradrenaline was washed out and the muscle left in Krebs-Henseleit solution for 30 min. The response to noradrenaline increased to 89% of

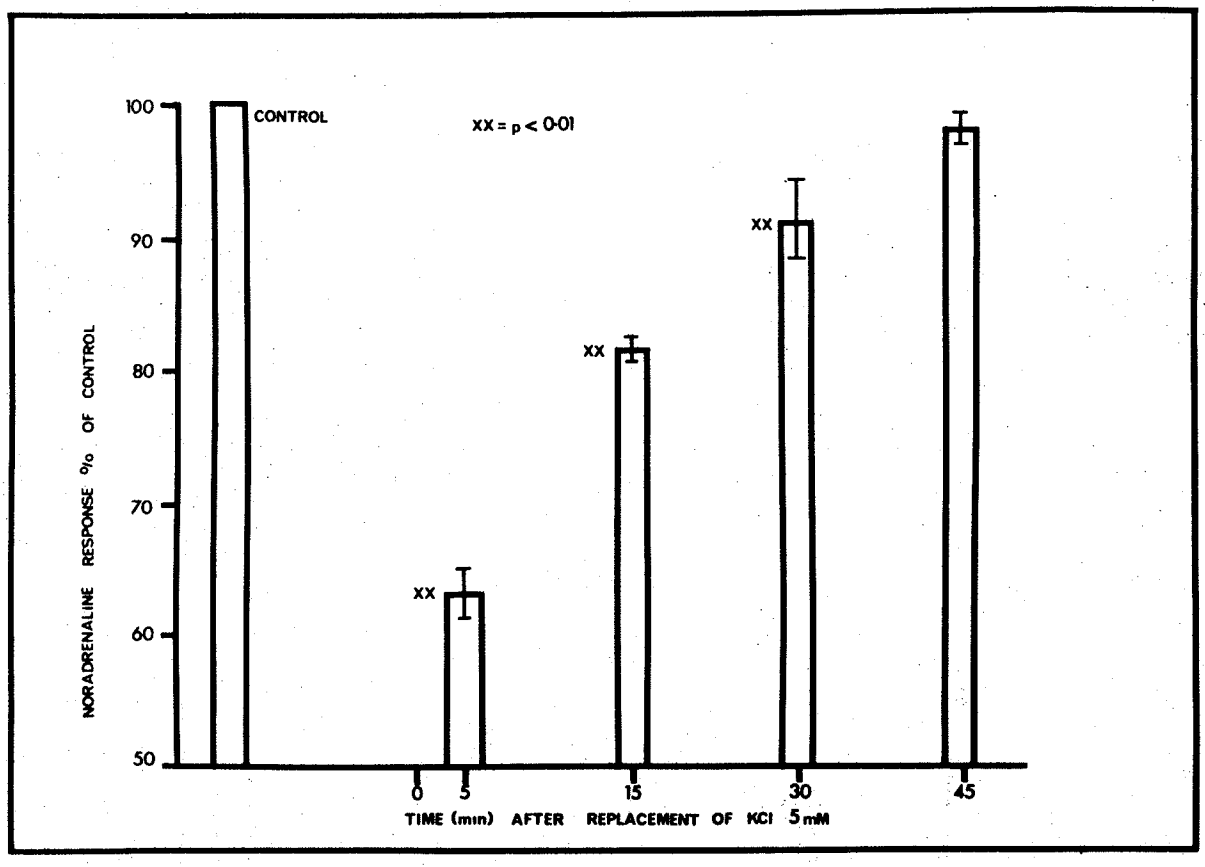


Fig. 33. Time course of loss of inhibitory effect of KCl on the response of spleen to noradrenaline after the muscle had initially been stimulated with noradrenaline, 10^{-6} g/ml, repeatedly in a K-free medium.

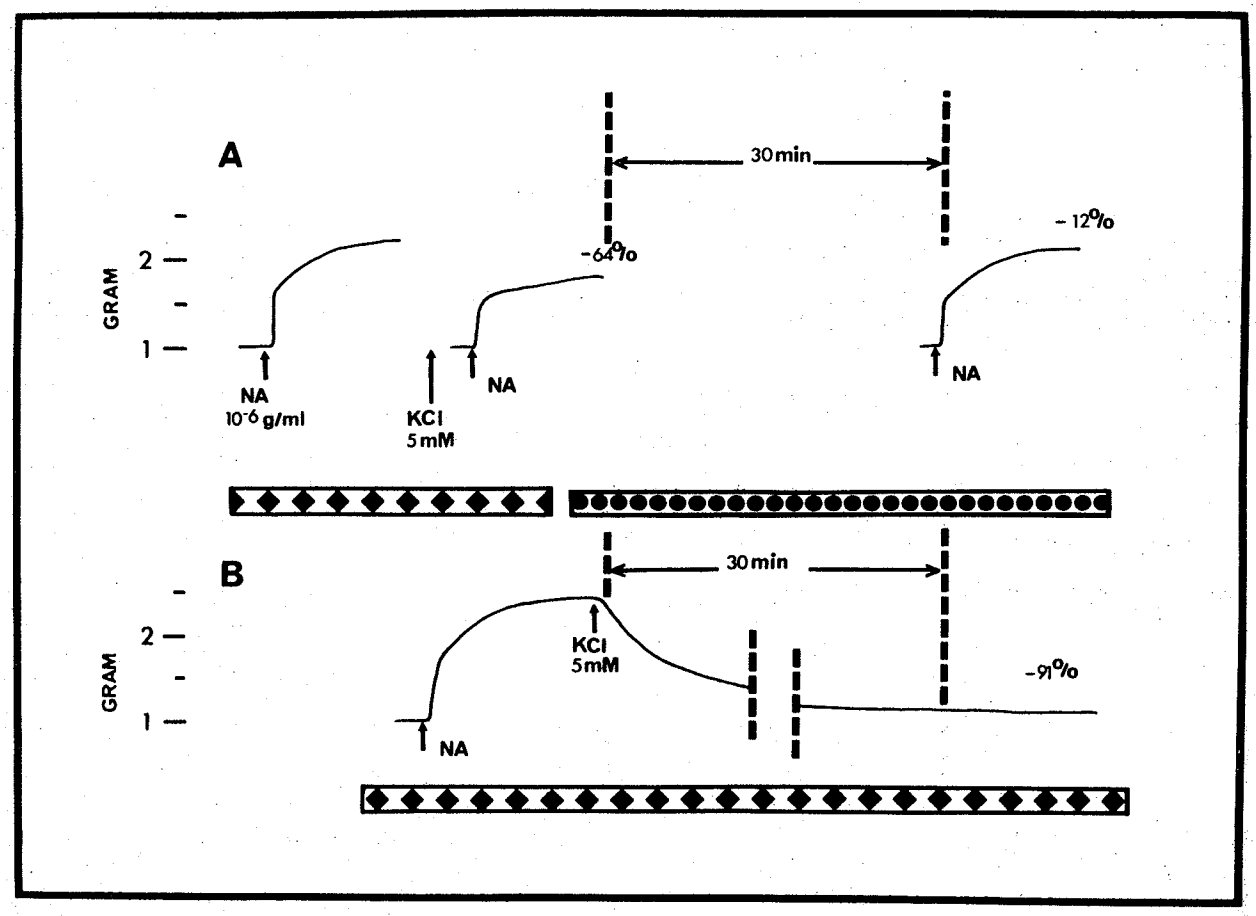


Fig. 34. Prevention by noradrenaline of recovery of spleen capsular muscle from inhibition caused by potassium.

A. Spleen strip was stimulated with noradrenaline (NA) in a K-free medium (◆). The second response obtained 2 min after addition of KCl to the medium was reduced to 64% of the control. The muscle then kept in Krebs-Henseleit medium (●) for 30 min recovered its sensitivity to NA.

B. Spleen strip stimulated with NA in K-free medium relaxed on addition of KCl. No recovery occurred in 30 min.

control. In a parallel experiment (Fig. 34B) a contraction to noradrenaline was obtained in a potassium-free medium and potassium chloride (5 mM) was added at the peak of the contraction. A prompt relaxation of the muscle ensued. Even though the potassium concentration was maintained for 30 min, there was no tendency for the muscle to regain its original tension.

c) Effect of different concentrations of potassium on response to noradrenaline in potassium-free medium

Paired muscles in standard Krebs-Henseleit solution were first stimulated with noradrenaline (10^{-6} g/ml) until constant responses were obtained. One muscle was then placed in potassium-free solution and the other strip remained in Krebs-Henseleit solution for comparison. Both muscles were stimulated again 15 min later. When the noradrenaline contraction reached a plateau, potassium chloride was added in a cumulative fashion to both strips. Concentrations of potassium chloride up to 4.2 mM relaxed the muscle immersed in potassium-free medium (Fig. 35A). A potassium chloride concentration of 6.3 mM did not alter the tension, but at a concentration of 8.4 mM the tension slightly increased. Thus potassium chloride had a dual, concentration dependent, effect on tension in potassium-free medium. Conversely, in a muscle suspended in Krebs-Henseleit solution (potassium concentration 5.4 mM), raising the external potassium concentration to 9.6 mM did not change the tension, but at a concentration of 11.7 mM a small contraction occurred (Fig. 35B).

In another experiment four spleen strips were stimulated with noradrenaline (10^{-6} g/ml) in potassium-free medium four times at 15 min intervals. When the fourth response reached a plateau, potassium chloride (2 mM) was added to the four baths (Fig. 36A). The muscle relaxed,

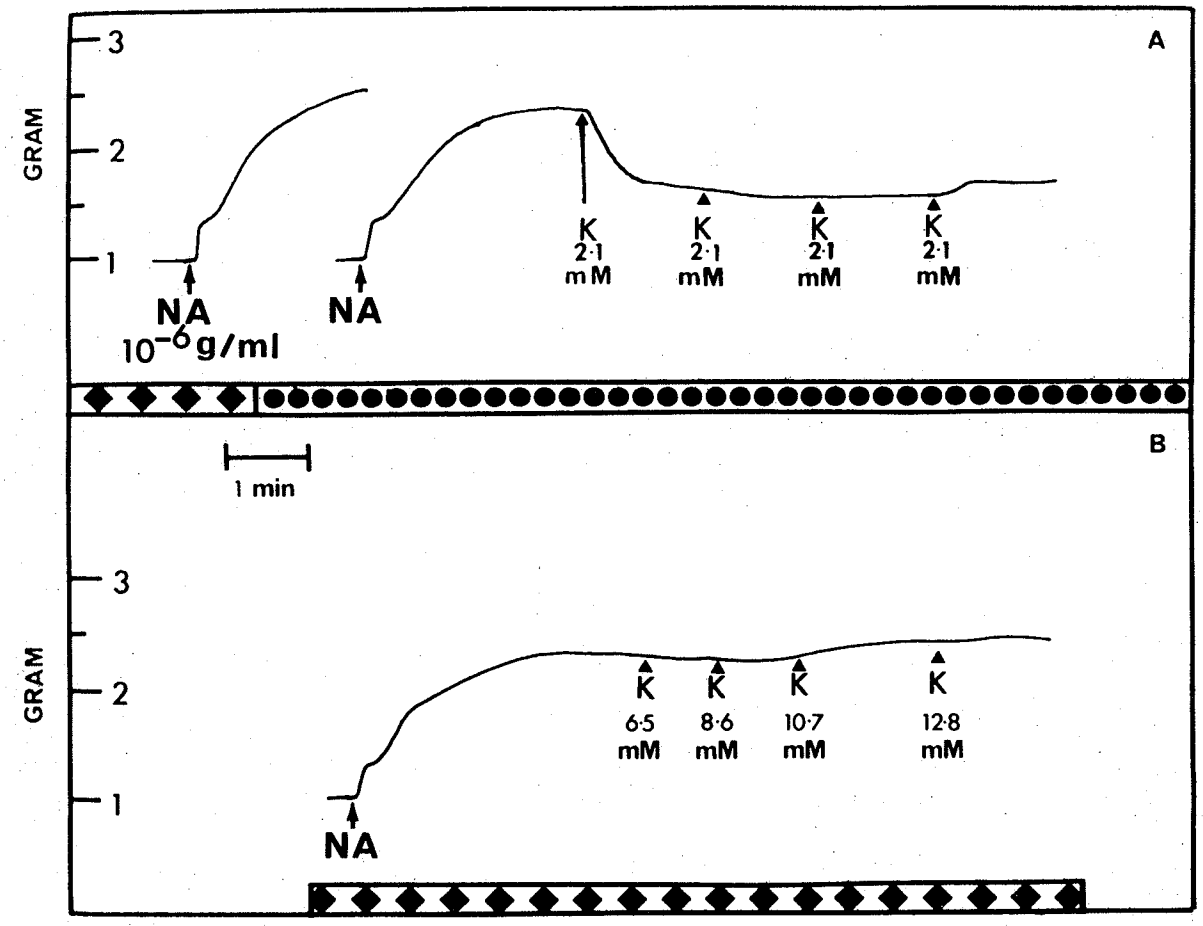


Fig. 35. Effect of KCl on response of spleen strips to noradrenaline, 10^{-6} g/ml, (NA).

A, in K-free medium (●); B, in Krebs-Henseleit solution (◆). KCl was added at K, with the concentration in the bathing solution as indicated.

reducing the active tension due to noradrenaline by 40%. The muscles were washed free of extracellular potassium chloride and challenged twice with noradrenaline. When the second response reached a steady level, the potassium chloride concentration in the baths was raised to 10 mM (Fig. 36B). A greater relaxation (90%) was seen. The responses with 60 and 140 mM of potassium chloride were similarly studied (Fig. 36 C and D). Potassium chloride (60 mM) produced only a 25% decrease in tension followed by a secondary increase which exceeded the control level by 30%. With 140 mM potassium chloride there was no relaxation. A quick but unsustained increase in tension was seen.

These results suggest that high concentrations of potassium can overcome the relaxant effect seen with low concentrations. The optimum concentration of potassium chloride for causing relaxation is between 6 and 10 mM.

It was hypothesised that some change occurred in the muscle when it was stimulated with noradrenaline in a potassium-free medium. This change is absolutely necessary for allowing potassium to subsequently exert its inhibitory effect. Noradrenaline is known to cause depolarisation of smooth muscle while inducing contraction. This would result in increased permeability of the membrane and downhill movement of sodium and potassium. In the absence of external potassium the membrane sodium pump is inhibited. Thus an ionic imbalance caused by noradrenaline may be perpetuated. On restoration of potassium the pump will resume its function. If the efflux of sodium is coupled with a similar influx of potassium then no net movement of charge will take place and the membrane potential will not change. However, if the pump operates for some reason in a fashion

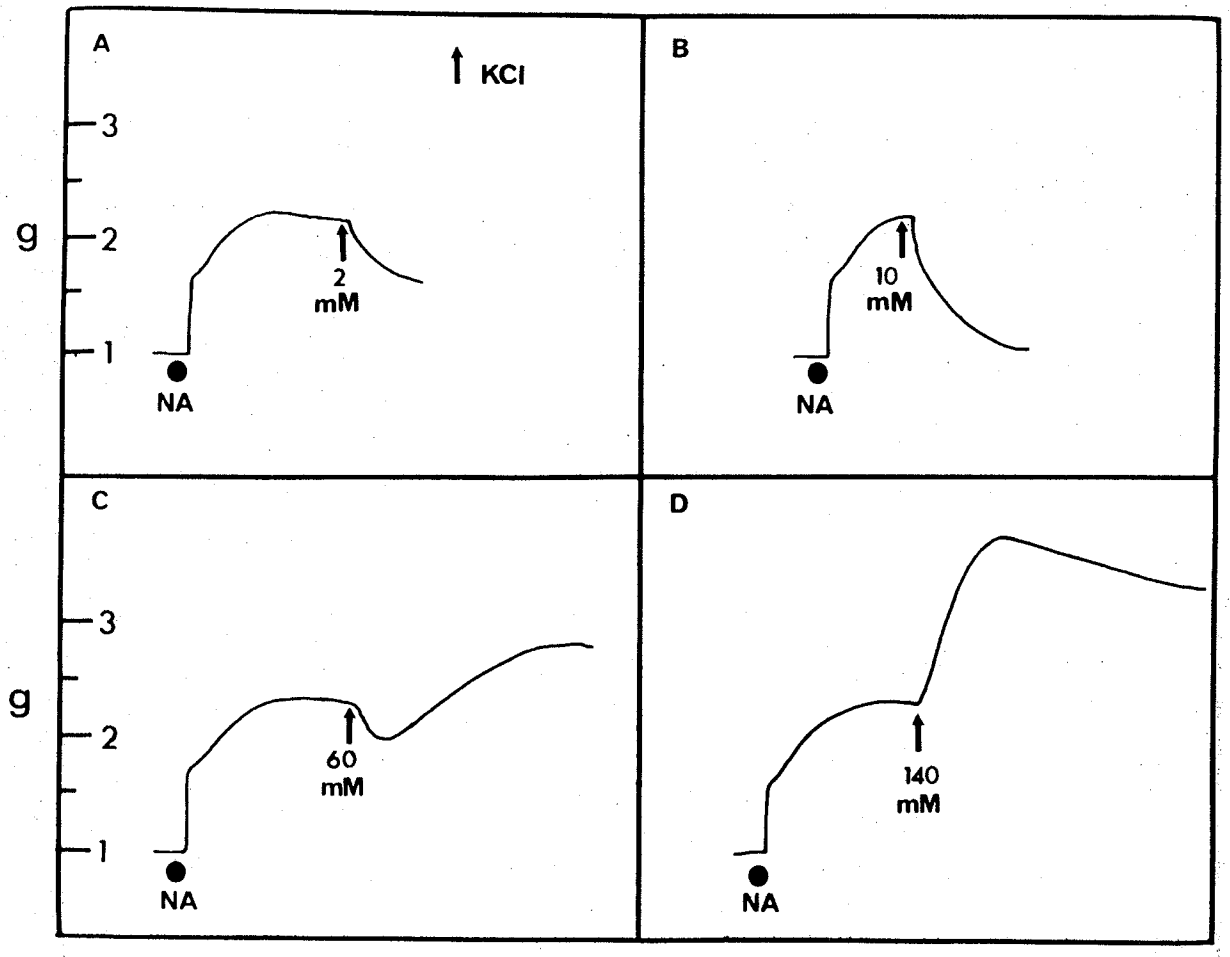


Fig. 36. Effect of potassium on response of reserpine treated spleen strips to noradrenaline obtained in a K-free medium.

Contraction due to noradrenaline (NA), 10^{-6} g/ml, KCl added as indicated.

in which the net efflux of sodium exceeds the influx of potassium then the membrane will hyperpolarise. This may be the cause for inhibition of responses to noradrenaline. However, if the muscle continues its incubation in Krebs-Henseleit solution then eventually the pump is expected to restore the internal potassium level as the sodium is pumped out. If the electrogenic nature of the pump is due to the increase in the internal sodium concentration or a decrease in internal potassium or both then restoration of normal internal concentrations of these two ions during the continued presence of potassium will lead to recovery from inhibition. However, if the muscle continues to be acted upon by noradrenaline during the presence of potassium, then in spite of the activation of the pump by potassium in an electrogenic fashion and the consequent relaxation of the muscle, the tendency of this pump to restore normal ionic concentration may be overcome by the maintained downhill ion movements as a consequence of the action of noradrenaline. Thus the inhibition will not show any tendency to subside. Other causes of inhibition have also to be tested. For example, an increase in potassium or chloride permeability or a decrease in sodium permeability by increasing external potassium may be important factors. Finally potassium may alter tissue sensitivity to noradrenaline by a non-electrical mechanism. The following experiments were done to test the above proposals.

If stimulation of an electrogenic sodium pump is involved in decrease in sensitivity, then procedures which inhibit the sodium pump should prevent the decrease in sensitivity.

d) Effect of ouabain on potassium induced inhibition of noradrenaline responses in capsular smooth muscle

In six experiments spleen strips from reserpine-treated cats were stimulated in potassium-free medium with noradrenaline (10^{-6} g/ml) four times at 15 min intervals. When the fourth response to noradrenaline reached a plateau, potassium chloride was added to attain a concentration of 2 mM. A marked relaxation (48% of total contraction) occurred (Fig. 37A). The muscles were then washed free of noradrenaline in potassium-free medium and ouabain (10^{-9} g/ml) was added to the bath; after 15 min another response to noradrenaline was obtained. Potassium chloride (2 mM) was again added and relaxation noted. Noradrenaline was then washed out with potassium-free medium and a higher concentration of ouabain (10^{-8} g/ml) added and the previous procedure repeated. In the same way relaxation induced by potassium chloride was tested in the presence of 3×10^{-8}, 10^{-7} and 3×10^{-7} g/ml ouabain. Small concentrations of ouabain (3×10^{-9} and 10^{-8} g/ml) produced slight increases in relaxation but these were not statistically significant (Fig. 37B,C). Higher concentrations of ouabain (3×10^{-8}, 10^{-7} and 3×10^{-7} g/ml) inhibited the response to potassium chloride to 38 (p 0.05), 17.5 and 0% ($p < 0.01$) of the noradrenaline response respectively (Fig. 37D,E,F). Representative traces are shown in Fig. 38A and 39. In this experiment ouabain (3×10^{-7} g/ml) decreased the inhibitory effect of potassium chloride (2 mM) to a very small relaxation, which was then followed by a small contraction. Inhibition of potassium responses by ouabain was reversible, and the time for recovery varied from 30 - 45 min for small concentrations (3×10^{-8} g/ml) to more than 90 minutes for the maximum blocking dose of 3×10^{-7} g/ml of ouabain.

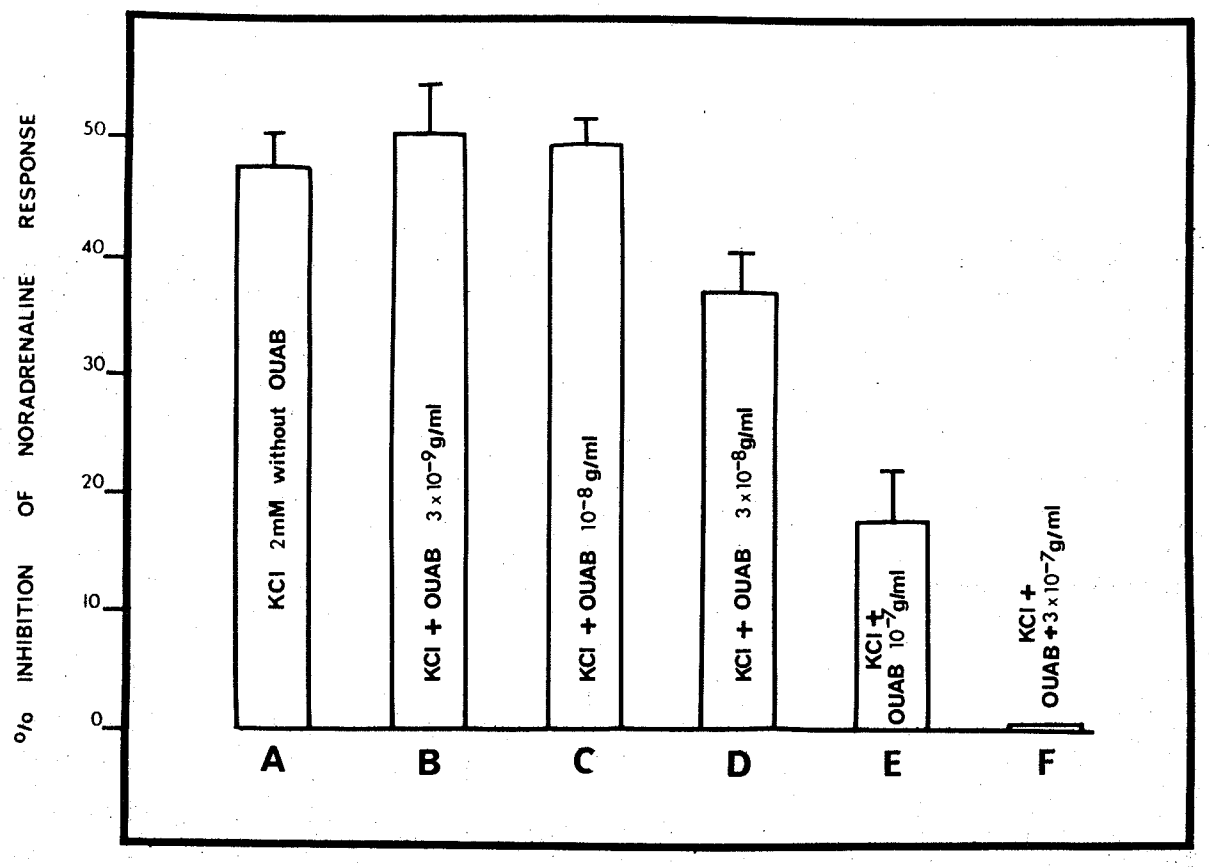


Fig. 37. Effect of ouabain on the relaxation of spleen due to potassium.

Strips were stimulated with noradrenaline (10^{-6} g/ml) in K-free medium. KCl (2 mM) was added to the bath at the peak of contraction and the ensuing relaxation expressed as a percentage of the active tension produced by noradrenaline.

The blockade by ouabain could not be overcome by increasing the concentration of potassium chloride. Instead, an increase in tension resulted depending on the concentration of potassium chloride.

e) Effect of substitution of lithium chloride for sodium chloride on potassium-induced inhibition of responses to noradrenaline

When lithium chloride is substituted for sodium chloride, it enters the intracellular water. However, the sodium pump is unable to pump out lithium. As a result, tissues loaded with lithium fail to hyperpolarise when the sodium pump is stimulated (Tamai and Kagiyama, 1968; Thomas, 1969).

In five experiments spleen strips from reserpine-treated cats were first stimulated four times with noradrenaline (10^{-6} g/ml) in a potassium-free medium. After the fourth response reached a plateau, potassium was added in a cumulative fashion to obtain concentrations of 0.3, 0.5, 0.9 and 1.2 mM in the medium. Each increase in concentration was made after the relaxation due to the previous concentration was complete. The tension at each of these points was expressed as a percentage of the initial tension due to noradrenaline. Noradrenaline was then washed out in a potassium-free bathing medium. Finally the above potassium-free solution which contained sodium was replaced with a potassium-free solution in which lithium chloride was substituted for sodium chloride. There was a prompt but small relaxation. The strip gradually contracted thereafter to bear a tension of 1.25 - 1.4 g after 30 min. The strips were again stimulated with noradrenaline (10^{-6} g/ml) and when the response had reached a plateau potassium chloride was again added in a cumulative manner. As can be seen in Fig. 38B and 40,

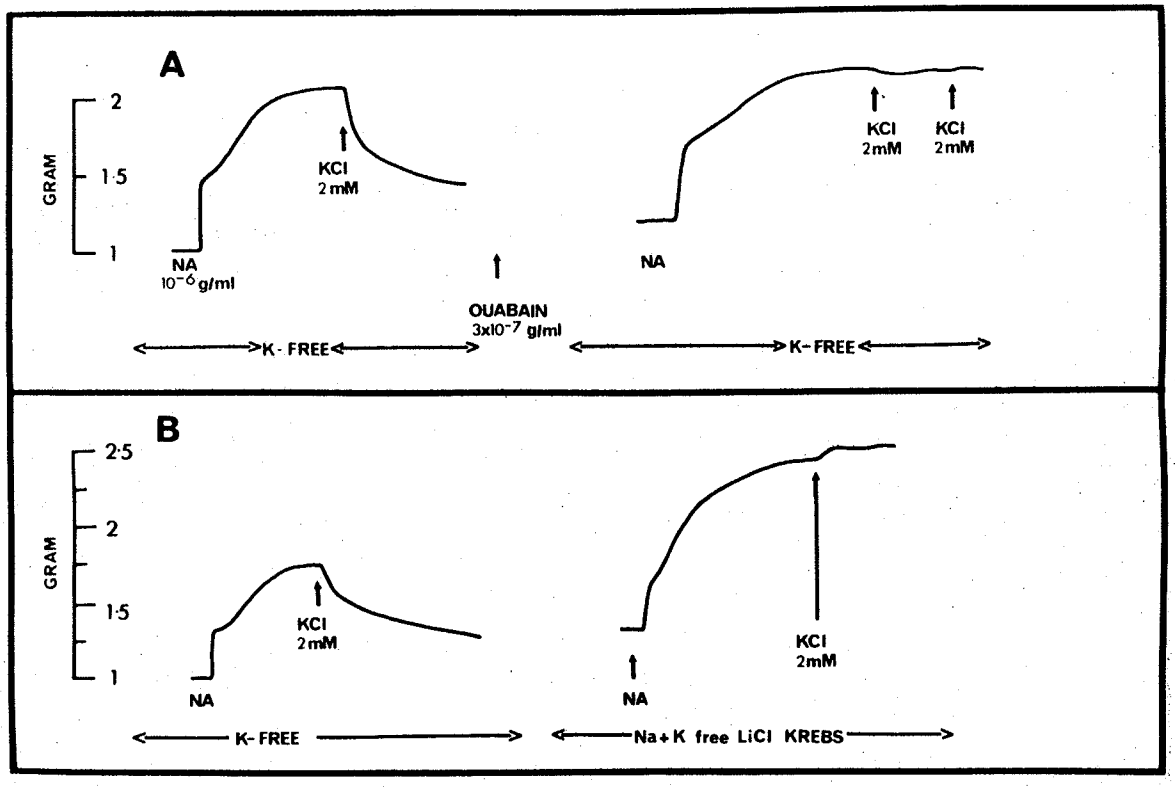


Fig. 38. Effects of ouabain and lithium chloride on relaxation due to potassium chloride.

Contractions in potassium-free solution due to noradrenaline, 10^{-6} g/ml, in strips from a cat treated with reserpine, 1 mg/kg, 24 hours beforehand. KCl added as indicated to inhibit the contraction. Ouabain added or K-free NaCl containing solution (K-free) replaced with a K-free solution in which NaCl was replaced with LiCl (Na + K-free LiCl Krebs) as indicated.

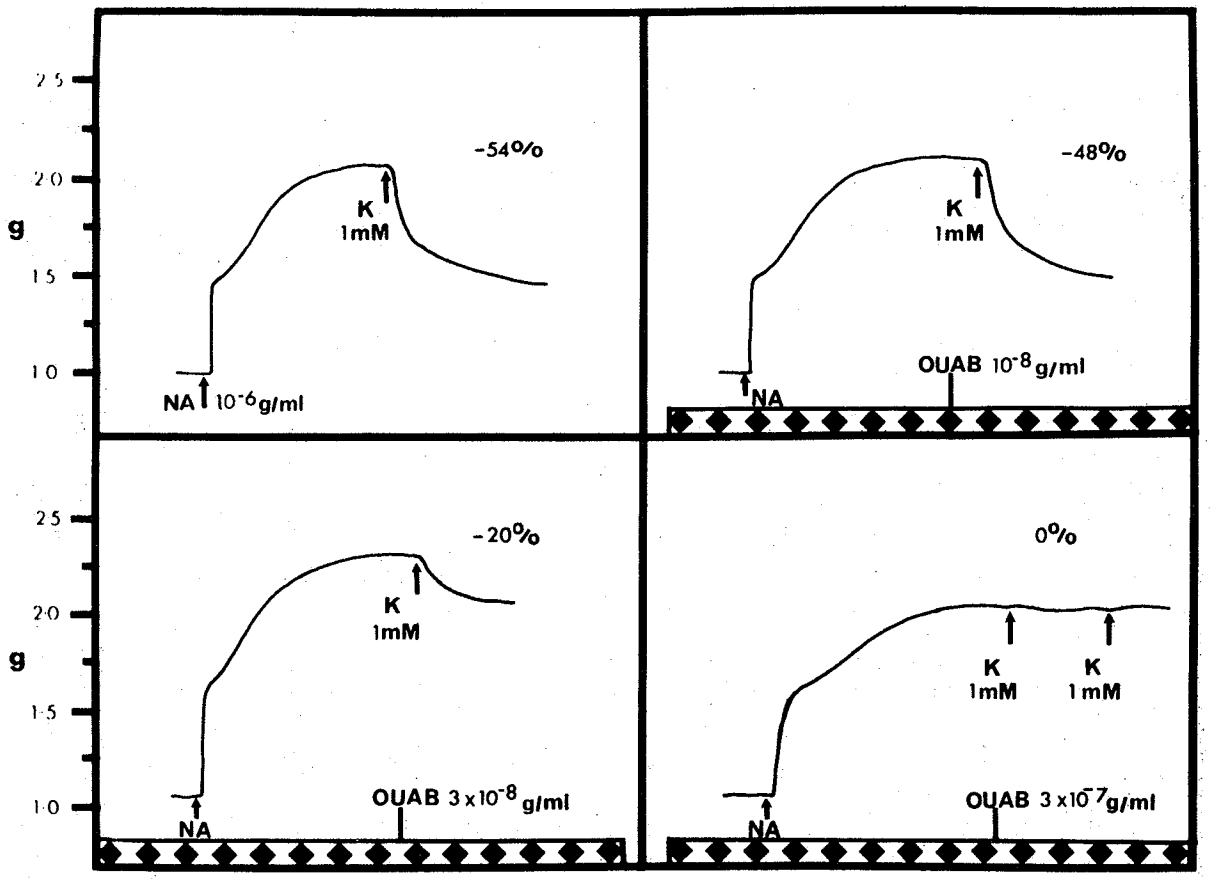


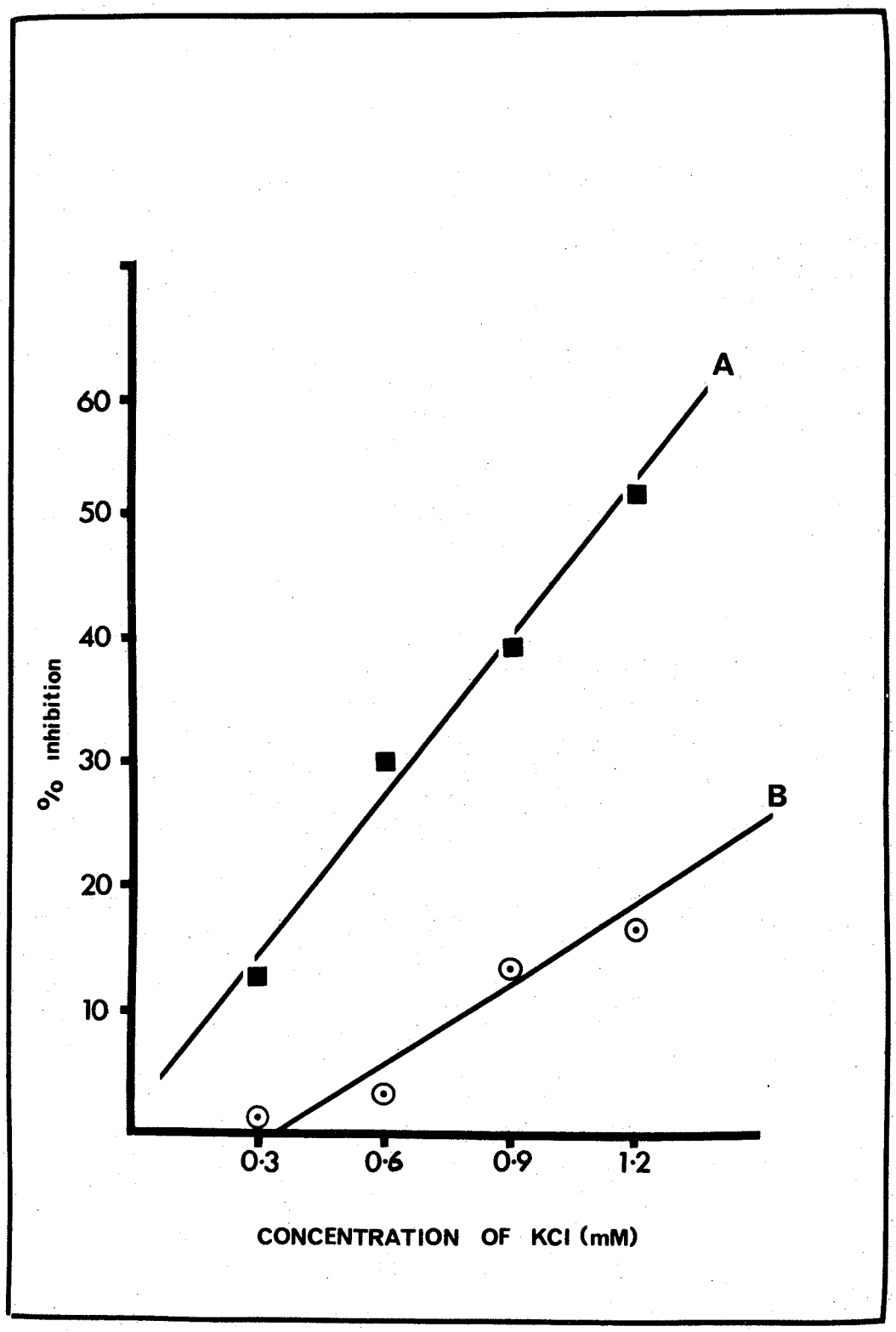
Fig. 39. Effect of ouabain on relaxation induced by KCl.

Responses obtained with noradrenaline, 10^{-6} g/ml (NA) in K-free medium. KCl, 2 mM, added at K.

A. Control response; B, C and D responses in the presence of ouabain 10^{-8}, 3×10^{-8} and 3×10^{-7} g/ml respectively. Numbers above each record indicate the inhibition expressed as a percentage of the peak active tension.

Fig. 40. Effect of substitution of lithium chloride for sodium chloride on the relaxation of spleen strips stimulated with noradrenaline in potassium-free medium.

Spleen strips from reserpine treated cats were stimulated with noradrenaline, 10^{-6} g/ml. At the peak of contraction the potassium concentration of the medium was increased to 0.3, 0.5, 0.9 and 1.2 mM. The inhibition of the noradrenaline-induced contraction was expressed as percentage of the active tension produced by noradrenaline (■). The same strips were then placed in a potassium-free medium in which all the sodium chloride was replaced by LiCl. The strips were then made to contract with noradrenaline and potassium concentration increased in steps as before (⊙).


A
B
60
50
40
30
20
10
% inhibition
0·3
0·6
0·9
1·2
CONCENTRATION OF KCl (mM)

the relaxation after each concentration of potassium was significantly reduced due to lithium substitution. More prolonged incubation of the muscle in (Na + K free) lithium-containing solution reduced the potassium chloride response even more. Fig. 38B shows complete blockade of the potassium chloride (2 mM) relaxation after incubation for 60 min in the lithium-containing solution. As in the experiment with ouabain (3×10^{-7} g/ml), potassium chloride (2 mM) now produced a small contraction instead.

f) Effect of reduction of temperature on relaxation induced by potassium chloride

Biological processes depending upon active or enzyme-dependent processes are very sensitive to changes in ambient temperature. On the other hand mechanisms depending on changes in membrane resistance are believed to be less sensitive to changes in temperature (Rall and Gilman, 1970). The effect of reducing ambient temperature on relaxation induced by potassium chloride was therefore hoped to shed some light on the nature of the process involved.

Four experiments were performed to test the effect of temperature. Spleen strips were first stimulated four times with noradrenaline (10^{-6} g/ml) in potassium-free medium at 37°C. The ambient temperature was then reduced to 23°C and 15 min later another response to noradrenaline was obtained (Fig. 41C). Addition of potassium chloride (4.2 mM) to the bath after the contraction had reached a plateau produced no change in tension. The potassium and noradrenaline were then washed out with potassium-free solution, the temperature raised to 33°C and administration of noradrenaline and potassium chloride repeated. Similar tests were made at 37°C. At 33°C, potassium produced a relaxation which amounted to 53% of the initial

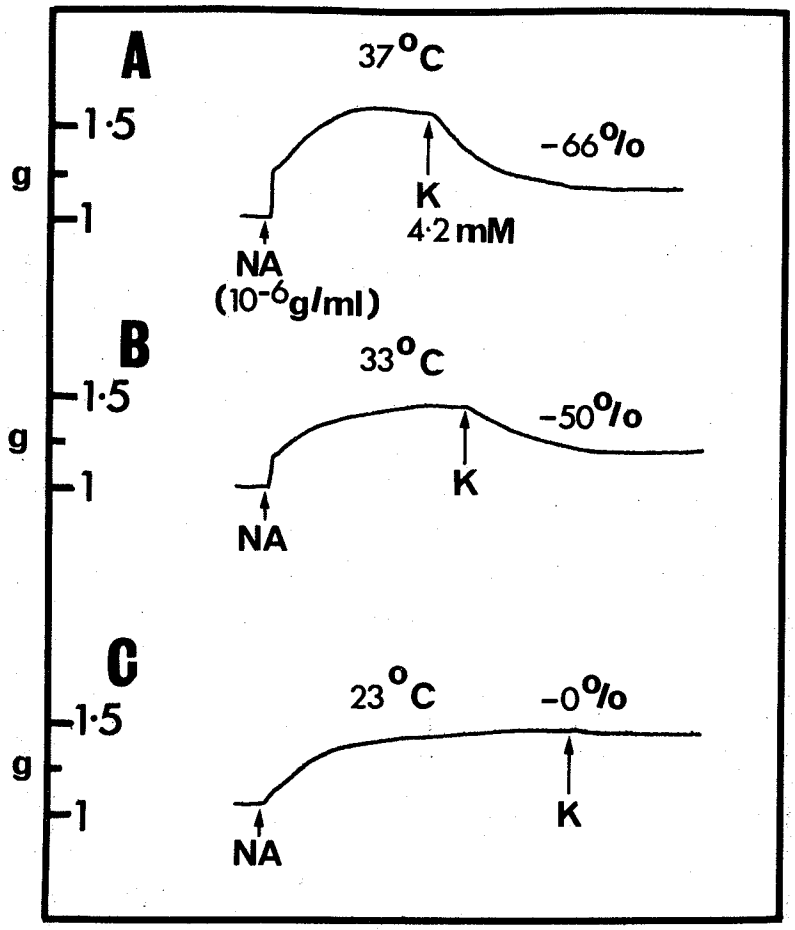


Fig. 41. Effect of temperature on relaxation due to KCl.

Noradrenaline, 10^{-6} g/ml, (NA) added to potassium-free solution bathing spleen strips at various ambient temperatures.

KCl, 4.2 mM, was added as indicated.

contraction while at 37°C this increased to 69%. Each of these values was significantly ($p < 0.01$) different from the others. The potassium-induced relaxation showed a high temperature dependence and between 27°C and 37°C it had a Q_{10} of 3.3.

The above findings, namely blockade of potassium-induced relaxation by low concentrations of ouabain or substitution of external sodium chloride by lithium chloride, along with the marked temperature dependence of the relaxation process, is very highly suggestive of an active process being involved, most likely the sodium pump. Earlier it was hypothesised that such a pump was probably made electrogenic by either an increase in internal sodium or a decrease in internal potassium or both. This was attributed to the action of noradrenaline. If such a mechanism exists then relaxation of spleen strips by potassium should also occur if ionic changes similar to the above are produced by a different procedure. It has long been known that incubation of smooth muscle in cold potassium-free solution for several hours results in downhill ion movements as a result of diminished activity of the sodium pump. It was therefore considered worthwhile to store spleen strips in a potassium-free medium at 4°C for 24 hours. The strips were then incubated in potassium-free medium at 37°C for 30 min. Ionic analysis of these strips showed a considerable increase in intracellular sodium and loss of intracellular potassium. The muscles were then stretched until they exerted a tension of 1 g. Experiments were done to find out the effect of potassium on the resting tension and on the increase in tension produced by noradrenaline. The effects of ouabain, lithium substitution, low temperature, substitution of an impermeant anion isethionate for the external chloride were also

studied. Since marked ionic changes occurred in the spleen strips during cold storage it was hoped that the potassium-induced relaxation could possibly be correlated with changes in ionic composition of the muscle.

g) Effect of potassium chloride on resting tension and responses to noradrenaline in sodium-loaded spleen strips

In three experiments, the sodium loaded spleens were stretched to 1 g tension while suspended in a potassium-free solution at 37°C. After 30 min, they were stimulated with noradrenaline (10^{-6} g/ml) twice at 15 min interval. The two phases of contraction were sometimes not as clear as in normal spleen strips. Fig. 42 depicts a single typical experiment. Potassium chloride (5 mM) added to the potassium-free bathing medium reduced the resting tension. Maximum relaxation occurred in about 5 - 6 min. Removal of potassium shortly after the tissue began to relax resulted in a partial restoration of tension. These results are somewhat different from those obtained from fresh spleen strips incubated in potassium free medium where the resting tension did not change. The response to noradrenaline (10^{-6} g/ml) was markedly diminished when tested 2 min after addition of potassium chloride. The decrease was mainly in the slow phase. If the muscle was then kept in the potassium-containing medium for 70 min after noradrenaline was washed out, sensitivity to noradrenaline increased; the inhibition of the response to noradrenaline not only decreased, but in fact the response was larger than the control response previously elicited in the potassium-free medium. The muscle was again washed free of potassium and 15 min later when noradrenaline was tested, there was a small further increase in the noradrenaline response. Thus this preparation showed the same kind of brief inhibitory

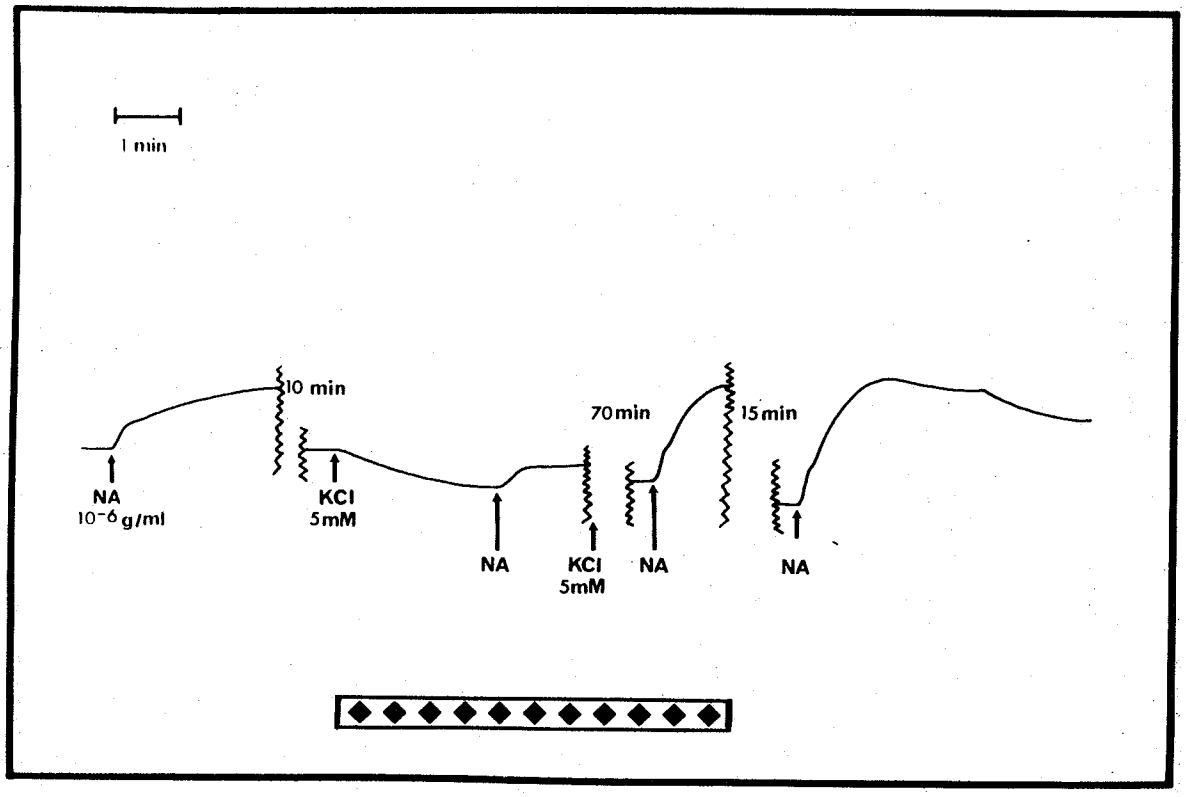


Fig. 42. Effect of KCl on the tension and responsiveness to noradrenaline (NA) of sodium loaded spleen strips.

The strips were suspended in a K-free medium. Noradrenaline, 10^{-6} g/ml, was added at NA, and KCl, 5 mM, as indicated. Note the initial short lasting inhibition followed by recovery of response due to NA in the presence of KCl. Removal of KCl produced further improvement in response to NA.

effect of potassium as seen in the normal spleen strips in earlier experiments.

h) Effect of ouabain and lithium on potassium induced relaxation of smooth muscle and on inhibition of response to noradrenaline

In two experiments, four sodium loaded spleen strips obtained from reserpine treated cats were stretched to 1 g tension in potassium-free medium. They were then exposed to potassium chloride for 1 min and the ensuing relaxation noted (Fig. 43A). The spleen strips were quickly washed free of potassium and allowed to regain the initial tension. Half the spleen strips were changed to a solution containing lithium chloride but no sodium or potassium, and the other strip remained in potassium-free solution. The change to the solution with lithium chloride produced a transient biphasic change in tension: a contraction followed by relaxation. The relaxation due to potassium chloride (2 mM) decreased 70% in strips placed in the solution containing lithium chloride while the responses in the strips that remained in potassium-free solution were unchanged.

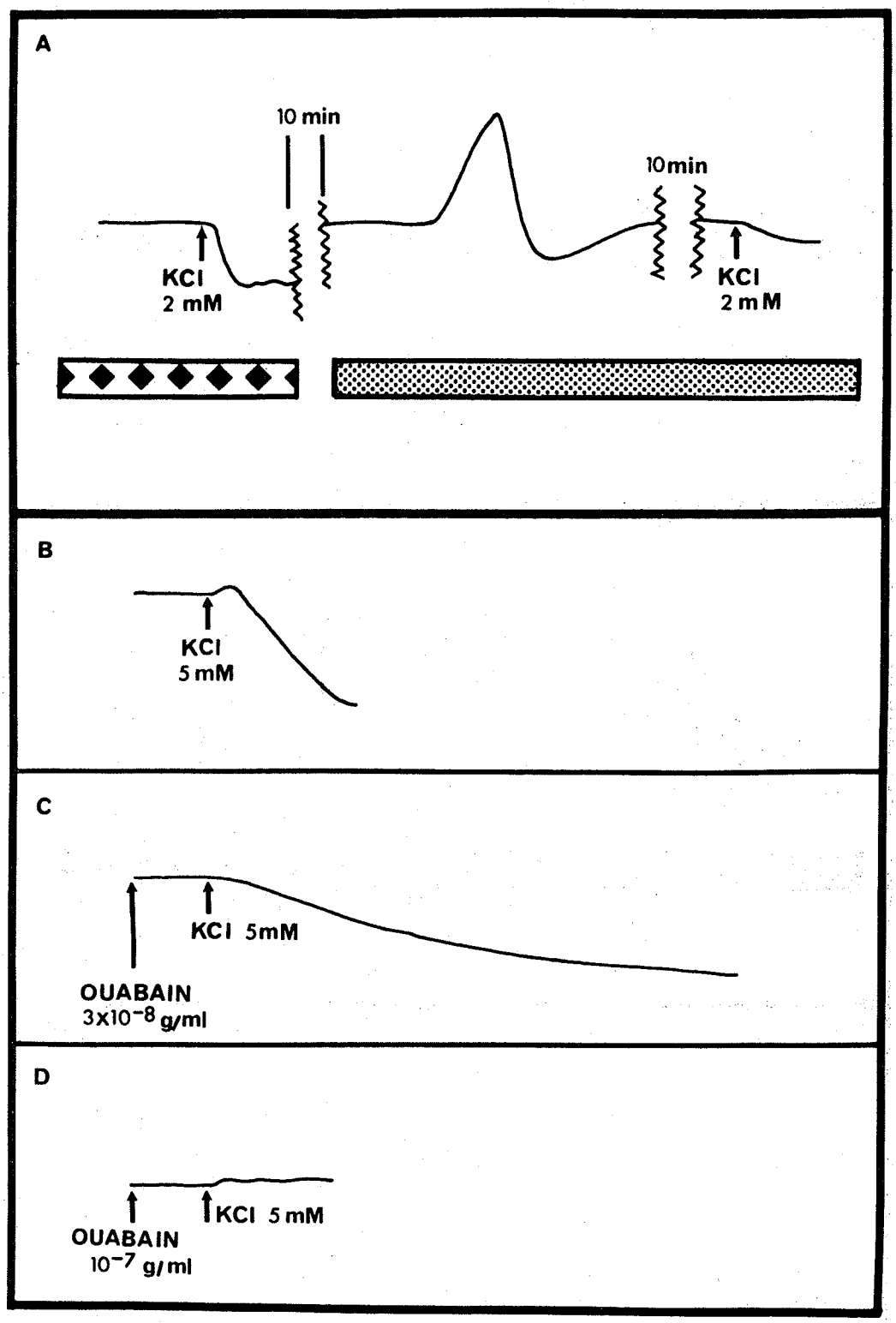
In similarly designed experiments the response of sodium loaded reserpine treated strips to potassium chloride (5 mM) was first determined (Fig. 43B). The strips were then washed free of potassium chloride and exposed to ouabain (3×10^{-8} g/ml) for 15 min. Potassium chloride (5 mM) then caused a relaxation which was only slightly less than in the control response but the rate of relaxation was much slower (Fig. 43C). The strips were again washed with potassium-free solution and exposed to ouabain (10^{-7} g/ml) for 15 min. Potassium chloride now produced a small contraction instead of a relaxation (Fig. 43D). As in the earlier experiments done on normal spleen strips, the blocking effect of ouabain could

Fig. 43. Modification of the relaxant effect of potassium (KCl) on sodium-loaded spleen strips.

A. The first response due to KCl was elicited in a K-free sodium chloride containing modified Krebs-Henseleit medium (◆). On washing KCl out, the tension rose to normal. 10 min later the bathing medium was changed to a K-free medium in which sodium chloride was replaced by lithium chloride (▒▒). A transient biphasic change in tension occurred. 10 min later application of KCl resulted in a smaller relaxation.

B. Control response of a sodium-loaded spleen strip to KCl in a K-free sodium chloride containing solution. KCl was washed out after peak relaxation occurred.

C, D. Reduced responses of same strip in the presence of ouabain 3×10^{-8} and 10^{-7} g/ml respectively.


A
10 min
10min
KCl
2 mM
KCl
2 mM
B
KCl
5 mM
C
KCl 5mM
OUABAIN
3x10⁻⁸ g/ml
D
KCl 5 mM
OUABAIN
10⁻⁷ g/ml

be removed slowly by repeated washes.

As described above, responses to noradrenaline were inhibited when sodium-loaded muscles relaxed under the influence of potassium. The following experiment was done to see if this inhibition could also be blocked by ouabain. Three experiments were done on pairs of sodium-loaded strips suspended in potassium-free medium (Fig. 44). Responses to noradrenaline (10^{-6} g/ml) were obtained first. One of the paired muscles was then treated with ouabain (10^{-7} g/ml) for 15 min. When potassium chloride (5 mM) was now added to media bathing both muscles, the muscle treated with ouabain exhibited a small contraction whereas the untreated muscle relaxed (mean relaxation 0.54 g). Noradrenaline was added to both baths. The response in the ouabain-treated strip increased 18% over the control value instead of decreasing by 80% as in the strip which was not treated with ouabain. Returning the untreated muscle to a potassium-free medium restored the response to noradrenaline but the ouabain-treated tissue did not change its response in potassium-free medium. This suggests that lack of inhibition of noradrenaline response in this tissue was mainly due to blockade of the sodium pump by ouabain.

i) Effect of temperature on potassium-induced relaxation of sodium-loaded spleen strips

Four sodium-loaded spleen strips were incubated in potassium-free medium at 18°C and stretched to exert a tension of 1 g. Potassium chloride (5 mM) was added to each. No change in tension resulted. Potassium was then washed out and the ambient temperature increased to 24°C. After 15 min potassium chloride was reintroduced. In this way the response to potassium at 30°C and 37°C was also measured (Fig. 45). The Q_{10} of

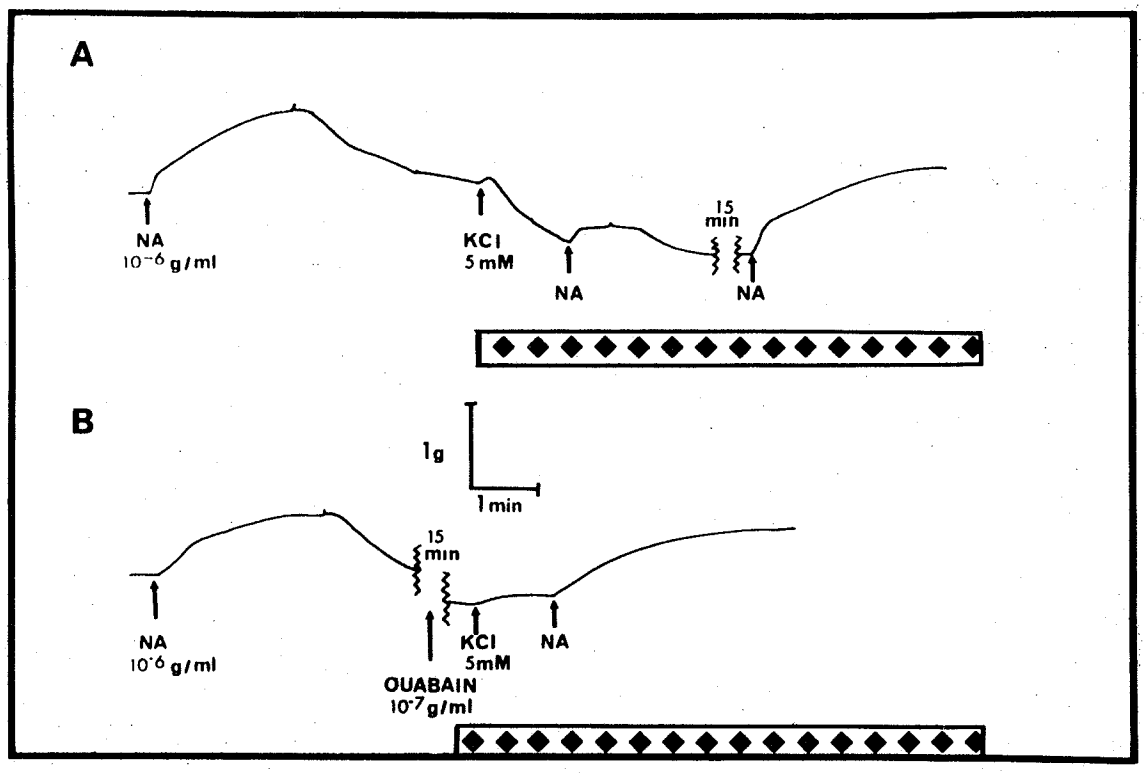


Fig. 44. Effect of potassium on sodium loaded spleen strips bathed in potassium-free medium.

Responses to noradrenaline, 10^{-6} g/ml, (NA) in spleen strips from cats treated with reserpine, 1 mg/kg, 24 hours beforehand. KCl, 5 mM, was added to the K-free bathing solution during the period indicated by (◆). A, without ouabain; B, with ouabain, 10^{-7} g/ml, added as indicated.

relaxation from 20°C to 30°C was 4.1. This high value in sodium-loaded muscles is somewhat greater than that for potassium induced relaxation in normal muscles incubated in a K-free medium.

j) Effect of reduction of external chloride on potassium induced relaxation

If it is assumed that potassium induced relaxation of a muscle which has been made to contract initially with noradrenaline is due to an increase in membrane potential, it is conceivable that increased inward pumping of chloride can be responsible either alone or in conjunction with an electrogenic sodium pump for the same. On the other hand a different situation is also possible if the cause of increase in membrane potential is an electrogenic sodium pump. If a certain amount of chloride accompanies the sodium this is likely to reduce the magnitude of increase in membrane potential. Removal of external chloride will then be expected to decrease potassium induced relaxation if a chloride pump contributes to the hyperpolarisation whereas depletion of internal chloride will be expected to reduce its shortcircuiting effect on the electrogenic sodium pump thereby increasing the potassium induced relaxation depending on the individual mechanism that is prevailing. These possibilities were tested as follows:

Four sodium-loaded spleen strips were suspended in potassium-free solution at 37°C and stretched for 30 min to exert a tension of 1 g. They were then exposed to potassium chloride (0.7 mM) and the relaxation was measured as a percentage of the initial tension. The muscles were quickly washed free of external potassium chloride and allowed to regain the initial tension. The bathing medium in three baths was now replaced

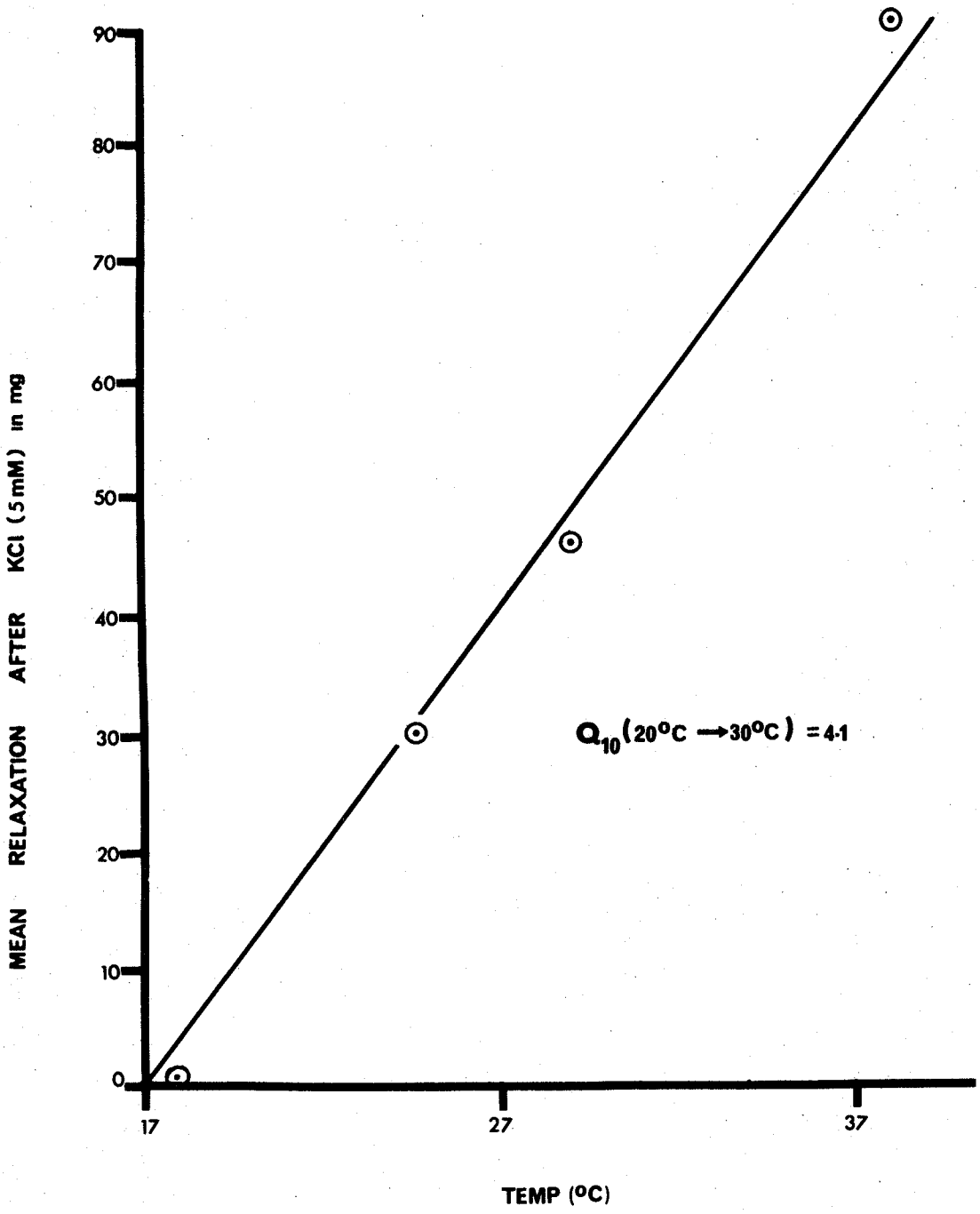


Fig. 45. Effect of temperature on the relaxant effect of KCl on sodium loaded spleen strips.

by a potassium-free medium in which sodium chloride was replaced by sodium isethionate. A small amount of chloride (2.5 mEq/l) in the form of calcium chloride still remained. In this medium, the muscles temporarily gained a very small amount of tension, which returned to normal after 15 min. All four muscles were again tested with potassium chloride (5 mM) and the percentage relaxation of the muscles exposed to low chloride, potassium-free medium was compared with the relaxation of the same muscle in a medium containing a greater amount of chloride. The fourth tissue which served as a control relaxed equally both times. The relaxation in low chloride solution was 11.4% greater than the relaxation in the medium containing the normal amount of chloride. This difference was not statistically significant.

k) Effect of potassium on the ionic contents of sodium-loaded spleen strips

Forty five spleen strips were loaded with sodium by incubation in potassium-free medium at 4°C for 24 hours. They were then divided into five groups and incubated in potassium-free medium at 37°C for 30 min. A sixth group consisted of five normal spleen strips incubated for 30 min in Krebs-Henseleit solution at 37°C. Two groups of sodium-loaded spleen strips were treated with ouabain (3×10^{-7} g/ml) during the last 15 min of the preincubation period and throughout the rest of the experiment.

After the preincubation was over, the normal spleen strips and strips from one of the groups of sodium-loaded muscles were taken out for estimation of ion contents. Potassium chloride (5 mM) was added to the remaining muscles. After 6 min, one group of untreated and another group of ouabain-treated strips were taken out for analyses. Two hours later

the remaining tissues were taken out. The results are shown in Fig. 46. The cold stored muscles had gained 110.1 mEq of sodium and lost 76.9 mEq of potassium per litre of intracellular water ($p < 0.01$). Incubation in a potassium chloride containing solution for 6 min resulted in a negligible change in intracellular potassium concentration but the sodium concentration decreased by 39.9 mEq/l, which was statistically significant ($p < 0.05$). This decrease did not occur in ouabain-treated tissues. Incubation in potassium chloride-containing medium for two hours nearly restored the ionic concentrations (differences from control statistically not significant), but this was prevented by ouabain.

It was mentioned earlier that an increase in membrane potential can be produced not only by stimulating an electrogenic pump but also by altering ionic conductances. For example, an increase in potassium permeability will tend to bring the membrane potential closer to the potassium equilibrium potential which has normally a higher value. The most acceptable way of distinguishing between the pump and the conductance mechanisms is to estimate the potassium equilibrium potential on the basis of the intra- and extracellular potassium activities (or concentrations) and measure membrane potential during the relaxation produced by potassium. If the membrane potential exceeds the potassium equilibrium potential then the hyperpolarisation is due to a pump, whereas if it does not, then a change in conductance is more likely. Additionally if the muscle cell is hyperpolarised beyond the potassium equilibrium potential by passing a current across the membrane and if extracellular potassium now depolarises the muscle instead of hyperpolarising it, then a pump mechanism can be excluded. An attempt was made to measure membrane potential changes in the

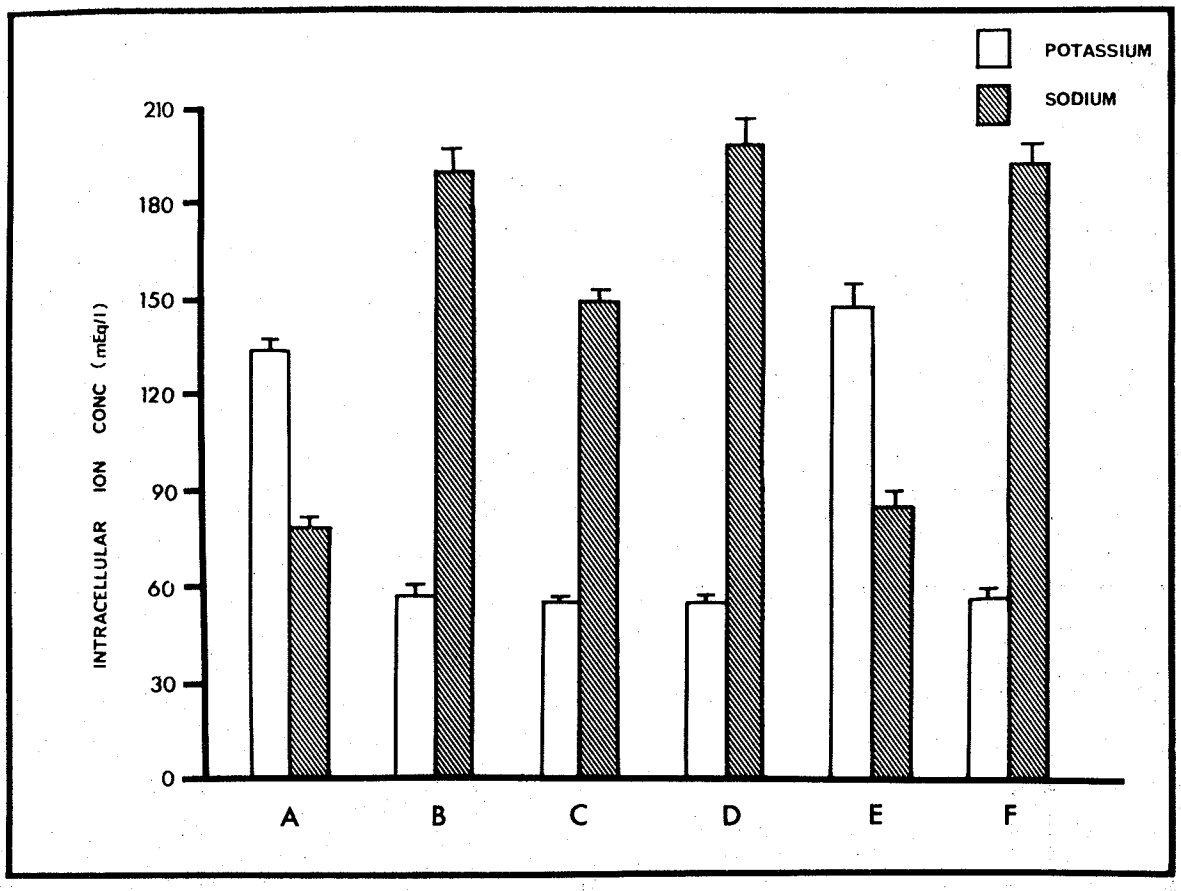


Fig. 46. Intracellular sodium and potassium concentrations in normal spleens and in cold stored spleens exposed to potassium chloride (5 mM).

A. Control value in untreated spleens; B, control value in spleens stored at 4°C in a K-free medium (cold stored); C, cold stored spleen + KCl for 6 min; D, ouabain (10^{-6} g/ml) treated cold stored spleen + KCl for 6 min; E, cold stored spleen + KCl for 2 hr and F, ouabain treated cold stored spleen + KCl for 2 hr.

spleen capsular smooth muscle. However, this had to be abandoned because of the difficulty in getting good impalements with the high resistance glass microelectrodes that have to be used for small smooth muscle cells. The presence of connective tissue around the muscle cells and the small size of the muscle cells are probably responsible for our failure to do electrophysiological studies on the splenic capsular smooth muscle.

An indirect approach was therefore tried. Barium ions are known to decrease potassium permeability in smooth or skeletal muscles (Sperelakis and Tarr, 1965; Sperelakis et al., 1967) and this has been suggested as a mechanism for its ability to depolarise muscle cells. It was reasoned that if the mechanism of relaxation of splenic capsular smooth muscle by potassium was induced by an increase in potassium permeability, then this should be modified by prior administration of barium.

1) Effect of potassium chloride on spleen strips in the presence of barium chloride in potassium-free medium

Two spleen strips were suspended in Krebs-Henseleit solution and another two in potassium-free medium. Barium chloride (2.5 mM) was added to the four baths. The response of the strips in Krebs-Henseleit solution was a small increase in tension (Fig. 47A). The responses occurred with a long latency (15 - 20 sec) and were initially rhythmic in the strips suspended in Krebs-Henseleit medium. In contrast, the strips suspended in potassium-free medium (Fig. 47B) contracted much more strongly, with no rhythmicity and with a shorter latency (4 - 6 sec). The responses of the strips to barium chloride in potassium-free medium were five times larger than those in Krebs-Henseleit solution. Addition of potassium chloride (1.4 mM) to both groups of strips resulted in relaxation which

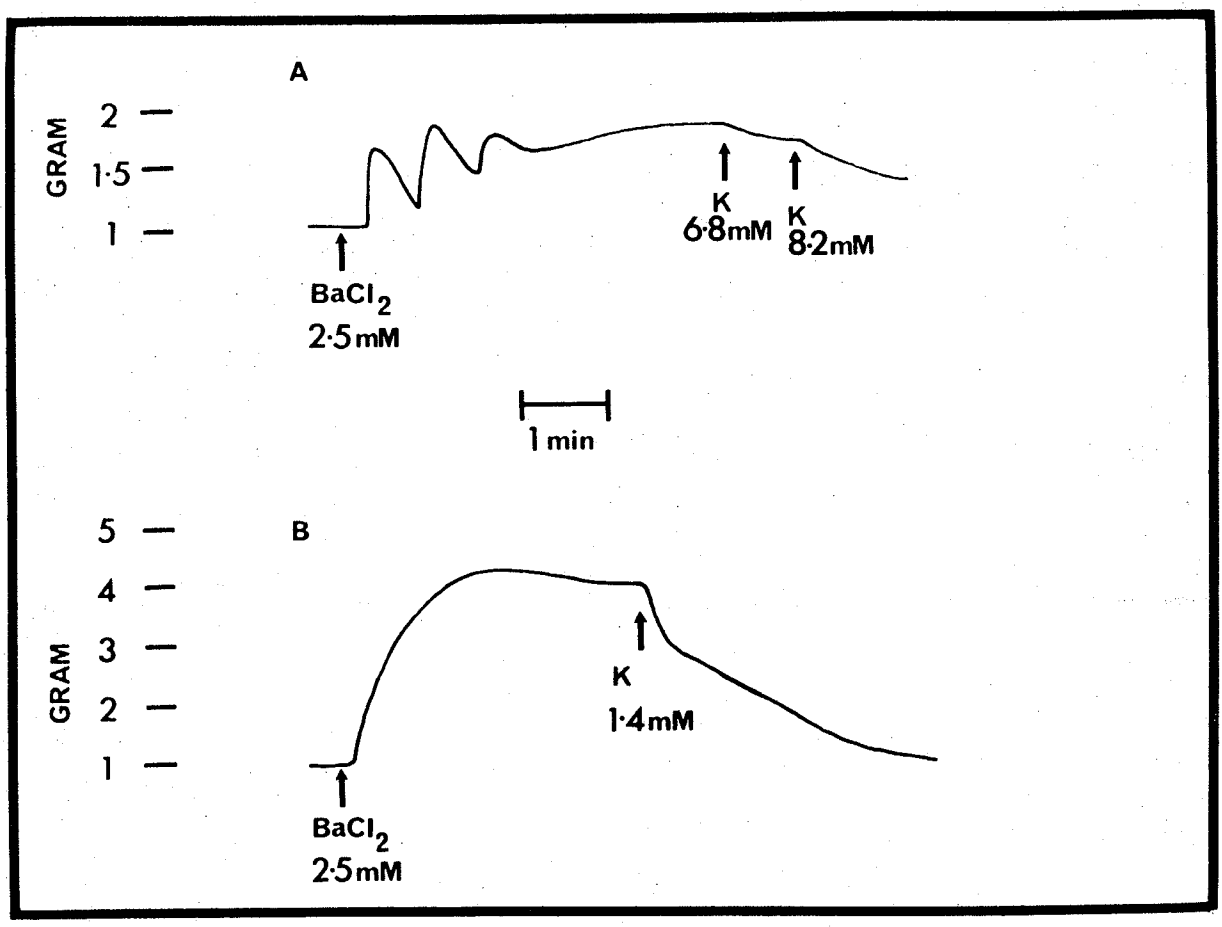


Fig. 47. Effect of external potassium on the response of spleen to barium.

A. Response to $BaCl_2$ in Krebs-Henseleit solution. At K external potassium chloride. Concentration was increased to 6.8 mM and 8.2 mM.

B. Response to $BaCl_2$ in same muscle after exposure to K-free medium. K, 1.4 mM was added as indicated.

was more marked in the strip immersed in potassium-free medium. These effects of potassium on tension induced by barium chloride were different from those on tension induced by noradrenaline. It may be recalled from Fig. 35B that increasing the potassium concentration in Krebs-Henseleit solution did not reduce the noradrenaline-induced increase in tension. Moreover, even in the tissue made to respond to noradrenaline in potassium-free medium the percentage relaxation due to potassium (2.1 mM) was less than the relaxation due to a much smaller concentration of potassium (1.4 mM) in the muscle which had been made to contract by barium chloride in potassium-free medium.

In four additional strips from separate cats, suspended in Krebs-Henseleit solution, responses to 2.5 mM of barium chloride were obtained. The concentration of potassium in the medium was then reduced from 5.4 mM to 0.3 mM and 15 min later the muscles were again stimulated with barium chloride. After the responses were obtained, barium chloride was washed out and the strips were then immersed in a potassium-free medium for 15 min. Barium chloride was added to the baths again. The mean increase in tension of strips in potassium-free medium was 5.04 ± 0.16 g. This was significantly different ($p < 0.01$) from the increase in tension in 0.3 mM potassium chloride (2.4 ± 0.134 g) and in 4.9 mM potassium chloride (1.03 ± 0.033 g).

If potassium-induced relaxation is due to an increase in potassium permeability and if the effect of barium in reducing potassium permeability is non-competitive, as has been suggested by Sperelakis and co-workers (1967), then one should expect a decrease in the ability of potassium to relax tissues treated with barium. The failure to elicit

this and the unexpected increase in responsiveness of the tissue to potassium suggests that in the presence of barium chloride the electrogenic sodium becomes more sensitive to potassium chloride or alternatively, potassium relaxes these muscles by a different mechanism in addition to or in the absence of an effect on the electrogenic sodium pump. This was investigated next.

m) Effect of ouabain on the relaxation induced by potassium chloride in muscles stimulated with barium chloride

Six spleen strips were incubated in potassium-free medium. Thirty minutes later they were stimulated with barium chloride and the relaxation on increasing potassium chloride concentration of the medium in a cumulative manner from 0.1 mM to 5 mM was noted.

Potassium chloride and barium chloride were washed out and after incubation for another 15 min in potassium-free solution the strips were stimulated with noradrenaline (10^{-6} g/ml) and the relaxation noted. The muscles were again washed with potassium-free solution and ouabain (3×10^{-7} g/ml) was added to all the baths. Fifteen minutes later, noradrenaline was repeated and addition of potassium chloride now failed to produce any relaxation. As in earlier experiments, a slight contraction occurred, instead. After another wash in potassium-free solution and with ouabain still present, the strips were finally stimulated with barium chloride (2.5 mM) and at the height of contraction, potassium chloride (5 mM) was added and the relaxation noted. In strips not treated with ouabain the tension decreased by 85.35 ± 3.3% of the initial increase due to barium chloride and to lesser extents in the presence of smaller concentrations of potassium (Fig. 48). In the presence of ouabain the

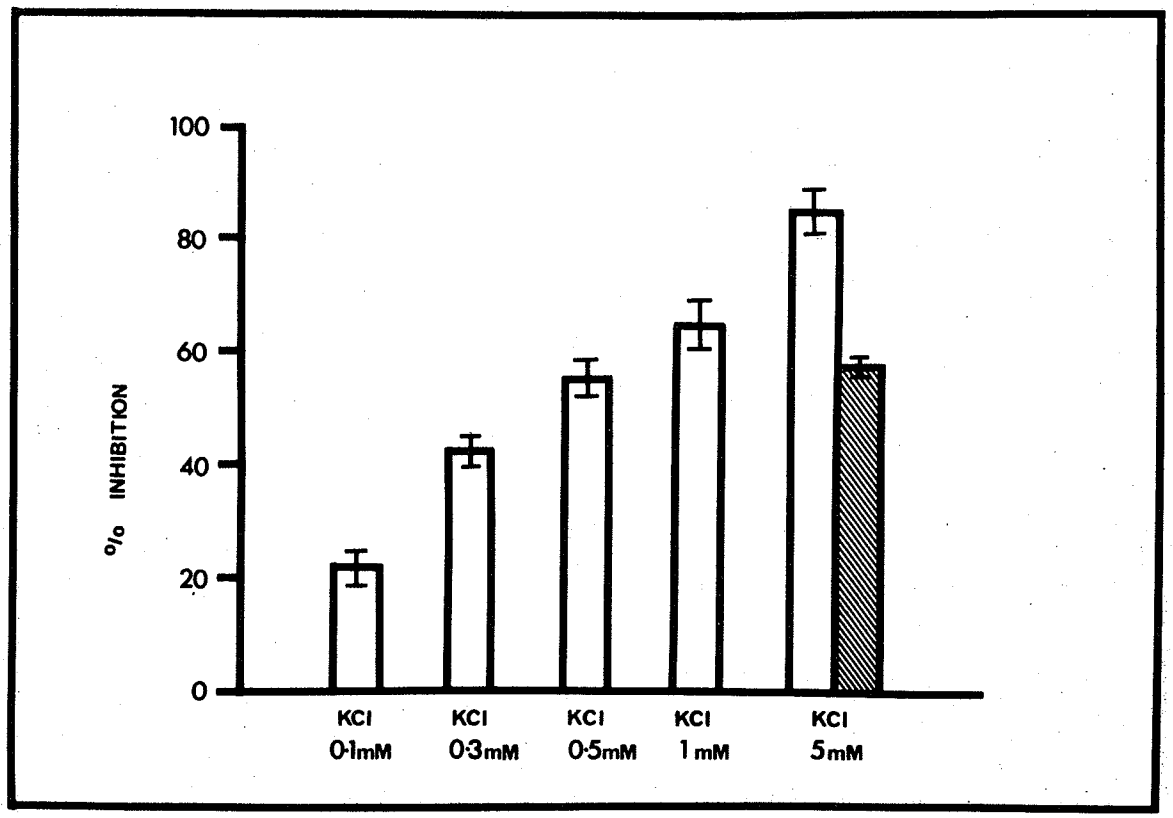


Fig. 48. Inhibition by KCl of the tension produced by $BaCl_2$ (2.5 mM) in a K-free solution.

KCl was added after the contraction induced by $BaCl_2$ had reached a plateau. Open bars show the relaxation, expressed as a percentage of the active tension produced by $BaCl_2$. Hatched bar shows a decrease in KCl induced inhibition in the presence of ouabain (10^{-6} g/ml).

relaxation due to potassium chloride (5 mM) was only 58 ± 1.38% of the active tension produced by barium chloride. This effect of ouabain was highly significant ($p<0.01$). Thus the ouabain sensitive component of relaxation, which most probably represents the contribution of the sodium pump, is 29% of the active tension produced by barium. Since a similar concentration of potassium chloride produces a 64% decrease in the active tension produced by noradrenaline in a potassium free medium and also because this effect is entirely blocked by ouabain, it appears that the ouabain sensitive relaxing mechanism of potassium in the presence of barium (29%) is less than that in the presence of noradrenaline (64%). The reason for this difference will not be clear until a possible inhibitory effect of barium on the sodium pump is ruled out. Even though the use of barium did not provide clear cut evidence regarding the possible role of permeability changes in mediating relaxation due to potassium, it became obvious that more than one mechanism may be involved. Nevertheless, in the presence of noradrenaline, the predominant mechanism for relaxation by potassium is stimulation of the sodium pump.

XIV. Effect of reserpine, cocaine and 6-hydroxydopamine on the two phases of contraction due to noradrenaline

Up to this point the effect of agents which depress smooth muscles on the two phases of noradrenaline were studied. Several procedures have an opposite effect on smooth muscles and cause supersensitivity to various agonists. It was therefore proposed to study the effect of three such procedures in the spleen - namely treatment with reserpine, cocaine or 6-hydroxydopamine.

a) Effect of reserpine

Administration of reserpine (1 mg/kg) to the cat 24 hr earlier produces an unspecific supersensitivity of the spleen strips to catecholamines and other agonists. This is accompanied by a depletion of endogenous catecholamines. The supersensitivity was measured on the basis of a shift to left of the dose response (isotonic contraction) curve and an increase in the maximum response of the spleen. It is generally believed that supersensitivity produced by reserpine is due to an effect of the drug on some postsynaptic structure or mechanism (Davidson, 1970). In six experiments spleen strips from a reserpine treated and a control tissue were paired and stimulated with various concentrations of noradrenaline. Duplicate samples from each control and reserpine treated tissue were analysed for their catecholamine content and the mean content was found to be 2.94 ± 0.3 g and 0.042 ± 0.004 μg/g tissue wet weight respectively. The depletion of catecholamines due to reserpine amounted to 98.5% of the control value. The threshold concentration of noradrenaline in the control tissue was 10^{-8} g/ml and maximum response was obtained with 3×10^{-5} g/ml. The isometric contractions produced by smaller concentrations of noradrenaline had proportionately smaller slow phases compared to the rapid phase. In fact in some tissues an initial fast phase was followed by a gradual decrease in tension similar to some earlier experiments in which the spleen strips were stimulated with noradrenaline in the presence of EGTA (15 mM). However, on increasing the concentration of noradrenaline, the slow phase became more prominent. The rapid phase also increased, though less in proportion to the slow phase (Fig. 49). With very high concentrations of the agonist sometimes the upper limit of the fast phase

merged indistinguishably with the slow phase. In such cases responses were obtained 15 sec after exposing the strips to EGTA (15 mM) - a procedure that has previously been shown to dissociate the fast phase from the slow phase. Reserpine treated strips had a threshold of 3×10^{-9} g/ml and contracted maximally in response to 10^{-5} g/ml noradrenaline. The dose response curve was shifted to the left but no change in the maximum was seen in the curve. Isometric responses thus differ from isotonic contractions of reserpine treated spleen strips in which the maximum response is higher. When the ratio of slow to fast phases in reserpine treated preparations was compared with those in control tissue it was found that for any given concentration of noradrenaline the ratio was slightly higher in the reserpine tissue. This difference was not statistically significant at the 0.05 level except for 3×10^{-7} g/ml noradrenaline. However, when the responses of reserpine treated strips to this concentration were compared with nearly equivalent responses in control strips due to 10^{-6} g/ml, no statistical significance could be found. A very curious observation in reserpine treated tissues was the presence of rhythmic contractions of small amplitude superimposed on the slow phase of responses obtained due to noradrenaline (3×10^{-8} to 3×10^{-7} g/ml). This phenomenon will be more fully described later.

Based on previous interpretation of the significance of the two phases of noradrenaline response, it seems that both stores of calcium are probably not differentially affected during the supersensitivity produced by reserpine.

b) Effect of cocaine and 6-hydroxydopamine

Both cocaine and 6-hydroxydopamine cause supersensitivity to

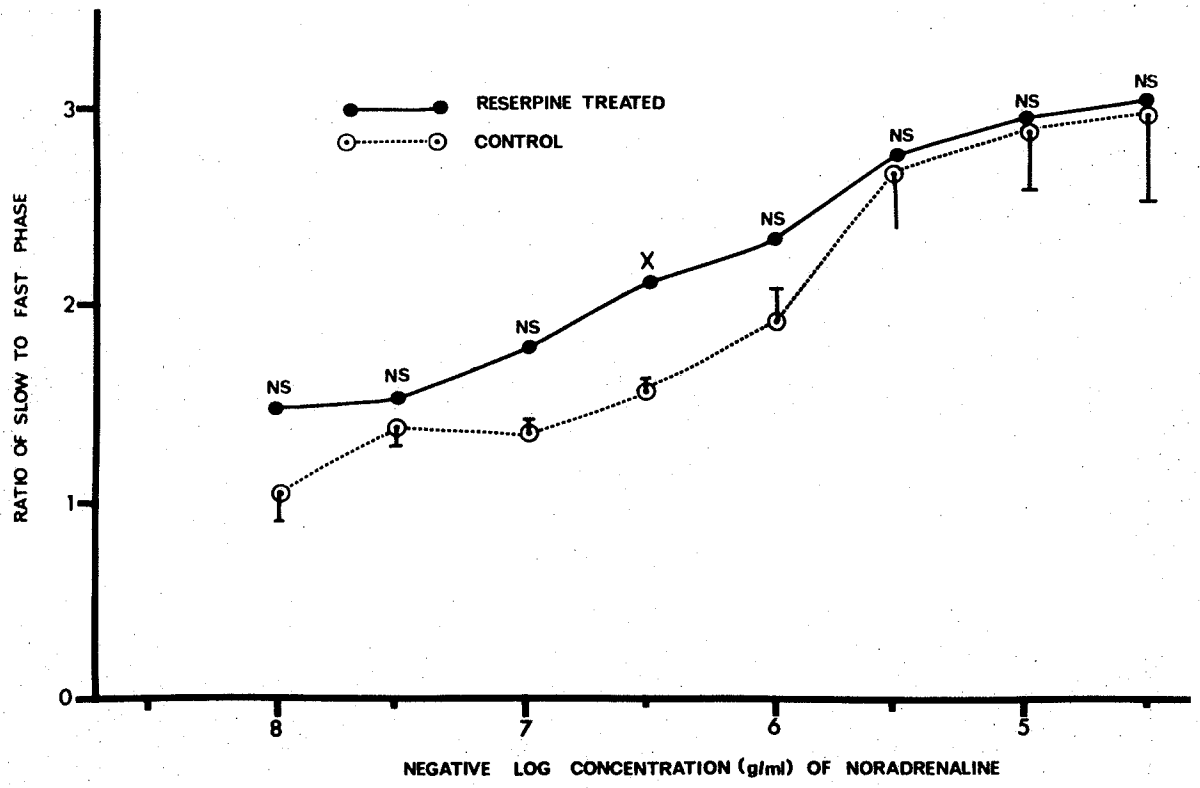


Fig. 49. Effect of reserpine on the phases of contraction of spleen strips due to noradrenaline.

Ratios of slow to fast phases of contraction produced by different concentrations of noradrenaline in spleen strips of cats treated 24 hrs prior to experiments with reserpine (1 mg/kg), (●), and in strips from untreated animals, (⊙). The ratios obtained in the two groups of spleen strips were not significantly different for any concentration of noradrenaline except 3×10^{-7} g/ml ($p < 0.05$), indicated by (X).

noradrenaline in the spleen (Davidson, 1970; Mailhot, 1970). Both the agents decrease neuronal uptake of noradrenaline, the former by an action on the amine pump and the latter by causing selective degeneration of the sympathetic postganglionic nerve terminals. Potentiation of catecholamines by both these agents is generally believed to be due to the blockade of uptake, although evidence favouring an alternative postsynaptic action of cocaine is mounting (Davidson, 1970). It is believed that the latter may involve an enhanced efficiency of utilisation of calcium stores for contraction (Greenberg, 1968; Kasuya and Goto, 1969). A basic effect such as this is an attractive mechanism for explaining the ability of cocaine to potentiate responses of agonists other than sympathomimetic amines. On the other hand, potentiation of noradrenaline responses in 6-hydroxydopamine treated spleen is specific. The responses of the spleen to isoprenaline, a poorly transported sympathomimetic amine in the heart (Iversen, 1967), and to histamine are not potentiated (Mailhot, 1970). It was therefore of interest to study the effect of these two agents on the two phases of noradrenaline responses.

In six experiments cocaine (10^{-5} - 3×10^{-5} g/ml) decreased the threshold concentration of noradrenaline from a control value of 10^{-8} g/ml to 10^{-9} g/ml. The maximum responses to noradrenaline were not different from the control but were obtained with a smaller concentration of 3×10^{-6} g/ml of the agonist. Qualitatively similar results were obtained in spleen strips from 6 cats treated with 6-hydroxydopamine 24 hr before experimentation. Such spleens had a catecholamine content of 0.19 ± 0.04 μg/g tissue wet weight, a decrease in 93.5% over control values. The shift in the dose response curve was roughly 70% of that obtained with cocaine. Marked rhythmicity

superimposed on the slow phase was seen in the response to noradrenaline (10^{-8} - 3 x 10^{-7} g/ml) in these tissues. This is sharply in contrast with normal tissues which as a rule did not show any rhythmicity.

The most characteristic change in the relationship between the ratio of slow to fast phases and the concentration of noradrenaline was an increase in the ratio at low concentrations compared to that in control tissues (Fig. 50). As the concentration of the agonist increased, the ratio fell to a level which resembled that in the control strips. The differences between the ratio for any concentration of noradrenaline from 10^{-8} to 10^{-6} g/ml in the control and cocaine or 6-hydroxydopamine treated tissues were significant ($p < 0.05$). The ratios in response to 3 x 10^{-6} and 10^{-5} g/ml of noradrenaline were not significantly different. When the ratios of the two phases in responses of equal magnitude in the two groups of tissues were compared rather than ratios in response to equivalent concentrations of the agonists, the differences were again found to be significant except in the case of ratios obtained with 10^{-6} g/ml and higher concentration of noradrenaline in the cocaine or 6-hydroxydopamine treated tissue and the corresponding responses in control.

The main effect of cocaine or 6-hydroxydopamine on the spleen is to increase the contribution of slow phase to any given response to noradrenaline.

XV. Comparison of the effects of noradrenaline and isoprenaline in spleen strips

Both noradrenaline and isoprenaline cause spleen strips to contract by acting on alpha-adrenoceptors (Bickerton, 1963). We were therefore surprised when at the beginning of this study (Fig. 1) it was

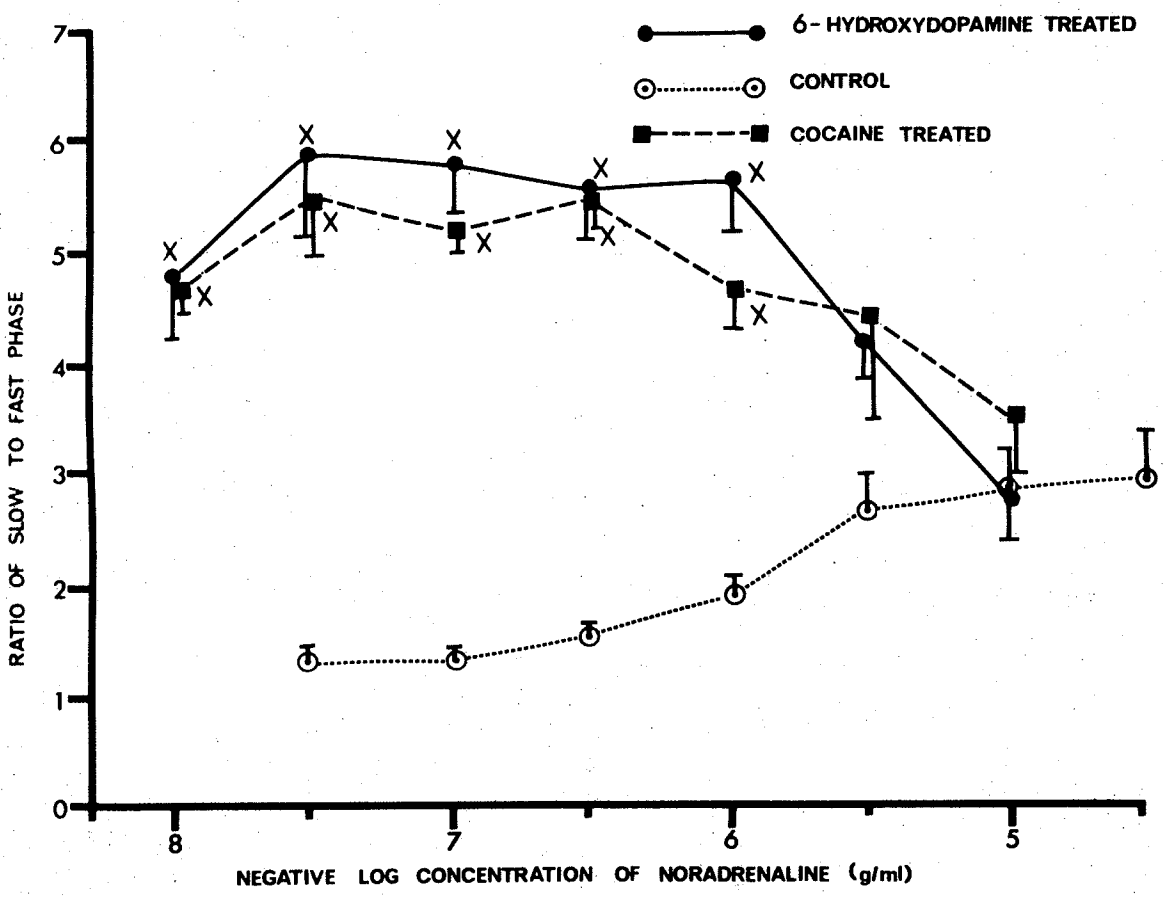


Fig. 50. Effect of 6-hydroxydopamine and cocaine on the phases of contraction due to noradrenaline.

The ratio of slow to fast phases of contraction of spleen strips to various concentrations of noradrenaline;

(⊙), in untreated strips (control); (■), in the presence of cocaine, 10^{-5} g/ml; (●), in strips from cats treated with 6-hydroxydopamine (35 mg/kg) 24 hr beforehand. Each ratio marked with (X) is significantly different from the corresponding ratio in the control curve ($p < 0.05$). No significant difference was observed between cocaine and 6-hydroxydopamine treated strips.

observed that equal responses to these two agents consisted of entirely different proportions of slow and fast phases. This was investigated in greater detail in this series of six experiments.

Compared to noradrenaline (10^{-8} g/ml) the threshold concentration of isoprenaline was greater (10^{-6} g/ml). The responses to isoprenaline had a larger proportion of slow to fast phase compared to responses with noradrenaline. Fig. 51 shows the ratio of slow to fast phases of response to nearly equal responses to isoprenaline and noradrenaline as a function of the corresponding concentration of agonist employed. The ratios were significantly different ($p < 0.05$) for the three smaller concentrations shown. The ratio of slow to fast phases in response to isoprenaline were larger with smaller doses and decreased progressively with increase in agonist concentration to approach the values obtained in responses due to high concentrations of noradrenaline. Thus the curve obtained with isoprenaline differed qualitatively from that obtained with noradrenaline in control strips but resembled those obtained with the same agent in tissues treated with cocaine or 6-hydroxydopamine.

In another three experiments the ratios of slow to fast phases of equal responses to noradrenaline (7×10^{-8} g/ml) and isoprenaline (3×10^{-5} g/ml) when compared in a 6-hydroxydopamine treated tissue did not show much difference. Fig. 52C shows a typical experiment. For comparison equivalent responses to the two agonists in strips from two untreated control spleen strips were obtained and the typical record shown in Fig. 52B. The two responses had markedly different ratios. It thus seems that the differences in the ratio of slow to fast phases of noradrenaline and isoprenaline in normal spleen strips are due to some mechanism that is affected by pretreatment with 6-hydroxydopamine.

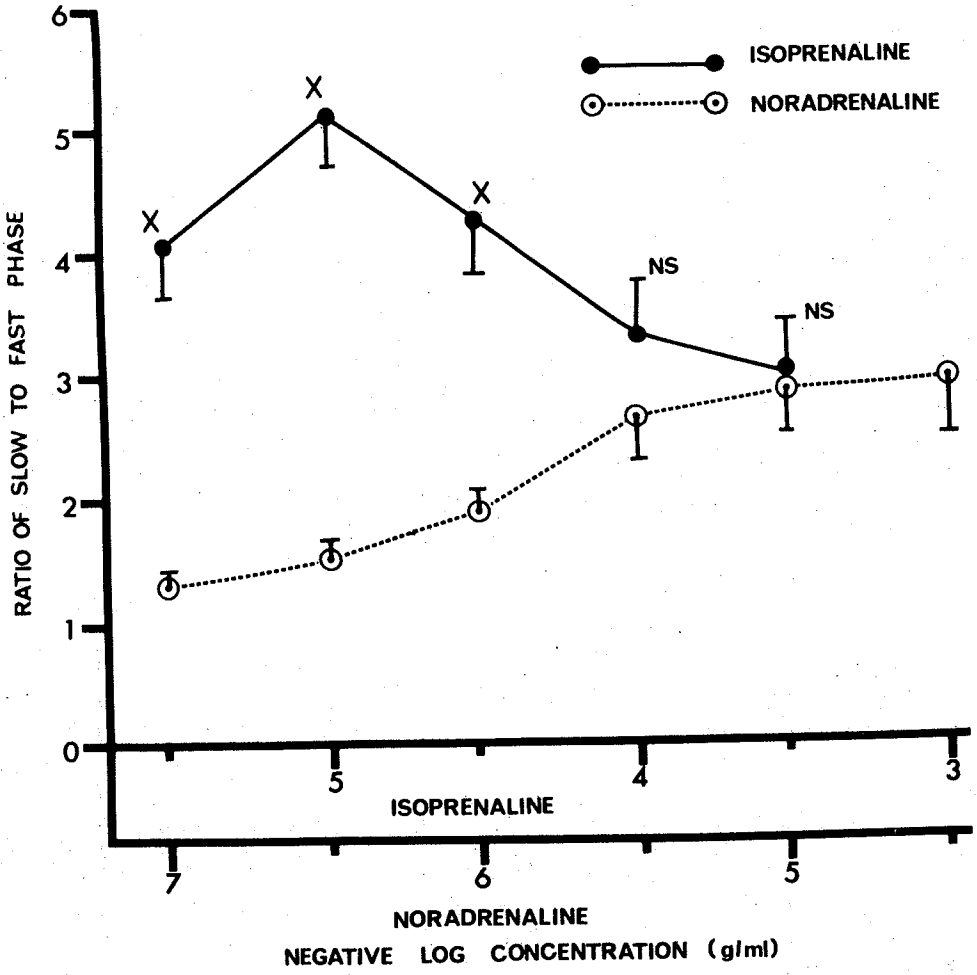


Fig. 51. Comparison of responses of spleen strips to noradrenaline and isoprenaline.

Ratios of slow to fast phases of responses after various concentrations of isoprenaline (●), and noradrenaline (⊙). The ratios in contractions of similar magnitude after isoprenaline and noradrenaline differed significantly ($p < 0.05$ shown at X) at the lower concentrations. The scales of concentration of the two agonists have been arranged so that contractions at the same point of the abscissa are nearly equal.

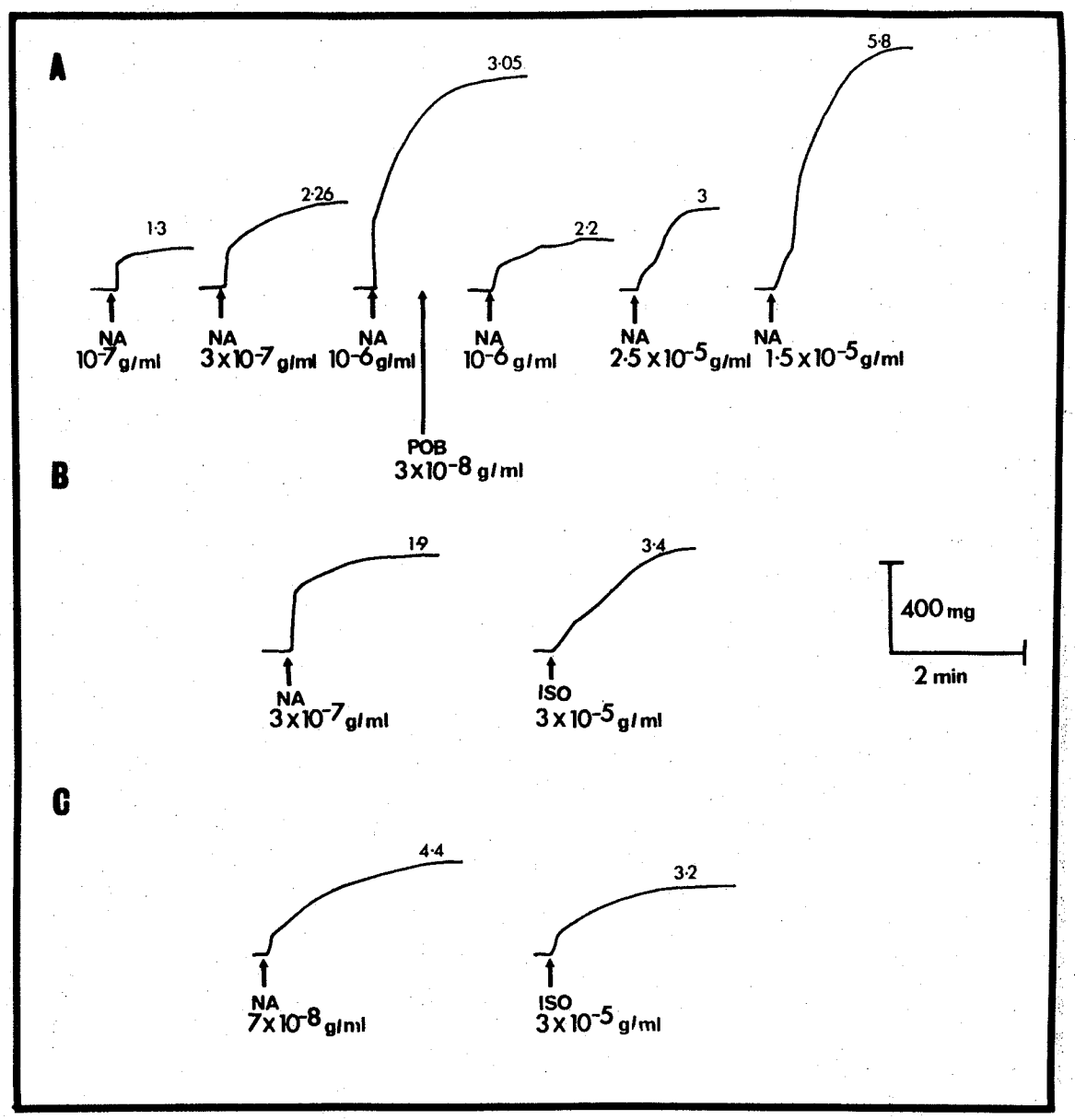


Fig. 52. Variation in the ratio of slow to fast phases of contraction with noradrenaline and isoprenaline; the effects of phenoxybenzamine and of treatment with 6-hydroxydopamine.

A. Contraction of spleen strip due to different concentrations of noradrenaline (NA) before and in the presence of phenoxybenzamine (POB).

B. Contractions of untreated spleen strip to noradrenaline (NA), isoprenaline (ISO).

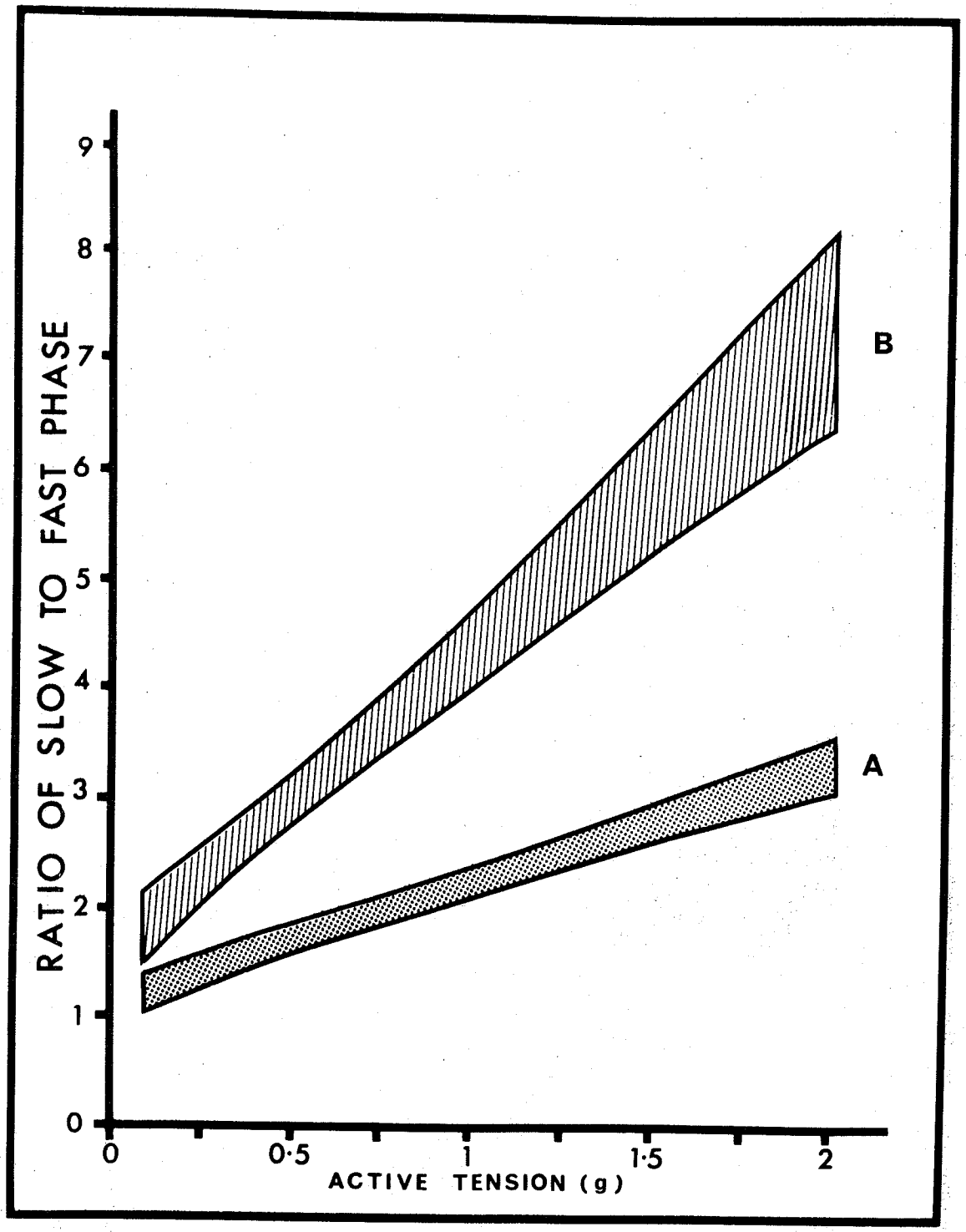
C. Responses to NA or ISO in spleen strips from a cat treated with 6-hydroxydopamine.

XVI. Effect of phenoxybenzamine on the response due to noradrenaline

Phenoxybenzamine is a non-equilibrium antagonist of the alpha-adrenoceptor in the spleen. Consequently if a single type of receptor exists for noradrenaline then inactivation of this receptor population should affect the response but not the proportion of slow to fast phases. This, surprisingly, was not found to be the case, (Fig. 52A). It can be seen that before exposure to phenoxybenzamine, responses to 10^{-7}, 3×10^{-7} and 10^{-6} g/ml of noradrenaline showed increasing responses having ratios of 1.3, 2.26 and 3.05 respectively. The tissue was exposed to phenoxybenzamine (3×10^{-8} g/ml) for 5 min followed by several washes with Krebs-Henseleit solution. The tissue became about 10 times less sensitive to noradrenaline. However, comparable responses after phenoxybenzamine had larger ratios of slow to fast phases. In 6 such experiments, spleen strips were stimulated with several concentrations of noradrenaline before and after treating the strips with phenoxybenzamine (3×10^{-8} g/ml). The results have been shown in a different way compared to the results obtained in the previous two sections. Since in normal tissues the ratio of slow to fast phases increased with increasing concentration, it was possible that the increased ratio of the two phases after phenoxybenzamine was due to the higher concentrations of noradrenaline necessary. It was therefore found more convenient to compare the ratio of slow to fast phases for any given value of active tension in normal (Fig. 53A) or phenoxybenzamine treated tissues (Fig. 53B). The shaded areas represent the two regression lines surrounded by their respective 95% confidence limits. For any given active tension produced by noradrenaline, the ratio of slow to fast phases was significantly higher in phenoxybenzamine treated

Fig. 53. Effect of phenoxybenzamine on the ratio of slow to fast phases of responses to noradrenaline.

Ratio of slow to fast phases of response to different doses of noradrenaline are plotted as a function of the total active tension at which they were measured: in the presence (striped) or absence (stippled) of phenoxybenzamine (3×10^{-8} g/ml). The active tension is a function of the dose of noradrenaline employed. The area within each zone denotes the 95% confidence limit for each regression line.


RATIO OF SLOW TO FAST PHASE
0
1
2
3
4
5
6
7
8
9
B
A
0
0·5
1
1·5
2
ACTIVE TENSION (g)

tissue. These results suggest that a simple blockade of a single population of alpha-adrenoceptors is probably not the only result of phenoxybenzamine treatment. It is interesting to note that the increase in ratio of slow to fast phases caused by phenoxybenzamine for any given active tension induced by noradrenaline is similar to the effect of cocaine or 6-hydroxydopamine even though phenoxybenzamine inhibits responses of spleen to noradrenaline while the latter agents cause supersensitivity.

XVII. Rhythmic contractions evoked in spleen strips

a) Effect of cocaine, reserpine and 6-hydroxydopamine pretreatment

Normal spleen strips in vitro never showed spontaneous rhythmic contractions. When such a spleen was stimulated with different amounts of noradrenaline, as a rule no rhythmicity was observed. However, in the presence of cocaine 10^{-5} - 3 x 10^{-5} g/ml or in spleen strips obtained from cats pretreated with reserpine (1 mg/kg) or 6-hydroxydopamine (35 mg/kg) 24 hr before the experiment, small concentrations of noradrenaline (varying from 10^{-8} - 3 x 10^{-7} g/ml) produced two phases of contraction but, in addition, rhythmic contractions superimposed on the slow phase were also seen in a majority of 6 - 10 experiments done after each procedure. A typical experiment in a spleen strip obtained from a cat treated with reserpine exhibited rhythmic contractions in the presence of 3 x 10^{-8} or 10^{-7} g/ml of noradrenaline (Fig. 54A). With the smaller concentration of noradrenaline the contractions occurred once every minute. The frequency was greater with the higher concentration of noradrenaline. When the muscle was stimulated with 3 x 10^{-8} g/ml of noradrenaline in the presence of cocaine (10^{-5} g/ml) in addition, the amplitude as well as rate of rhythmic contractions increased compared to the control reserpine

treated tissue. Increasing the concentration of noradrenaline in a tissue exhibiting rhythmic contraction gradually increased the frequency of contraction, but with concentrations above 10^{-7} g/ml there was a marked decrease in the amplitude of the rhythmic contractions. Usually with concentrations of noradrenaline above 3×10^{-7} g/ml, the rhythmic contractions seemed to fuse into one another and eventually disappear. The responses shown in Fig. 54B were obtained in the presence of cocaine (10^{-5} g/ml) with various concentrations of noradrenaline. The 3rd response represents the stage where the rhythmic contractions were becoming fused. Further increase in noradrenaline concentration resulted in the typical 2 phase response without rhythmic activity.

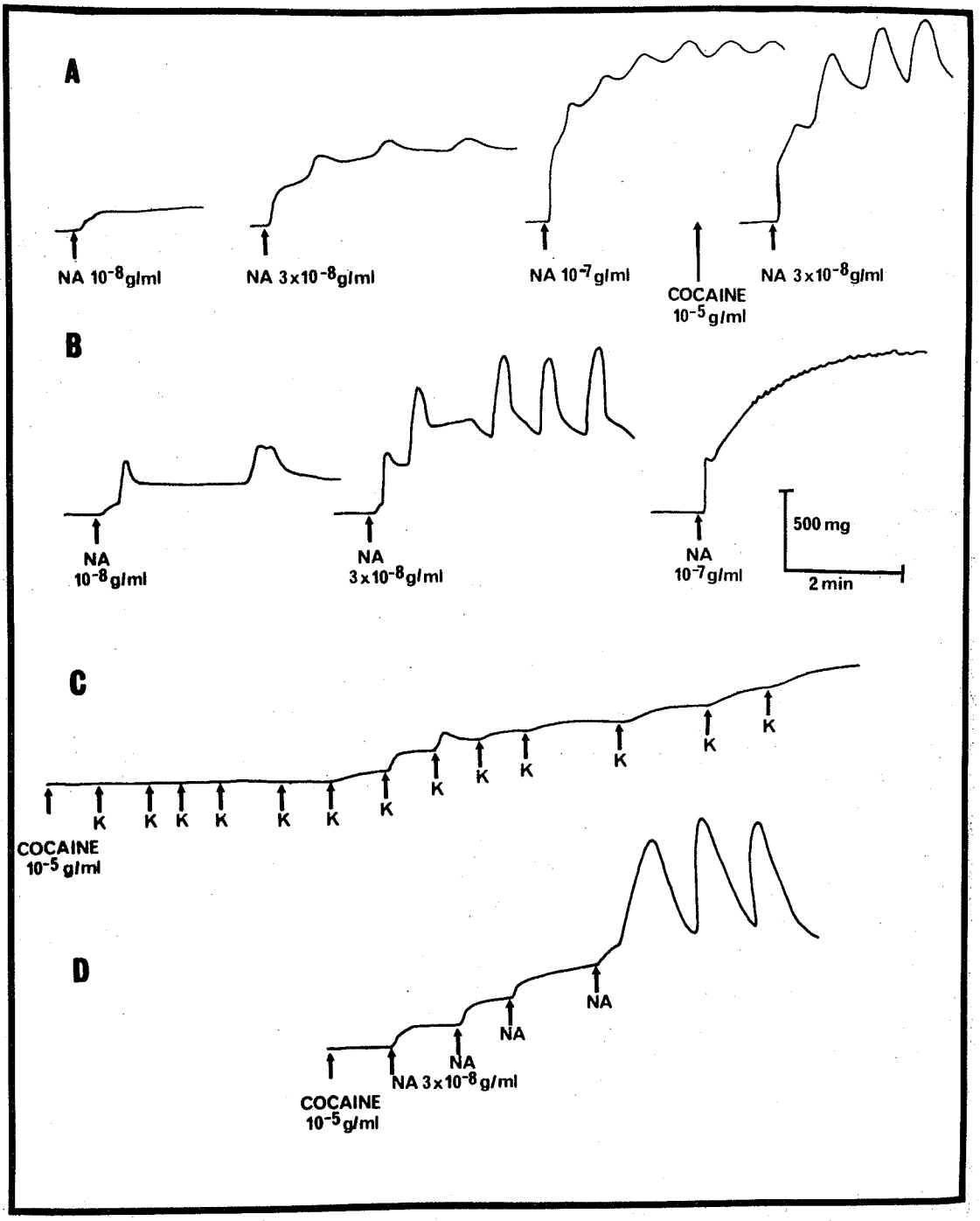
The induction of rhythmic contraction did not appear to be due merely to increase in tension due to the agonist. In 4 experiments, pairs of reserpine treated spleen strips were exposed to cocaine (10^{-5} g/ml). One of them was stimulated by cumulatively increasing concentrations of potassium chloride (Fig. 54C) while the other was stimulated with noradrenaline (Fig. 54D). In the presence of noradrenaline (1.2×10^{-7} g/ml) larger rhythmic contractions were elicited. On the other hand when equivalent contractions were produced by potassium, no rhythmicity was induced. Increasing the potassium concentration to cause a contraction larger than that due to noradrenaline (not shown) still did not give rise to rhythmic contractions. The behaviour of the spleen to elevated external potassium differs from that of some vascular smooth muscles which do exhibit rhythmicity in response to this ion (Johansson and Bohr, 1966).

Fig. 54. Effect of noradrenaline or potassium in spleen strips after treatment with cocaine or reserpine.

A. Effect of different concentrations of noradrenaline (NA) on spleen strips of reserpine treated cats. Unlike responses in untreated spleen strips, in these strips rhythmic contractions were superimposed on the slow phase. Cocaine caused further potentiation in the response to NA (3×10^{-8} g/ml) along with increase in amplitude of rhythmic contractions.

B. Effect of cocaine on spleen strip from normal cats. The strip was first exposed to cocaine, 10^{-5} g/ml. Increasing concentrations of noradrenaline (NA) caused at first, increase in the amplitude of the fast and slow phases as well as the amplitude and frequency of rhythmic contractions. NA (10^{-7} g/ml) the rate of rhythmicity became faster but the amplitude was reduced. At still higher concentrations (not shown) the rhythmic contractions disappeared.

C and D. Paired cocaine treated strips obtained from same spleen as in B. KCl, 2.1 mM, (K) added cumulatively in C. No rhythmic contractions were seen. Noradrenaline, 3×10^{-8} g/ml, (NA) added cumulatively in D. Note rhythmic contractions.


A
NA 10⁻⁸g/ml
NA 3x10⁻⁸g/ml
NA 10⁻⁷g/ml
COCAINE 10⁻⁵g/ml
NA 3x10⁻⁸g/ml
B
NA 10⁻⁸g/ml
NA 3x10⁻⁸g/ml
NA 10⁻⁷g/ml
500 mg
2 min
C
K
COCAINE 10⁻⁵g/ml
D
NA
NA
NA
NA 3x10⁻⁸g/ml
COCAINE 10⁻⁵g/ml

b) Effect of increase in external potassium concentration on the rhythmicity induced by sympathomimetic amines in spleen strips

During the study of rhythmicity induced by noradrenaline, it was seen that rhythmicity could not be maintained for more than 5 - 10 min. The active tension gradually decreased and rhythmicity eventually subsided. At this point replacement of the bathing fluid with a fresh solution of noradrenaline and cocaine restored the rhythmic responses. This suggested that destruction of the drugs was occurring. However, when rhythmicity was induced by a relatively specific alpha-adrenoceptor stimulant phenylephrine, the effect persisted for as long as 45 min. In several experiments, therefore, phenylephrine was used instead of noradrenaline.

Spontaneous rhythmicity observed in various smooth muscles is believed to be myogenic and associated with action potentials which arise in pacemaker cells and are propagated over a large mass of smooth muscles in a concerted fashion (Somlyo and Somlyo, 1968a). A detailed search of the literature failed to reveal any description of rhythmicity in spleen strips in vitro. Nor could data be found concerning the electrophysiological correlates of contractility of the spleen. We have so far not succeeded in making direct microelectrode recordings of transmembrane potential changes in the splenic smooth muscle during rhythmicity. Under these circumstances we were forced to employ an indirect approach to study the effect of changes in membrane potential on rhythmicity. The experiment shown in Fig. 55A is typical of 3 experiments on strips from reserpine treated cat in which rhythmic contractions were obtained with phenylephrine

(2×10^{-5} g/ml) and cocaine (10^{-5} g/ml). Increasing the potassium concentration of the bathing medium from 5.4 to 8.4 mM abolished rhythmicity but did not change basal active tension. An increase in the concentration of phenylephrine to 4×10^{-5} g/ml by addition of the required amount to the bath resulted in an increase in basal active tension and resumption of rhythmic activity. The potassium concentration was raised again to 11.4 mM and rhythmicity disappeared. A further increase in phenylephrine concentration to 6×10^{-5} g/ml raised the basal active tension but did not restore rhythmicity and further increase in potassium concentration to 14.4 mM caused an additional increase in tension but no rhythmicity. Thus elevation of potassium had a dual effect - an initial suppression of rhythmicity without changing basal tension and a subsequent increase in basal tension. In two experiments, after addition of cocaine (10^{-5} g/ml) and phenylephrine (2×10^{-5} g/ml) the muscle exhibited rhythmic contractions initially. However, as the basal active tension reached a maximum for the dose employed, the rhythmicity disappeared. Increasing the concentration of potassium to 8 mM produced a slight decrease in tension and resumption of rhythmic contractions (Fig. 55B). Further elevation of potassium concentration to 14 mM abolished rhythmicity (not shown) as the basal active tension increased. Fig. 55C shows the results of another experiment in which, after rhythmicity was induced by phenylephrine in the presence of cocaine, progressive increase in potassium concentration resulted in a progressive decrease in the amplitude of the rhythmic contraction - with a slight decrease in basal active tension but with higher concentration (11.2 mM) of potassium accompanied by an increase in basal active tension back to the level before adding potassium. Any further increment in

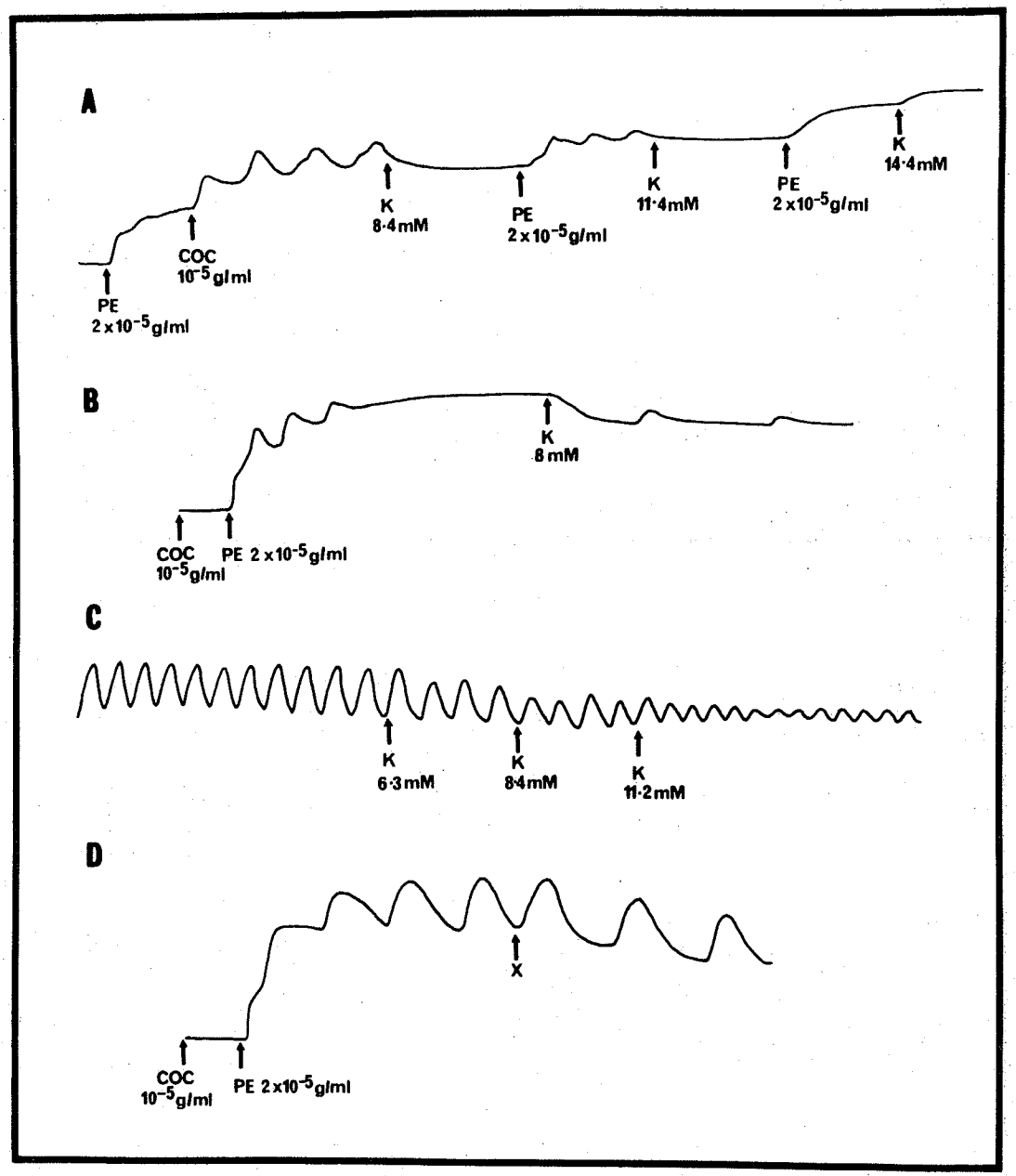
Fig. 55. Rhythmicity in spleen strips.

A. Rhythmicity due to cocaine (COC) after contraction was induced by phenylephrine (PE). Addition of KCl (K) to obtain a final concentration of 8.4 mM stopped rhythmic activity with slight decrease in basal active tension. Addition of PE restored rhythmicity which was blocked by an increment in K concentration. Further addition of PE increased tension but did not restore rhythmicity. Increasing K concentration to 14.4 mM further increased tension but did not restore rhythmicity.

B. Addition of PE in the presence of COC caused an increase in tension. Rhythmic contractions superimposed on the slow phase finally disappeared. Addition of KCl decreased tension and restored rhythmic contractions.

C. Effect on rhythmicity induced by COC (10^{-5} g/ml) + PE (2×10^{-5} g/ml) of addition of K to the bathing medium.

D. After rhythmicity was induced by COC + PE, the level of the bathing medium was lowered at X so as to expose half the muscle to air. The amplitude of contractions remained unchanged although the basal active tension decreased slightly.


A
PE
2 x 10⁻⁵ g/ml
COC
10⁻⁵ g/ml
K
8·4 mM
PE
2 x 10⁻⁵ g/ml
K
11·4 mM
PE
2 x 10⁻⁵ g/ml
K
14·4 mM
B
COC
10⁻⁵ g/ml
PE 2 x 10⁻⁵ g/ml
K
8 mM
C
K
6·3 mM
K
8·4 mM
K
11·2 mM
D
COC
10⁻⁵ g/ml
PE 2 x 10⁻⁵ g/ml
X

potassium concentration abolished rhythmicity while increasing the basal active tension. In Johansson and Bohr's (1966) study, elevation of potassium (30 mM) was also found to decrease rhythmicity of strips obtained from subcutaneous arteries when the initial active tension was high. This was associated with a decrease in frequency of rhythmic contractions. When the initial active tension was low, addition of potassium resulted in an increase in tension and rhythmicity; the latter never occurred in any experiment on spleen strips. The present experiments indicate that rhythmic contractions are produced only by a limited range of concentrations of sympathomimetic agents and these are affected by alteration in the extracellular concentration of potassium.

c) Conduction of activity in rhythmically acting spleen strips

Rhythmic contractions of smooth muscle generally indicate single unit behaviour. This means that activation of smooth muscle cells of one part of the tissue should result in propagation of excitation to other parts of the tissue and activation of the cells there. The following experiment was designed to test this point. Rhythmic contractions were induced in pairs of spleen strips by addition of cocaine and phenylephrine. After the contractions had become uniform, the medium in one of the baths was lowered so that only half of the strip was exposed to the solution while the other half was exposed to warm moist air above the medium. If the pacemakers initiating the rhythmic contractions existed in the lower half of the strip (bathed by the medium) and if the rhythmic strip was capable of conducting the excitatory wave of depolarisation arising from the pacemakers, then the upper half of the strip should still contract rhythmically and the total magnitude of the rhythmic contraction should

not be altered. On the other hand if the conduction failed, the amplitude of rhythmic contractions should be reduced by about half. Finally, if the pacemaker cells were in the region of the strip exposed to air, the rhythmic contractions might be completely abolished as the limited amount of the agonist in the solution present on the muscle as a thin film was metabolised by enzymes. In this case, even though the muscle exposed to the medium could conduct an impulse, no rhythmicity would be seen. Thus a decrease or abolition of rhythmicity does not necessarily rule out single unit behaviour of the muscle. On the other hand persistence of the undiminished rhythmic contractions would strongly support this possibility. When the level of the medium surrounding the rhythmically contracting strips was lowered to expose half of the strip to air, the basal active tension invariably decreased steadily. In 2 out of 6 experiments the amplitude of rhythmic contractions was maintained, in 1 experiment the amplitude was diminished, and in the remaining 3 muscles the rhythmicity was abolished. Fig. 55D shows the result of the experiment in which the rhythmicity did not diminish in amplitude.

d) Myogenic response

Most known single unit muscles exhibit a peculiar phenomenon known as myogenic contraction. In this, a muscle when stretched rapidly responds by an increase in tension or shortening. Spleen strips from 5 different cats under 1 g resting tension returned every time in over 20 tests to their original level of tension slowly after rapid stretch (Fig. 56A, 1st and 2nd responses). The strips were then exposed to cocaine (10^{-5} g/ml) and noradrenaline (10^{-8} g/ml). The strips showed either a simple increase in tension and no rhythmicity or an initial rhythmicity

and increase in tension which gradually disappeared. If the strips were rapidly stretched at this stage the muscle developed a transient increase in tension over and above the resting tension of 1 g (Fig. 56A, 3rd and 4th responses). This occurred in every one of nearly 30 tests. In other strips in which the rhythmicity was regular and maintained, stretch was applied before an expected contraction. Again an active increase in tension occurred and a long pause was seen before the next spontaneous contraction occurred (not shown). When cocaine and noradrenaline were washed out (Fig. 56A, 5th and 6th responses), myogenic responses could not be elicited in 90% of the tests.

e) Effect of altered calcium concentrations and of substances altering calcium utilisation on the rhythmic contractions in spleen

Calcium ions are believed to play an important part in carrying the ionic current responsible for action potentials in smooth muscle. Whether the ions are derived from the extracellular (? loosely bound store) or from the tightly bound store is still not clear. The effect of decrease in external calcium on rhythmicity was therefore studied as follows: Rhythmic contractions were produced by noradrenaline in 3 spleen strips treated with cocaine (10^{-5} g/ml). When the contractions became regular, EGTA (1 mM) was added to the bathing medium. This was expected to reduce the free Ca^{++} concentration of the medium to approximately 1.5 mM. Such reduction of available calcium has been shown by the experiments described earlier not to affect either phase of contraction due to noradrenaline. EGTA did not appreciably influence the basal active tension of the strips. However, the rhythmic contractions almost completely disappeared

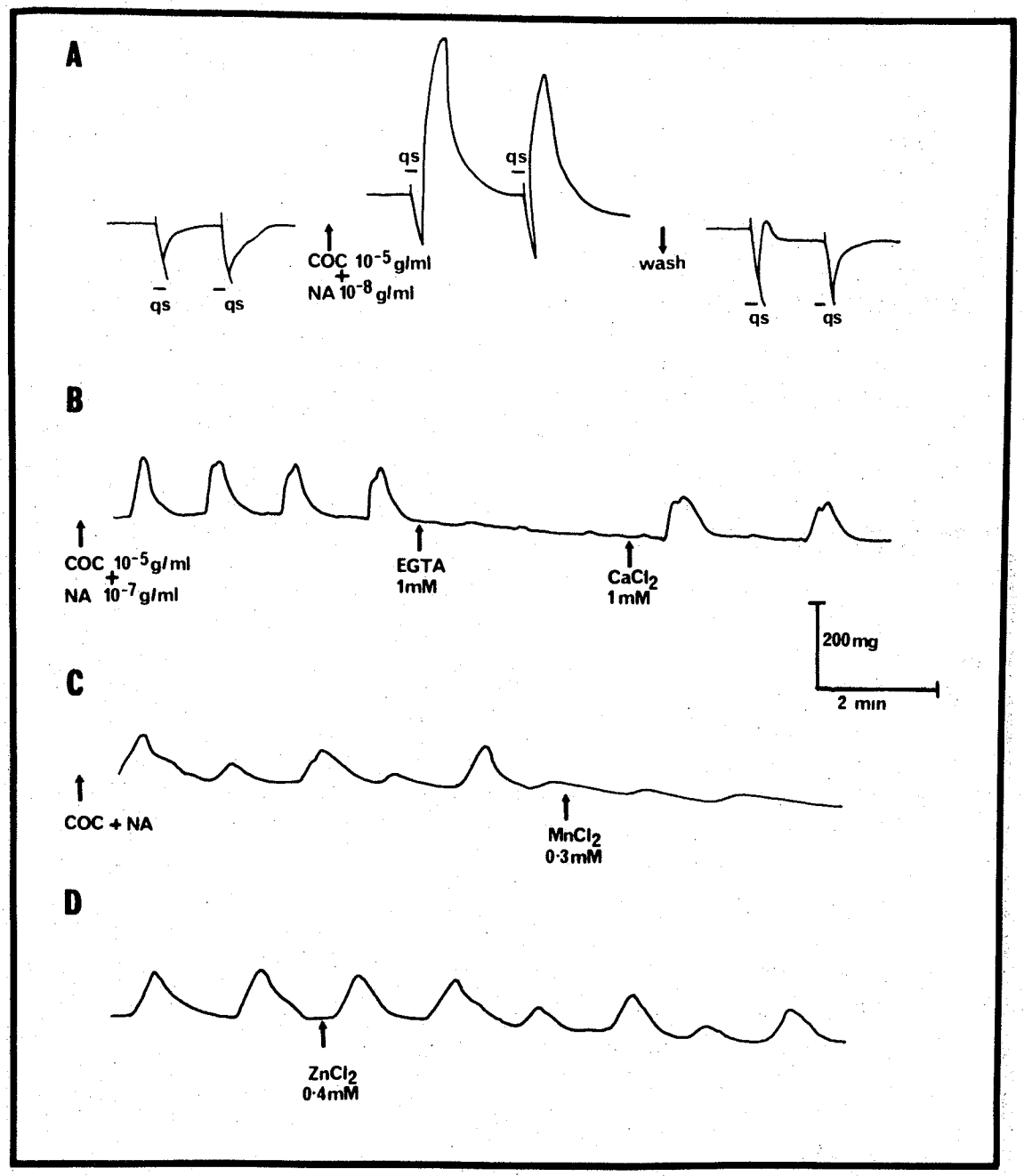
Fig. 56. Rhythmic contraction of spleen.

A. Quick stretch (qs) applied to resting spleen strip did not cause rebound increase in tension. After cocaine (COC) and noradrenaline (NA) a slight increase in tension but no rhythmic contractions were seen. qs now caused a marked rebound increase in tension. Washing out the drugs abolished rebound contraction due to qs.

B. Rhythmicity was induced in a spleen strip by noradrenaline (NA) in the presence of cocaine (COC). Addition of EGTA nearly abolished rhythmicity but had very slight effect on basal active tension. Small abortive rhythmic increase in tension was seen. Addition of $CaCl_2$ restored rhythmicity contractions.

C. Rhythmicity induced as above was diminished by $MnCl_2$.

D. Rhythmicity induced as above. $ZnCl_2$ had a very slight inhibitory effect.


A
qs
qs
qs
qs
COC 10^{-5} g/ml
+
NA 10^{-8} g/ml
wash
qs
qs
B
COC 10^{-5} g/ml
+
NA 10^{-7} g/ml
EGTA
1mM
$CaCl_2$
1 mM
200 mg
2 min
C
COC + NA
$MnCl_2$
0·3mM
D
$ZnCl_2$
0·4mM

(Fig. 56B). The residual contractions appeared to be tiny and abortive and had a frequency similar to the contractions before addition of EGTA. Addition of calcium chloride (1 mM) restored rhythmic activity.

Similarly manganese chloride, which preferentially affects the utilisation of loosely bound calcium for producing the slow phase of contraction in the spleen due to noradrenaline, markedly diminished the rhythmic contraction in each one of 4 experiments (Fig. 56C). On the other hand zinc chloride in a concentration of 0.4 mM, which preferentially affects tightly bound calcium stores, had a much smaller effect on the rhythmic responses (Fig. 56D) in a similar number of experiments as the above. A higher concentration of 1 or 2 mM decreased rhythmicity every time (not shown). Such high concentrations, however, also affect the utilisation of loosely bound calcium to some extent,as seen earlier.

f) Effect of tetrodotoxin

Action potentials produced due to an increase in membrane permeability to sodium are abolished by tetrodotoxin,without many exceptions. Increasing amounts of tetrodotoxin were added to two spleen strips made rhythmic by cocaine (10^{-5} g/ml) and phenylephrine (2×10^{-5} g/ml). Concentrations of tetrodotoxin up to 5×10^{-6} g/ml failed to abolish rhythmicity (Fig. 57A) in 3 experiments. This concentration is 50 times greater than that required to abolish action potentials in the guinea-pig diaphragm (Kuriyama et al., 1966).

g) Effect of temperature and hypertonicity on rhythmic contractions

After rhythmicity was produced in 3 strips using the procedure mentioned above, the temperature of the bath was lowered in steps. Fig. 57B shows the effect of moderate lowering of temperature by 7°C. This resulted

in a decrease in the frequency and amplitude of contractions. These effects were reversed on restoring the temperature of the medium to 37°C.

Solutions made hypertonic by sucrose are well known to abolish conduction rhythmicity in single unit muscles (Dewey and Barr, 1968; Tomita, 1966b). Addition of sucrose (120 mM) to the medium bathing the rhythmically contracting spleen strips abolished activity.

h) Effect of inhibition of sodium pump on rhythmicity

Daniel and Chapman (1963) have suggested that rhythmic fluctuation in the activity of an electrogenic sodium pump may be responsible for oscillations in the membrane potential. It is conceivable that such fluctuations may be responsible for rhythmic contractions. Accordingly the effect of removal of external potassium and of the consequent inhibition of the sodium pump on rhythmicity was studied (Fig. 57D). After the spleen strips were made rhythmic with cocaine and noradrenaline, the Krebs-Henseleit bathing medium was changed to a prewarmed, K-free medium containing the same concentrations of cocaine and noradrenaline as the previous solution. The tension increased and rhythmicity disappeared in each one of three experiments. Addition of 5.4 mM potassium chloride caused a decrease in tension and resumption of rhythmic activity.

These results suggest that spleen strips which ordinarily do not behave as single unit muscles can be made to do so by several procedures. The rhythmicity does not seem to depend on sodium influx but a loosely bound source of calcium seems to be necessary. Reduction of external potassium concentration, which does not increase noradrenaline responses in normal spleen strips, increases the tension and abolishes rhythmicity when the latter is present. This suggests that an increased activity of

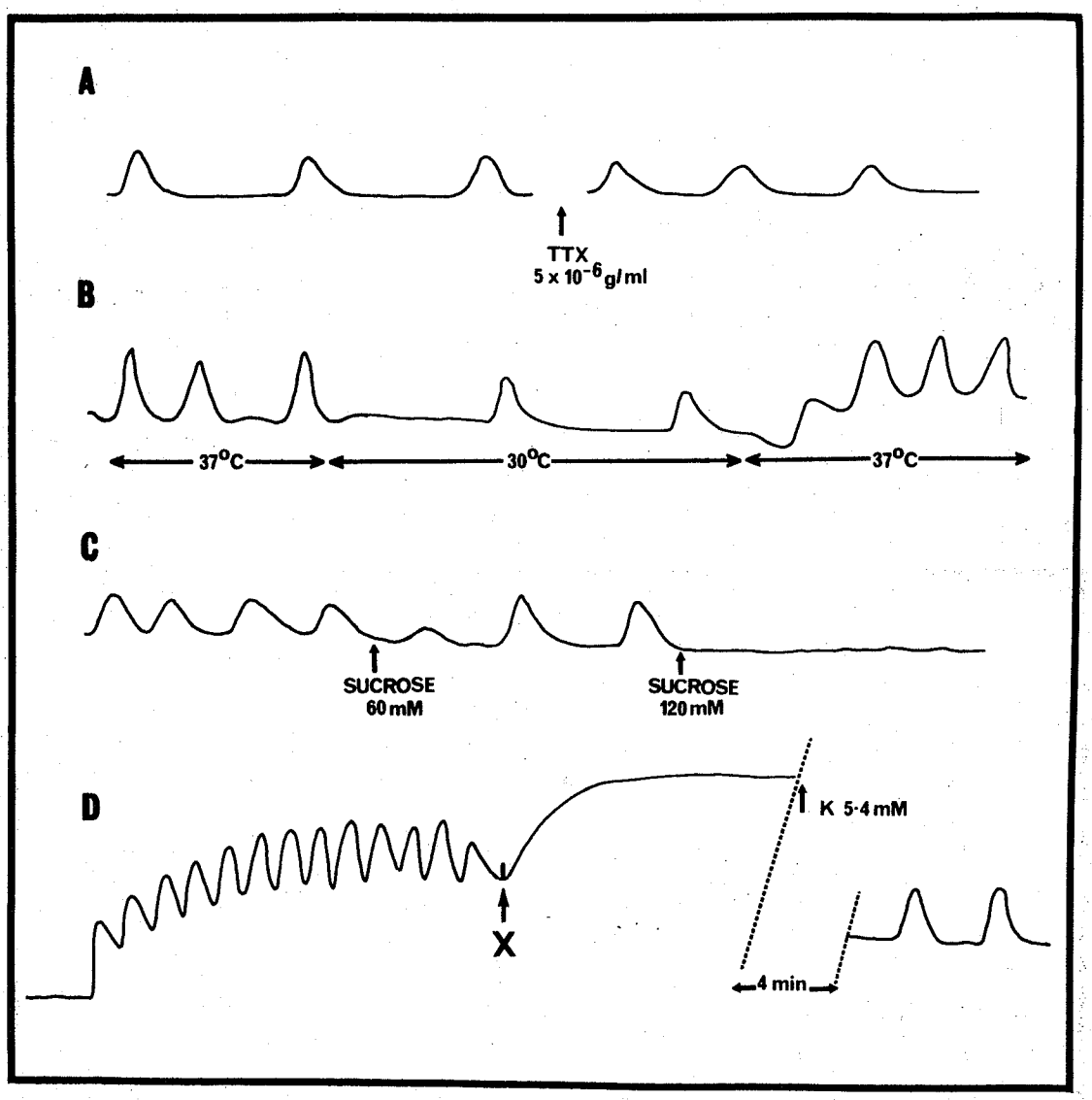
Fig. 57. Rhythmic contraction of spleen.

A. Rhythmic contraction induced in cocaine (10^{-5} g/ml) treated spleen by noradrenaline (3×10^{-8} g/ml). Tetrodotoxin (TTX) did not block the rhythmic contractions.

B. Rhythmic contractions obtained as above. Reduction of temperature from 37°C to 30°C decreased frequency of rhythmic contractions. Further lowering to temperature to 23°C (not shown) stopped rhythmic contractions.

C. Rhythmic contractions elicited as above and abolished by addition of sucrose (120 mM).

D. Rhythmic contraction produced in cocaine (10^{-5} g/ml) treated spleen by noradrenaline (10^{-7} g/ml). Replacement of the Krebs-Henseleit bathing medium with a K-free solution containing the same concentration of noradrenaline (X) resulted in an increase in tension and abolition of rhythmic contractions. Restoration of normal potassium concentration (5.4 mM) caused a decrease in tension and restoration of rhythmic contractions.


A
TTX
5 x 10⁻⁶ g/ml
B
37°C
30°C
37°C
C
SUCROSE
60 mM
SUCROSE
120 mM
D
K 5·4 mM
X
4 min

an electrogenic sodium pump may be prevailing in such tissues.

i) Electron microscopic examination of spleen capsule

The absence of spontaneous rhythmicity, myogenic response and also the lack of rhythmicity when stimulated with agonists in normal spleen suggest that it is probably functioning as a multiunit smooth muscle. On the other hand under the influence of cocaine, reserpine or 6-hydroxydopamine, the same tissue assumed a behaviour characteristic of single unit muscles. Dewey and Barr (1962) showed that the close functional communication between single unit smooth muscle cells was due to the presence of tight junctions between adjacent cells. These areas of membrane fusion or close membrane apposition were called nexuses. If splenic muscle ordinarily functions like a multiunit smooth muscle then it should be devoid of nexuses. It was therefore surprising to find that 'tight junctions' did exist among adjacent smooth muscle cells in the spleen capsule (Fig. 58). Various kinds of these junctions appeared to exist. The most common was one in which adjacent muscle cells extended processes which interdigitated. In some regions of these processes, the membranes appeared to make a very close contact. Less commonly the membranes of adjacent cells abutted against each other. Under higher magnification the two membranes appeared fused to each other with the loss of one layer.

In spite of these tight junctions, there was an abundance of connective tissue which surrounded the muscle cells extensively. This feature is typical of many multiunit muscles. The above picture suggests that the splenic capsular smooth muscle in the cat possesses the morphological correlates of a single as well as multi unit muscle even though functionally the former cannot be shown, in vitro, except under artificial

Fig. 58. Electron micrograph of spleen smooth muscle cells.

Smooth muscle cells marked a and b are closely apposed (in the region shown with an arrow) to form a nexus. Processes from cells marked b and c show extensive interlocking (x 35000).

The inset (lower right hand corner) shows the region of the nexus in greater magnification (x 54500). The membranes of adjacent cells appear to be fused.

1986

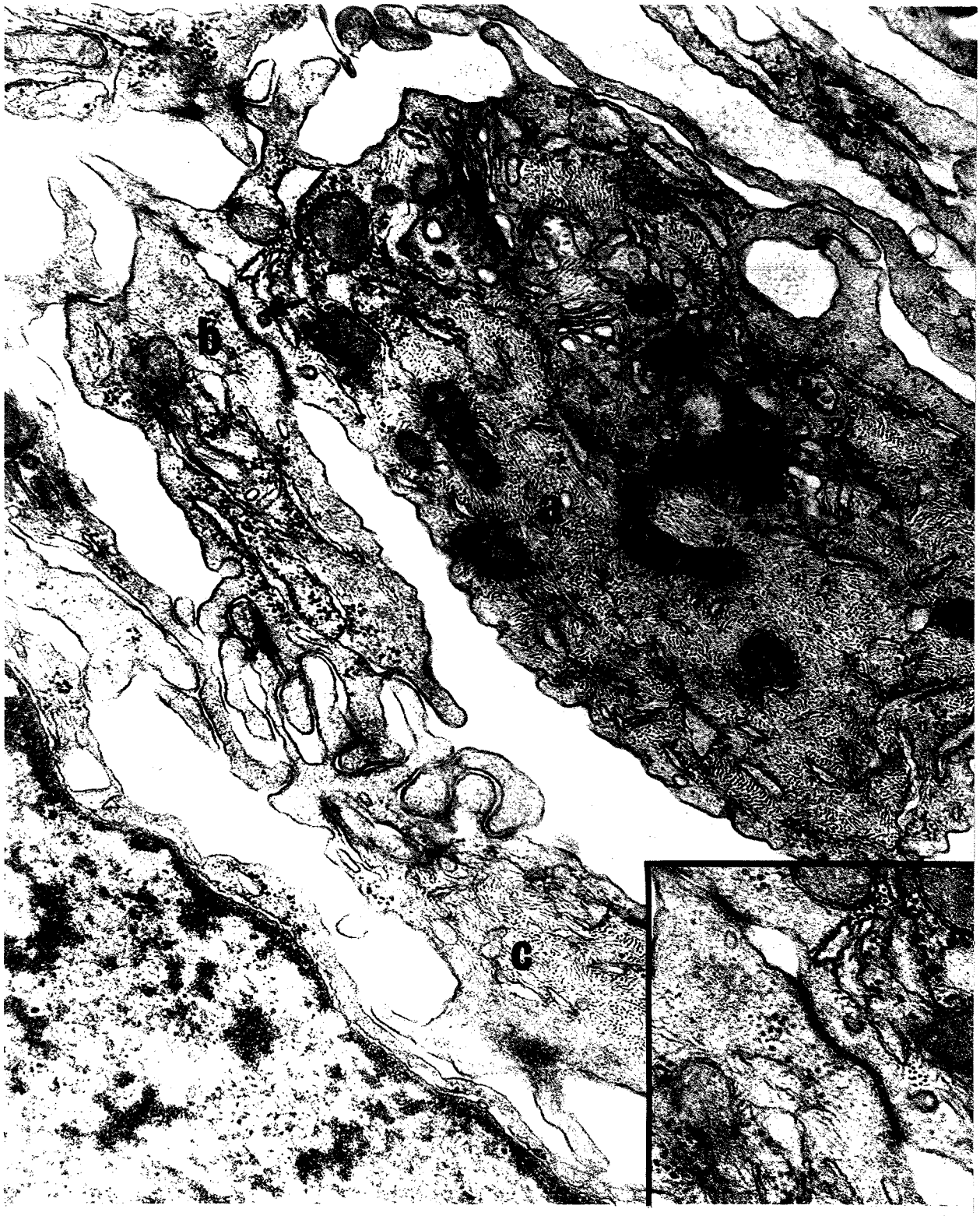


Fig 58

conditions. Whether such situations result in formation of more tight junctions or in a primary decrease in the resistance between existing junctions or secondarily as a result of an increased insulation of the perijunctional region around the nexus is not known. This certainly seems a fruitful area for further research.

DISCUSSION AND CONCLUSIONS

To maintain continuity and to explain the logical progression of this investigation, several relevant facts arising from the experiments have been discussed earlier in the 'RESULTS' section. Only those discussions that have a more general bearing on the problems investigated will be dealt with in greater length in this section.

Role of calcium in splenic capsular smooth muscle contraction and effect on the various phases of contraction

The importance of calcium ions in mediating contractility of muscles in general is well accepted, starting from the classic papers of Ringer (1880; 1882) on the effect of this ion on the heart. The demonstration by Heilbrunn and Wiercinsky (1947) that intracellular injection of calcium into skeletal muscle of the frog caused contraction is one of the most direct demonstrations of the role of calcium. More recently the study of Ridgeway and Ashley (1967) in which an increase in the calcium stimulated chemoluminescence of intracellularly injected aequorin in the giant muscle fibre of the barnacle was seen during muscle contraction, was a further confirmation of relationship between intracytoplasmic free calcium and muscle contraction. The necessity of calcium in smooth muscle contraction due to a variety of stimuli has been established in experiments where addition of calcium to glycerinated muscle fibres caused contraction (Filo et al., 1965) or the removal of calcium from the external medium resulted in loss of responsiveness of smooth muscles to stimuli (Waugh, 1962).

It has long been known that removal of external calcium does not decrease muscle responsiveness to all stimulants equally. For example, removal of external calcium abolished the responses to potassium

in the guinea-pig taenia coli (Durbin and Jenkinson, 1961), rabbit aortic strip (Hudgins and Weiss, 1967), rat uterus (Edman and Schild, 1962) faster than the responses to acetylcholine, noradrenaline and acetylcholine. This led to the proposal that different agents made available either loosely bound (extracellular) or tightly bound (intracellular) pools of calcium (Daniel, 1963; Waugh, 1962; Hinke, 1965; van Breemen et al., 1966; Hiraoka et al., 1968). Daniel (1965) proposed that the various functional pools of calcium might operate independently of one another or in a coupled manner in series. Our initial results with the spleen, in which stimulation with noradrenaline, isoprenaline and histamine resulted in isometric contractions which consisted of an initial fast and a subsequent slow phase, prompted us to investigate the mechanism of production of these two different phases.

The presence of two distinct phases of contraction with noradrenaline and isoprenaline which act on the alpha-adrenoceptors in the cat spleen (Bickerton, 1963) and histamine which acts on a different kind of receptor (Innes, 1962) suggested that the phenomenon was fairly general. Moreover, the absence of two phases in contraction produced by potassium - an agent which, unlike noradrenaline, does not have much effect on the tightly bound calcium pool (Hinke, 1965) suggested that an analysis of the different phases of contraction might result in a possible correlation with the manner of utilisation of the various calcium pools for contraction. Before going further it was necessary to eliminate the possibility of the two phases being artefacts of uneven mixing of the concentrated drug with the bathing medium. This possibility was easily ruled out when it was seen that noradrenaline premixed with Krebs-Henseleit

solution also produced two phases.

Another source of objection can be the lack of an absolutely non-compliant material to connect the spleen strips to the transducers in these experiments. Such a situation would lead to distortion of the recorded mechanical effects. However, if this were the case, it is the rapid phase which should be attenuated most. In the experiments in which the effect of different concentrations of noradrenaline on the two phases were studied, it was found that the smaller contractions produced by low concentrations of the agonist consisted of a proportionately larger amount of fast phase compared to responses obtained with higher concentrations of the agonist. This shows that the two phases of contraction are not mechanical artefacts due to the connecting terylene thread and indeed if there is any distortion of the fast phase due to the thread, it is insufficient to mask the phase to any significant extent.

With the two phases of contraction established as a reflection of some functional aspect of spleen strips it was necessary to consider if the different phases represented rates of contraction of a population of similar muscle cells or whether there were two populations of muscle cells. If the latter was true and if these two populations were not homogeneously distributed, then spleen strips cut in different directions would have shown differences in the proportion of the two phases. Since we found no such difference in our experiments to test this point we therefore cannot determine whether the cause is a single population of muscle cells or two populations distributed homogeneously. The production of greatest active tension change in the longitudinally cut strips correlates with the histological finding that most of the muscle cells were

aligned longitudinally.

Muscle contraction is a result of a series of events culminating in a response of the contractile proteins. It was clearly necessary to determine whether the two phases of contraction were due to factors related to the responsiveness of the contractile proteins or some earlier step or steps in the process. It is well known that the active tension produced by muscle increases when the resting tension muscle is increased up to a certain limit. This is most likely due to change in the position of actin and myosin filaments in relation to each other (Huxley and Peachey, 1961; Gordon et al., 1966).

Spleen strips subjected to resting tensions ranging from 0.5 g to 3 g produced the largest active tension in response to noradrenaline with the resting tension at 1 g; the active tension was considerably less when resting tension was either increased or decreased further. In spite of marked changes in active tension production, the relative proportions of slow and fast phases did not change significantly. This suggests that these were limited not by the contractile proteins but by some earlier step(s).

The factors regulating the magnitude of fast and slow phases, which appear to be related to some early step in the chain of events causing contraction could be different rates of utilisation of calcium from multiple pools or different rates of attainment of equilibrium of drug concentration in the vicinity of the alpha-adrenoceptors (biophase) and the bath or both. Factors which can affect the concentration of an agonist such as noradrenaline in the biophase are the concentration gradient and the rate of inactivation of noradrenaline in the biophase.

The latter is believed to be limited mainly by the rate of re-uptake of the agonist into nerve endings (Iversen, 1967).

In order to test the first possibility, spleen strips were stimulated with noradrenaline in the presence of different low concentrations of external calcium. No significant change was seen in either phase even when the calcium concentration was reduced from 2.5 mM up to 0.8 mM. Further reduction of calcium concentration to 0.1 mM resulted in a preferential decrease in the slow phase. Finally, prolonged exposure to a calcium-free medium abolished the residual slow phase and also the fast phase. Elevation of intracellular free calcium is accepted as the prerequisite for contraction, and it is therefore expected that removal of calcium should decrease contractility. Since the slow phase is more readily affected by reduction of extracellular calcium, this phase seems to depend upon either the extracellular water as a source of calcium or an internal pool of calcium that is readily in equilibrium with the extracellular space. As a corollary, the fast phase of noradrenaline response probably relies on a pool of calcium that is not so easily affected by alterations in extracellular calcium concentrations.

In contrast, the contraction produced by high concentrations of potassium in reserpine treated spleen strips seems to have a single phase. It could be argued that if the potassium-induced contraction had two phases very close to each other they would be indistinguishable from a single phase. If this were true then stepwise reduction of external calcium should, by affecting any slow phase preferentially, dissociate the possible two phases. This never happened. Instead, the contractions progressively decreased in size without unmasking two phases. In experi-

ments where the slow phase of the response to noradrenaline was selectively decreased by exposing the muscle to the agonist after a brief reduction of external calcium with EGTA (15 mM), the residual response to noradrenaline was equal to or slightly greater than the fast phase of the control contraction obtained in Krebs-Henseleit solution. On the other hand the response to potassium was markedly decreased. The small residual response may either represent a slight effect of potassium on a tightly bound pool of calcium or an incomplete removal by EGTA of extracellular calcium.

Daniel (1963) proposed that the loosely bound calcium pool was not in the extracellular water but in a superficial site in the membrane, basing his conclusions on calculation of diffusion times and rates of loss of contractility of rat uterus to acetylcholine in a calcium-free medium. The presence of ground substance outside the cell membrane proper, makes this suggestion likely. This concept is supported to some extent by the present findings that the fastest rate of efflux of an extracellular marker, sucrose-^{14}C, was 1.8 min compared to that of ^{45}Ca which was 4.3 min. Again, when the loss of sucrose from the strips was considered with the loss of slow phase in a low calcium medium (0.1 mM), about 92% of the rapidly emerging compartment of sucrose-^{14}C had been lost at the end of 5 min in contrast to only 50% of the rapidly emerging compartment of ^{45}Ca. In strips exposed to a low calcium medium (0.1 mM) for 5 min the slow phase decreased by 48.2%. This correlates reasonably well with the 50% decrease in the rapidly emerging ^{45}Ca compartment, although it is possible that in a calcium-free medium the rate of loss of slow phase might have been somewhat faster. Nevertheless the discrepancy

between the rate of loss of ^{45}Ca and sucrose-^{14}C is considerable. In the 0.1 mM calcium containing solution, the predominant decrease in slow phase was accompanied by a decrease in total tissue calcium content. The decrease in calcium was greater than what could be accounted for by a decrease in the calcium content of the extracellular space only (i.e. net loss of tissue calcium occurred). It was estimated that a 61% decrease of the slow phase and 18% decrease of the fast phase was associated with a loss of 0.42 mM/kg tissue wet weight of calcium, after making allowance for the decrease in concentration of calcium in the extracellular space. An additional loss of 0.34 mM/kg tissue wet weight of calcium was associated with the loss of 82% of the initial fast phase and 39% of the initial slow phase when the strips were placed in a calcium-free medium. Making gross approximations, the pool of calcium responsible for the slow phase appears to be somewhat in excess of 0.42 mM/kg while the pool responsible for the fast phase is about 0.34 mM/kg.

Daniel (1965) estimated that the total calcium fraction related to contractility is 0.2 mM/kg tissue wet weight. Our estimates are higher than those in the above study. However, if the different pools of calcium were being independently lost into the extracellular space and if there were a pool that was unrelated to contractility, then it would be very easy to overestimate the fraction of calcium utilised for contraction. The calcium content of spleen strips (1.75 mM/kg tissue wet weight) incubated in Krebs-Henseleit solution are somewhat lower than that in the guinea-pig taenia coli, 2.5 mM/kg (Bauer et al., 1965) but similar to that of frog stomach muscle, 1.54 mM/kg (Bozler, 1963) and canine trachaelis muscle, 1.6 mM/kg (Kroeger, 1970). However, the calcium concentration

in the bathing media in the studies cited above varied by as much as 1.25 mM. This can cause a discrepancy of 0.25 mM/kg, if an extracellular space of 20% is assumed.

The method of measurement of the slow and fast phases is of considerable importance. The two phases were dissociated by measuring the contraction due to noradrenaline in the presence of EGTA (15 mM) and 2.5 mM calcium in the medium. This resulted in a contraction which was not sustained. The peak of this contraction was similar in magnitude to the fast phase of the contraction due to the same concentration of the agonist in Krebs-Henseleit medium alone. In another series of experiments the tissues were depleted of calcium until no response to noradrenaline occurred. On restoration of calcium (2.5 mM) in the presence of noradrenaline the preparation responded with a single phased contraction which was nearly equal in magnitude to the contraction that occurred in Krebs-Henseleit solution. The contraction occurring on restoration of extracellular calcium was assumed to be similar to the slow phase of the normal contraction to the agonist. When an algebraic summation of the peak of the fast phase and the peak of the contraction due to restoration of external calcium was done, the resultant was larger than the composite contraction experimentally obtained. It was therefore concluded that one of the two phases was probably transient. Since this conclusion agreed with the observation of the fast phase being transient in the presence of EGTA, for all subsequent calculations the slow phase was measured as the total active tension. This may be a slight overestimate but was justified from the standpoint of convenience without any great compromise in accuracy.

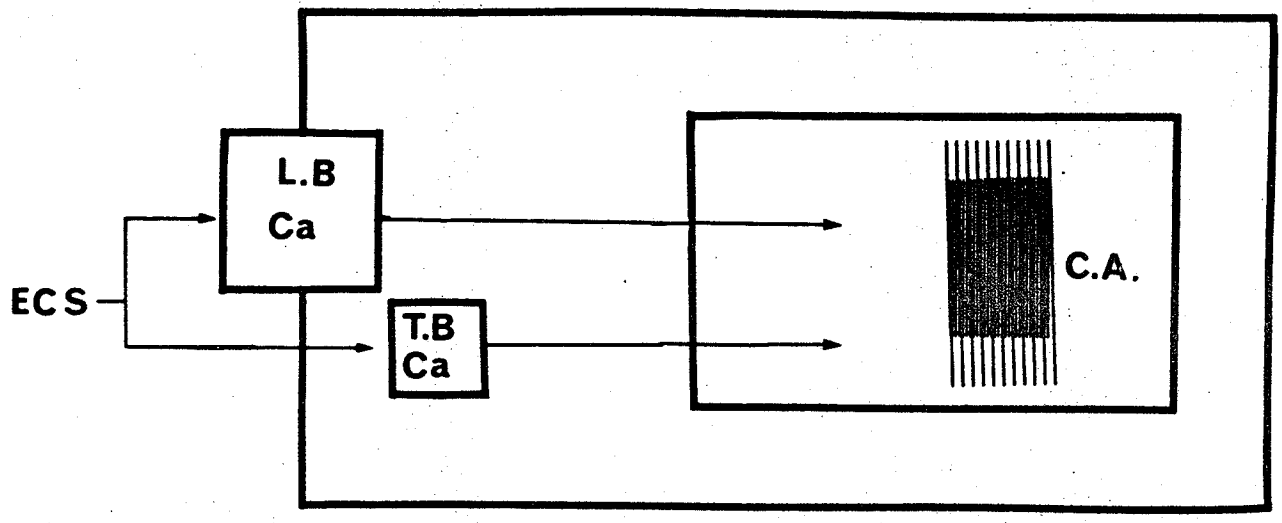
Relationship of contractility with multiple calcium pools

Two phases of contraction have been reported previously in rabbit ileum (West et al., 1951), guinea-pig taenia coli (Pfaffman et al., 1965; Karaki et al., 1967; Imai and Takeda, 1967a; Ferrari and Carpenedo, 1968). In these studies contractions were elicited with either acetylcholine or potassium. Imai and Takeda observed that the initial phase of the contraction of guinea-pig taenia coli was associated with spike-like electrical activity and this phase was more susceptible to reduction of external calcium than the second (tonic) phase which was associated with sustained depolarisation of the membrane. The tonic phase was abolished by prolonged depletion of calcium. These workers also found that divalent cations such as manganese inhibited the first phase preferentially. Subsequently these investigators (1967b) observed that the smooth muscle relaxant, papaverine, preferentially abolished the tonic phase while another agent, aminophylline, affected the initial phase. Ferrari and Carpenedo (1968) confirmed these observations with papaverine. West et al. (1951), using acetylcholine-induced contractions of rabbit ileum, and Pfaffman et al. (1964), using potassium-induced contractions of the guinea-pig taenia coli, observed that the tonic phase of contraction of either tissue was preferentially decreased by metabolic inhibition due to cyanide, dinitrophenol, iodoacetate, ouabain or anoxia. It was therefore postulated that the 'tightly bound' store of calcium that mainly contributed to the second (tonic) phase, depended on metabolism for proper functioning. Whether this pool was depleted during metabolic inhibition or was unable to deliver its content into the cytoplasm was not established.

In view of the above observations, it appeared peculiar that the two phases of contraction of spleen strips due to noradrenaline showed exactly the reverse relationship to reduction of external calcium compared to the above visceral smooth muscles. This diametrically opposite behaviour is seen not only with calcium depletion but also with substrate depletion and after treatment of the muscle with manganese or papaverine. The cause of this difference is not known. However, the peculiarity of the spleen is not unique. Other preparations, e.g. rabbit aorta (Brodie et al., 1959; Bohr, 1963; 1964), rat aorta (Godfraind and Kaba, 1969) and guinea-pig ureter (Chapman and Holman, 1968) also contract in response to agonists. Contractions due to adrenaline or noradrenaline in the aorta preparations and potassium in the guinea-pig ureter have two phases and the initial fast phase is more resistant to decrease in external calcium than the subsequent slow phase. It was very surprising that the guinea-pig ureter behaved so differently from the guinea-pig taenia coli even though they had similar electrophysiological correlates, both having a fast phase associated with action potentials and a subsequent slow or tonic phase associated with maintained depolarisation and no action potentials.

The main difference between the two phases of contraction in the rabbit aorta (Brodie et al., 1959) and the spleen capsular strip is in the slower responses in the former tissue. Also, there is a better separation between the two phases in the spleen. Bohr (1963) postulated that the fast component of adrenaline-induced contraction in the rabbit aorta was indicative of the muscle membrane excitability whereas the slow phase was limited by the excitation-contraction coupling process.

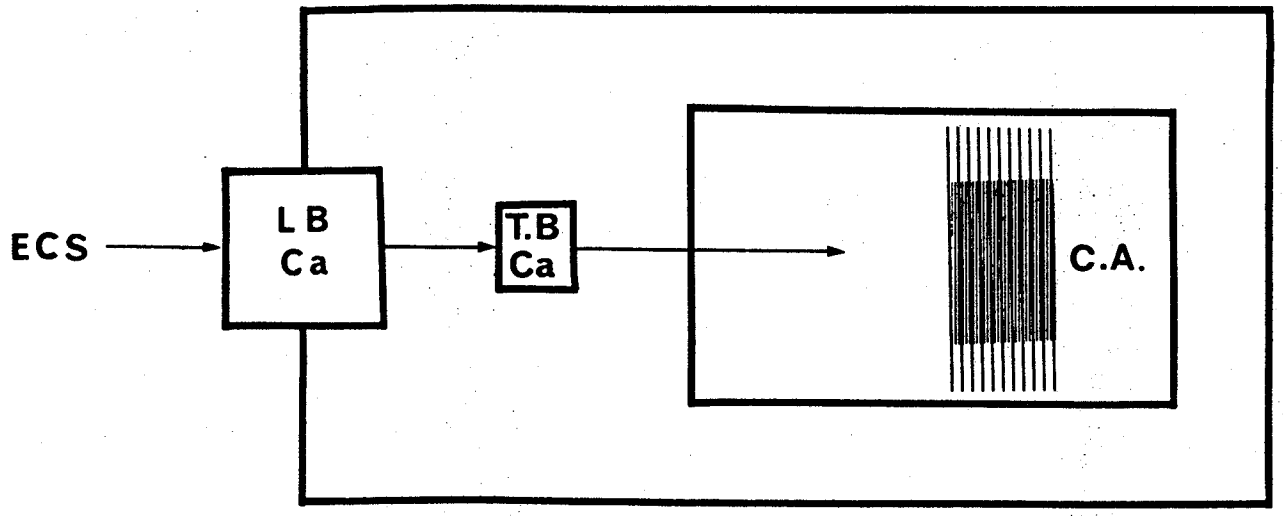
MODELS OF CALCIUM POOLS



PARALLEL MODEL

LB Ca - LOOSELY BOUND CALCIUM
TB Ca - TIGHTLY BOUND CALCIUM
C.A. - CONTRACTILE APPARATUS
ECS - EXTRACELLULAR SPACE

Fig. 59



SERIES MODEL

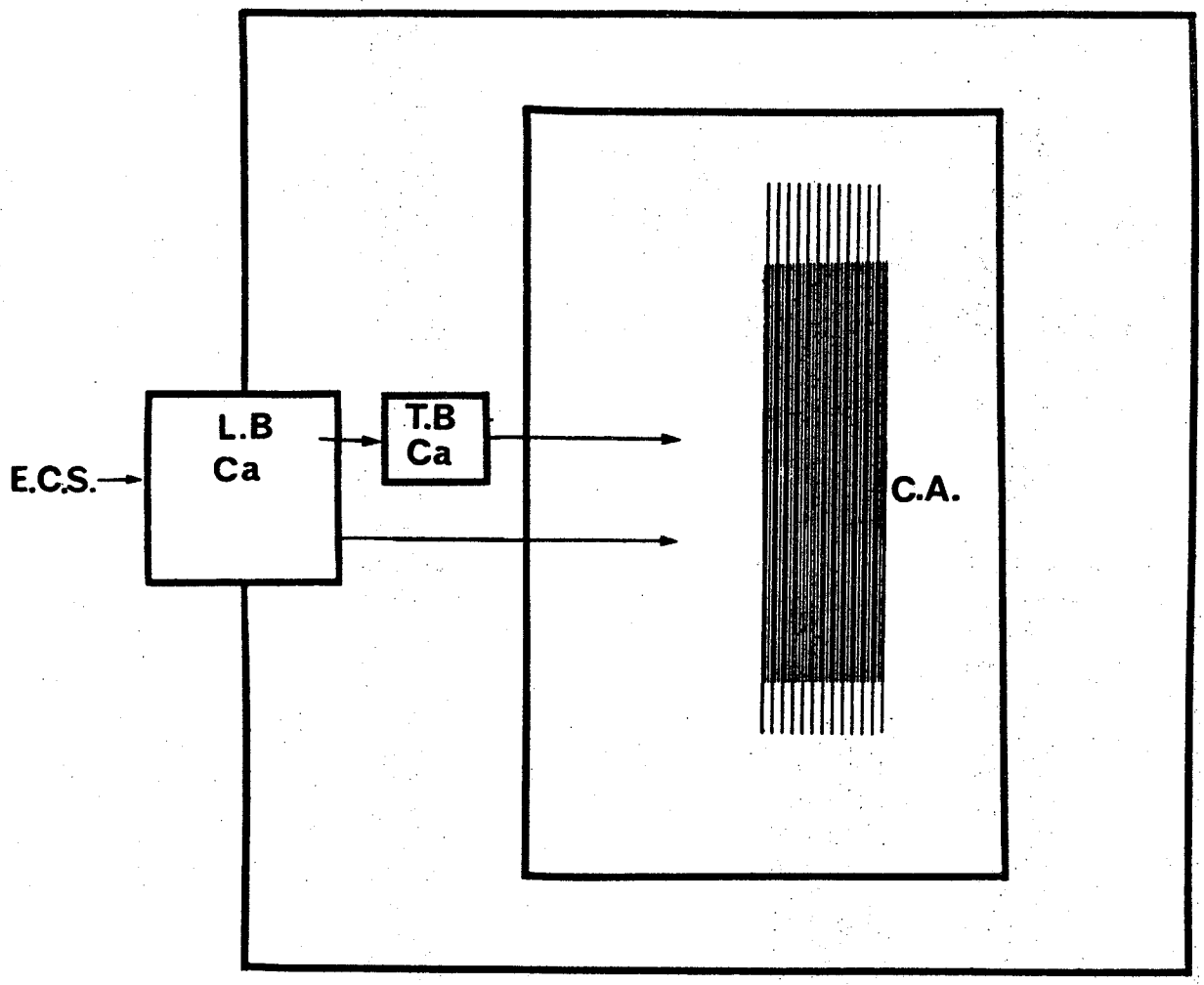
He envisaged the two processes to be consecutive in a single sequence of events. The present results on the effect of low external calcium tend to agree with Bohr's findings, although we do not agree with the interpretation that membrane excitability is related only to the fast phase. Evidence will be discussed later that in the spleen both phases of contraction due to noradrenaline represent membrane excitability although one may be more dependent on electrical changes in the membrane than the other. Daniel (1965) discussed various models of calcium pools. His 'series model' was mainly supported by numerous observations (Waugh, 1964; Hinke et al., 1964; Hiraoka et al., 1968) in which the response to potassium that was attributed to an extracellular or loosely bound calcium pool was always lost in a calcium-free medium before the response to noradrenaline or adrenaline, which mainly utilised a tightly bound pool of calcium. The converse was never shown to happen.

Proposed model of calcium pools in the spleen capsular smooth muscle

In the present study on the spleen strip, a variety of procedures preferentially reduced either phase of contraction due to noradrenaline and by inference this would reflect preferential effect on either the loosely or tightly bound pools of calcium. Thus manganese, diazoxide and clonidine affected the utilisation of loosely bound calcium mainly, whereas procaine, zinc, papaverine, aminophylline, nitrite and to some extent substrate depletion preferentially affected the utilisation of tightly bound calcium. These observations are more consistent with a 'parallel' arrangement of the two calcium pools. Higher concentrations of either class of the above agents affected both pools of calcium. This may either be as a result of an overlap in the effect of these

Fig. 60

MODEL OF CALCIUM POOLS IN CAT SPLENIC CAPSULAR SMOOTH MUSCLE



SERIES-PARALLEL MODEL

L.B.Ca - LOOSELY BOUND CALCIUM
T.B.Ca - TIGHTLY BOUND CALCIUM
C.A. - CONTRACTILE APPARATUS
E.C.S. - EXTRACELLULAR SPACE

preferentially acting agents on the two pools or due to a 'series-parallel' arrangement of the two pools. This was tested in the present study with the help of manganese. In the normal spleen strips manganese reduced the slow phase of the response to noradrenaline more than the fast phase (53.9% v/s 29.5%). However, in spleens which had been first depleted of calcium and then replenished in the presence of manganese chloride, the fast phase was reduced by manganese as much as the slow phase (56.25% v/s 56.20%). This suggests that the tightly bound calcium pool is filled via the loosely bound pool. In the normal spleen noradrenaline can independently release calcium from the tightly bound pool even though the release from the loosely bound pool is inhibited, hence the preferential decrease of the slow phase.

Other lines of evidence suggest that a 'series model' in itself is not acceptable and that a 'parallel' or 'series-parallel model' has to be considered. For example, Hinke (1965) found that after responses of rat ventral tail artery to noradrenaline and potassium were abolished in a calcium-free medium, substitution of barium restored potassium-induced responses more than those due to noradrenaline. Since potassium acts on the loosely bound pool of calcium, the above observation can be best explained by the parallel model. The mechanism by which barium (Hinke, 1965; Bohr, 1964; Daniel, 1963) or strontium (Bohr, 1964; Daniel, 1965; Hudgins, 1969) substitute for calcium is not known. Daniel (1965) proposed that these agents mainly substituted for calcium at the superficial site where calcium is loosely bound. This suggestion was made on the basis of the observation that responses to acetylcholine that were restored by strontium or barium in a calcium-free medium decayed very rapidly when these

substitutes were removed, even though the agonist was still present. On the other hand Hudgins (1969) found that the responses of rabbit aorta to potassium, noradrenaline or histamine that were restored by strontium in a calcium-free medium showed tachyphylaxis. This suggests that strontium may possibly be restoring responsiveness of muscles by releasing residual bound calcium. In the present study this possibility was examined more closely. The responses to potassium that were completely abolished in a calcium-free medium were markedly restored by strontium. The restored responses instead of having a single phase exhibited two phases. In the presence of EGTA (15 mM) in Krebs-Henseleit solution, the response to potassium was markedly diminished. However, in a calcium-free strontium containing medium with EGTA, potassium produced a larger response. Also the restored responses to potassium when strontium substituted for calcium diminished when the spleen strips were exposed to EGTA and were restored when the stores were first refilled with calcium and then the loosely bound pool of calcium was removed. These observations support the possibility of strontium not only substituting for calcium at the level of the contractile apparatus but also in releasing residual calcium from the tightly bound pool. It appears that potassium-induced depolarisation may permit strontium to gain access to the tightly bound pool. In the normally polarised muscles, strontium in the concentrations employed did not result in any contraction. Responses to noradrenaline were also reduced in the calcium-free medium. This decrease was less than that of responses to potassium. The diminished responses to noradrenaline were also partially restored by strontium but to a lesser extent than the responses to potassium. It remains to be seen

whether this is related to a lesser ability of noradrenaline to allow entry of strontium into the cell.

An interesting outcome of the use of the various substances which preferentially affected one of the two phases of contraction due to noradrenaline was the observation that two of the agents which reduced the slow phase are clinically used as antihypertensive agents. Diazoxide has been shown by Rubin et al. (1963a & b) to reduce systemic blood pressure by a predominant effect on the arteriolar calibre and had little effect on capacitance vessels. Wohl et al. (1967) showed that the dilator effect of diazoxide was mediated by antagonism of calcium. Unlike diazoxide, the hypotensive effect of sodium nitrite is accompanied by dilatation of capacitance vessels (Rubin et al., 1963a & b) which is responsible for the postural hypotension. Unlike diazoxide, in the spleen sodium nitrite preferentially reduced the fast phase of noradrenaline responses. While differences in the effect of calcium depletion on the responses of arterial and venous smooth muscles have been documented (Somlyo and Somlyo, 1968b) little is known about the differences between resistance (arterioles) vessels and veins. The above qualitative differences in the effects on the two phases of a single response in the spleen of agents known to act on different parts of the vascular tree suggests that additional study with more antihypertensive agents acting on smooth muscles may enable the spleen strip to be used for screening potentially good antihypertensive agents from others which may cause postural hypotension.

Radioactive ^{45}Ca flux studies

Studies of radioactive calcium movements in spleen strips revealed several important things. Tissues that were loaded with ^{45}Ca in

a ^{40}Ca-free medium lost their radioactivity in a ^{40}Ca-free medium in a manner that could be described by a sum of two exponentials having half times of 74.5 and 7.2 min. Of the radioactivity present in the muscle 67.8% came out at a slower rate whereas 32.2% came out faster. When efflux was conducted in the presence of 2.5 mM calcium, the half time of the slow component of efflux was decreased to 47 min and that of the faster component to 6 min. Also a fraction of the ^{45}Ca that came out slowly in the absence of external calcium appeared to emerge from the fast moving compartment. A similar ability of external calcium to increase efflux of ^{45}Ca from tissues has been shown by several workers (Hudgins and Weiss, 1969; Krejci and Daniel, 1970b; Goodman and Weiss, 1971). This may reflect a process of self-exchange. When ^{45}Ca efflux was compared with sucrose-^{14}C efflux, most of the sucrose came out from a fast compartment, and the half time was considerably less than that of the fast efflux of calcium. It is possible that a faster component of ^{45}Ca efflux resembling that of sucrose may have been missed because of lack of resolution of the measurement procedure, or that the ^{45}Ca emerging from the fast compartment was bound to the tissue to some extent. The most significant observation was the ability of noradrenaline to increase the efflux of ^{45}Ca at a time when most of the rapidly emerging isotope had left the tissue and what was left behind was the more tightly bound calcium fraction. The ability of the alpha-adrenoceptor blocking agent phentolamine to reduce this increase in efflux indicated that the release of bound ^{45}Ca was related to activation of the alpha receptors in the spleen. The observation that noradrenaline failed to increase the efflux of sucrose-^{14}C at the same time in another set of experiments suggested

that the release of ^{45}Ca by noradrenaline was not due to a squeezing effect of contraction. Finally, the increased ^{45}Ca influx was inhibited significantly by zinc or procaine. These agents also preferentially inhibit the fast phase of noradrenaline-induced contractions. On the other hand, manganese which preferentially inhibited the slow phase of contraction due to noradrenaline did not significantly diminish the increased ^{45}Ca efflux caused by noradrenaline.

An appreciable release of ^{45}Ca from spleen strips occurred when ^{40}Ca was added to the calcium-free medium in which efflux was being conducted. The release of this self-exchangeable fraction of calcium was not diminished significantly either by manganese or procaine. This indicates that the above fraction is different from the loosely or tightly bound pools of calcium that participate in contraction.

It was rather surprising that noradrenaline did not increase the influx of ^{45}Ca even though it was earlier established that the slow phase of the noradrenaline-induced response was very sensitive to reduction of external calcium. While we have no experimental findings to resolve this paradox, the inability of agonists to increase ^{45}Ca influx in the rat uterus has been reported in the past (van Breemen and Daniel, 1966; Krejci and Daniel, 1970a). On the other hand, in a large variety of tissues such as the circular muscle of the cat intestine (Sperelakis, 1962), rabbit aorta (Briggs, 1962) and guinea-pig taenia coli (Urakawa and Holland, 1964) an increase in influx is associated with contraction. Krejci and Daniel have suggested that in the rat uterus contraction may hinder the movement of substances from the extracellular space into the bathing medium. This appears to be an unlikely explanation for the

phenomenon observed in the spleen because noradrenaline did not significantly alter the efflux of sucrose. Another possibility is that during contraction calcium which is bound to ground substances outside the cell membrane (and therefore a part of the extracellular space) may be translocated inwards for contraction. Under these circumstances no change will be observed in the tissue calcium levels during contraction, as was seen in the spleen. However, even under these circumstances one would expect external ^{45}Ca to replace some of the ^{40}Ca that may be translocated inwards during contraction. The lack of increase in ^{45}Ca influx in the spleen during exposure to noradrenaline still remains a mystery.

Effect of depolarisation on contractility

In spite of the frequent observations made by many workers that smooth muscle contraction induced by many agents is associated with depolarisation of the membrane and often with action potentials there seems to be no doubt that contractions can also occur in depolarised tissues in the absence of any further change in membrane potential (see INTRODUCTION). Very little work has been done to find out the electrophysiological correlates of the two phases of contraction seen in various tissues. Imai and Takeda (1967a) and Chapman and Holman (1968) found that the initial fast phase of contraction in the guinea-pig taenia coli or ureter was associated with spikes while the subsequent slow or tonic phase was associated with steady depolarisation of the membrane and no action potentials. We did not study the electrophysiology of the splenic capsular smooth muscle. However, when the relation of existing membrane potential of the splenic muscle with the resulting contraction due to noradrenaline was examined by varying the potassium concentration of the

medium over a wide range several interesting findings were obtained.

A slight elevation of potassium concentration from 5.4 mM to 10 mM caused no increase in amplitude of contraction although the slow phase became faster. An increase to 30 mM potassium caused a small increase in resting tension as well as potentiation of both the fast as well as slow phases of response to noradrenaline. This increase can not be due to the change in resting tension of the muscle since it was higher than the optimum tension of 1 g. The most significant finding with higher concentrations of potassium (60 and 140 mM) was that the slow phase progressively diminished while the rapid phase remained slightly larger than normal. It was confirmed that the rapid phase of the response to noradrenaline still truly represented the utilisation of tightly bound calcium, when the presence of EGTA (15 mM) did not diminish it. The decrease in the slow phase when the muscle was depolarised suggests that it is dependent on depolarisation in the polarised muscle. On the other hand, the fast phase is resistant to any decrease under similar conditions. Potentiation of smooth muscle response to noradrenaline in the presence of moderate increases of potassium concentration has been noted earlier in the rabbit aorta (Bohr et al., 1958). Bevan and Osher (1963) warned that the potentiation observed could have been due to the effect of potassium on resting tension. This objection is not applicable to the present study because noradrenaline was added after any response to potassium had reached a steady level. If the slow phase of noradrenaline-induced response in the spleen is dependent upon depolarisation then a small increment in external potassium may decrease the threshold for noradrenaline. However, the mechanism of increase of fast phase by potassium is

not clear.

Analysis of the role of sodium pump in regulating contractility

Several workers have shown that contractility of smooth muscle to agonists decreases in the absence of potassium in the external medium. Barr et al. (1962) observed that dog carotid artery strips when stored in the cold for 36 - 50 hr gained sodium and lost potassium. Such strips when stimulated electrically in a potassium-free medium responded very feebly. However, restoration of potassium concentration of the medium to normal levels restored the responsiveness. Similarly Brodie et al. (1959) observed that in the absence of external potassium, the slow component of the response of rabbit aortic strips to adrenaline diminished first followed by the fast component. Restoration of potassium concentration led to recovery of the slow component even though the fast component became depressed further. The investigators did not mention the time course of the above changes. Results similar to the above showing the necessity of potassium for contraction have been presented by Paton (1961) and Karaki et al. (1967). Another phenomenon often observed when certain smooth muscles are exposed to potassium-free medium is an increase in tension (Leonard, 1957; Konold et al., 1968).

Surprisingly, spleen strips kept in a potassium-free medium for prolonged periods did not show any change in the resting tension and continued to respond to noradrenaline without any significant changes in the amplitude of the response or in the proportion of the two phases. However, when the muscles were returned to Krebs-Henseleit solution shortly before stimulation, the responses were diminished mainly because of a reduction of the slow phase. Responses to noradrenaline in Krebs-

Henseleit solution were diminished only if the muscle was stimulated while in the potassium-free medium. The inhibition progressively decreased in spite of maintenance of potassium in the bathing medium. It also disappeared rapidly if the muscle was returned to the potassium-free medium. Potassium, within a limited concentration range, relaxed spleen strips that had contracted in the presence of noradrenaline in a potassium-free medium. The inhibitory effect of potassium was abolished by inhibitors of the sodium pump such as ouabain. Substitution of external sodium chloride by lithium chloride and reduction of external temperature also abolished the effects of potassium. These findings as well as the known ability of potassium in the concentrations employed (10 mM) to activate the sodium pump powerfully (Skou, 1965) suggest that the inhibition of the slow phase of noradrenaline responses due to potassium may be attributed to the sodium pump. Similar observations were also made when the effect of noradrenaline was studied on sodium loaded spleen strips in the presence or absence of external potassium. In addition the phase of inhibition due to potassium, in these muscles, was associated with a loss of intracellular sodium that was in excess of the gain in potassium. This observation suggests an uncoupling of the sodium pump. Several investigators have shown that sodium loaded skeletal muscles (Cross et al., 1965; Mullins and Awad, 1965; Adrian and Slayman, 1966) and uteri (Taylor et al., 1970), while recovering from the cold in the presence of external potassium, become hyperpolarised due to the activity of an electrogenic sodium pump. While no measurements of membrane potentials have been made in the spleen it is possible that a phenomenon similar to the above may be happening. The preferential decrease of the slow phase

during such states again goes to support the conclusion that the loosely bound calcium pool is more dependent upon electrical phenomena in the membrane than the tightly bound calcium pool.

Several additional points arise from this investigation of the sodium pump in the spleen.

a) The sodium pump is electrogenic only when the internal sodium concentration of the muscle is high or the potassium concentration is low or both.

b) Substitution of external chloride by the non-penetrating large anion isethionate did not affect the relaxation produced by potassium in the sodium loaded muscle. This rules out the possibility of an inwardly directed chloride pump contributing to the inhibition or the shortcircuiting effect of chloride ions moving out of the cell thereby reducing the inhibition. In previous studies by Taylor et al. (1970) removal of chloride ions resulted in an increase in hyperpolarisation. This effect of isethionate is contingent upon adequate depletion of intracellular chloride (Rang and Ritchie, 1968). Whether such a state was achieved in the spleen in the present study is not known although it should be noted that incubation of the strips in the isethionate-containing chloride-free medium was longer than in the above studies.

c) Taylor et al. (1970) observed in the rat uterus that high concentrations of external potassium (up to 120 mM) did not shortcircuit the pump and hyperpolarisation was always seen. On the other hand, in the present study relaxation with increasing amounts of potassium of spleen strips made to contract by noradrenaline in a potassium-free medium increased up to a concentration of 10 mM. Further increment in potassium

concentration led to a decrease in the peak amplitude of relaxation and a secondary increase in tension (presumably due to the well known depolarising action of potassium). Taylor and co-workers proposed an effect of sodium pumping on potassium permeability to explain the absence of a short-circuiting effect of potassium on the electrogenic sodium pump. If this is true, it would appear that this mechanism does not exist in the spleen. The data obtained from this study cannot verify this assumption conclusively.

d) Unlike normal spleen strips stimulated in a potassium-free medium, the sodium loaded strips stimulated in a potassium-free medium with noradrenaline responded poorly to noradrenaline. Exposure to Krebs-Henseleit solution diminished the responses further as well as reducing the resting tension of the muscle. A similar reduction in resting tension was found by Barr et al. (1962) in cold stored carotid artery strips, but this was accompanied by an increased responsiveness to noradrenaline or electrical stimulation during exposure to potassium. In the present study when the sodium loaded spleen strips were kept in Krebs-Henseleit solution for a prolonged period, the initial inhibition of noradrenaline responses was reversed and eventually responses became larger than control responses in the potassium-free medium. However, when the preparation was again returned to a potassium-free medium, the contractions became even larger. This suggests that two processes were occurring simultaneously, one being a shortlasting inhibition of response to agonist due to the sodium pump and the other being a recovery of the sensitivity of the muscle to agonists by a different mechanism (probably recovery of intracellular potassium). The inability of Barr and co-workers to observe any inhibition of noradrenaline responses may have been because the inhibitory component

of the sodium pump was less than the restoration of responses due to recovery of internal potassium. It may be important to note that in the study done by Barr and co-workers the strips were stored in a potassium-containing medium in the cold. This might have resulted in a lesser degree of sodium enrichment than in the spleen strips which were stored in a potassium-free medium in the cold. These workers did not consider the possibility that the sodium pump might mediate a decrease in tension of cold stored carotid strips when exposed to potassium. Instead they explained it on the basis of an increase in internal potassium. It may be necessary to reinterpret their conclusions on the basis of a pump mediated phenomenon.

e) In tissues where hyperpolarisation is observed as a result of any procedure, several other mechanisms besides activation of an electrogenic sodium pump have to be considered. While Taylor et al. (1970) proposed that the hyperpolarisation in the rat uterus recovering from cold storage is due to an electrogenic sodium pump, Kao and Nishiyama (1969) argued that a similar phenomenon in the rabbit uterus is not due to this mechanism. Among other things an increase in potassium permeability can also give rise to an increase in membrane potential. On the basis of the finding of Hodgkin and Horowicz (1959) that potassium permeability of frog semitendinosus muscle was directly related to the external potassium concentration we argued whether the relaxation due to potassium of spleen strips previously stimulated with noradrenaline in a potassium-free medium could not be explained by a similar increase in potassium permeability. This argument was weakened very much at the outset when we failed to elicit any increase in resting tension when the spleen strips

were first exposed to potassium-free medium. This was expected to decrease permeability to potassium, cause depolarisation of the membrane and result in shortening of the muscle. The latter occurs in dog carotid artery (Barr et al., 1962) and in slow avian striated muscle (Somlyo and Somlyo, 1969) under similar conditions. It can be concluded from this that any effect of external potassium on potassium permeability, within the range of 0 to 5.4 mM, is not prominent enough. On the other hand, substantial relaxation was produced under appropriate conditions by potassium in concentrations less than 5 mM. Barium ions have been reported by several workers to inhibit the permeability of the membrane to potassium (Sperelakis and Tarr, 1965; Sperelakis et al., 1967). Also, barium suppresses relaxation induced by relaxants in the depolarised rat uterus (Schild, 1967). It was therefore considered of interest to see if the relaxant effect of potassium could be altered in spleen strips stimulated with barium in a potassium-free medium. The observation of an increase in contraction of spleen strips due to barium when external potassium is reduced, is unique. In earlier studies done on muscles other than spleen, the opposite was found to be the case, e.g. in the guinea-pig taenia coli (Karaki et al., 1967). However, in every case the tissue employed was one which did not also respond to other agonists in the absence of external potassium. Hence any mutual antagonism between barium and potassium could be easily masked by a more distal effect of low potassium in the sequence of events causing muscle contraction. The interaction between barium and potassium was readily observed in the spleen, since it continues to respond in the absence of external potassium. The relaxant effect of potassium was greater in contractions due to barium in potassium-free medium than in contractions

due to noradrenaline. Furthermore, elevation of external potassium did not relax spleen strips made to contract with noradrenaline in the presence of Krebs-Henseleit solution. Thus it seems that two different mechanisms exist by which potassium can relax splenic smooth muscle. However, the relaxation of muscle by potassium in the presence of barium was partly due to stimulation of the sodium pump since it could be partially blocked by ouabain. A similar concentration of ouabain completely abolished potassium-induced relaxation in the presence of noradrenaline. Our calculations indicated that the component of relaxation of the strips due to potassium in the presence of barium that could be attributed to stimulation of the sodium pump was less than the corresponding component in the strips stimulated with noradrenaline. The above results suggest that the predominant mechanism of inhibition of spleen strips due to potassium in the presence of noradrenaline is due to stimulation of the sodium pump since this could be completely blocked with ouabain. In the presence of barium, however, an additional mechanism exists, the nature of which is not resolved. It may be interesting to find out if competition occurs between barium and potassium for access to sites of calcium storage or to the contractile proteins, since both mechanisms have been proposed as an explanation for the ability of barium to cause contraction of smooth muscles (Daniel, 1964).

f) The sodium pump in normal muscles is not electrogenic as evidenced by the lack of change in resting tension of unstimulated muscles when exposed to potassium-free medium. This in turn may suggest that the membrane potential did not decrease. A contraction would have been expected on removing external potassium if an electrogenic sodium pump were

contributing to the resting membrane potential.

g) On a few occasions (Leonard, 1957; Dodd and Daniel, 1960), increased responsiveness to agonists has been reported to occur when the muscles are exposed to a potassium-free medium. It is possible that due to some factors of experimental handling of the tissue an initial accumulation of sodium might have made the sodium-pump electrogenic and the tissue less sensitive to agonists. On removal of potassium from the medium the inhibition due to the pump might have been removed resulting in enhancement of responses.

Role of agonist disposition in modifying contractions

It was mentioned earlier that two mechanisms for production of the two phases of contraction due to noradrenaline were considered. The first was concerned with the utilisation of different sources of calcium and this expectation was supported by the experimental results. A second factor that could influence the magnitude of the two phases could be the rate of increase of agonist concentration in the vicinity of the alpha-adrenoceptors. Several of our experiments bear on this possibility. The time taken for a response due to noradrenaline to reach a plateau level was longer in a muscle incubated in Krebs-Henseleit solution than when the calcium depleted muscle was first exposed to noradrenaline and then a response was induced by restoration of calcium concentration in the bathing medium. From this it was inferred that the rate at which the agonist concentration attained a steady state in the biophase and not the rate of availability of calcium from its various stores was the limiting step in the entire sequence of events which led to muscle contraction. Isoprenaline and noradrenaline, both full agonists acting on the same

alpha-adrenoceptors in the spleen, produced different proportions of fast and slow phases for contractions of similar magnitudes. This is unexplainable on the basis of the prevailing concepts of drug-receptor interactions where it is generally believed that the sequence of events following activation of the same receptor by full agonists is similar. However, it is known that different full sympathomimetic agonists for the alpha-adrenoceptors in the spleen may possess different affinities and different rates of inactivation. For example, isoprenaline has a lower affinity than noradrenaline, based on a higher value of ED_{50} (Davidson, 1970). Another difference between noradrenaline and isoprenaline is the rate of their disposal from the biophase by re-uptake into sympathetic nerve endings. In the rat heart the affinity of noradrenaline for the 'Uptake I' process in nerve endings is greater than that of isoprenaline (Iversen, 1967). If the same characteristics of the uptake process apply to the spleen, then for any given concentration of the two agonists in the bath, the steady state concentration of isoprenaline in the biophase will be influenced less by the uptake mechanism. The following results supported the concept that the proportions of the two phases of responses due to sympathomimetic agonists depended upon the magnitude of their inactivation by the uptake process:

a) The ratio of slow to fast phase increased with noradrenaline concentrations. If the events that follow receptor activation remain constant then the proportion of the two phases should not change no matter how many receptors are activated. Therefore an alternative explanation has to be found. Since the catecholamine uptake mechanism is saturable (Iversen, 1967) it should be less effective in limiting the amounts of

noradrenaline in the biophase when the concentration of this agonist in the bath is high. Brodie et al. (1959) observed similar changes in the ratio of slow to fast phases in the rabbit aorta with increasing doses of adrenaline.

b) With isoprenaline, the proportion of slow to fast phase was much higher than with noradrenaline for an equivalent increase in active tension. The low affinity of isoprenaline in the spleen does not appear to be responsible for the smaller fast phase because in spleen strips in which neuronal uptake of noradrenaline was impaired either with cocaine (Davidson and Innes, 1969; Mailhot, 1970) or by degeneration of sympathetic nerve endings with 6-hydroxydopamine (Mailhot, 1970) the responses to noradrenaline resembled the responses to isoprenaline in normal spleen strips. Cocaine has been shown by Mailhot (1970) not to alter the affinity of alpha-adrenoceptors in the spleen to the non-equilibrium blocking agent, phenoxybenzamine . If this can be extrapolated to assume that the affinity of the agonist, noradrenaline is also not altered by the above agent, then the mechanism for alteration in the ratio of slow to fast phases after cocaine or 6-hydroxydopamine cannot be explained on the basis of different affinities. On the other hand, since both these procedures diminish the inactivation of noradrenaline by uptake, it can be concluded that in the normal tissue uptake constitutes a more effective mechanism for inactivation of noradrenaline at a time when the slow phase is occurring than during the fast phase. This implies that the uptake process shows a lag in its onset or in its activation above a basal level consequent to the entry of noradrenaline into the biophase. While isoprenaline is generally believed not to be inactivated appreciably by

Uptake I mechanism in the rat heart (Iversen, 1967), Davidson and Innes (1970) measured the uptake of isoprenaline in reserpine treated spleen strips that were thicker than those employed in the present study and found that the content of this amine was appreciable. The uptake of noradrenaline tested similarly was greater than that of isoprenaline. While no information is available about the nature and kinetics of uptake of isoprenaline in the spleen it appears from the present study that uptake certainly does not constitute an important mechanism for inactivation of isoprenaline as it does for noradrenaline.

Cocaine and 6-hydroxydopamine, both caused supersensitivity of the spleen strips to noradrenaline. Another agent, reserpine, also causes supersensitivity in a variety of smooth muscles (Innes, 1960; Fleming and Trendelenburg, 1961). Hudgins and Fleming (1966) suggested that this was probably as a result of the action of reserpine at a site between the receptor and contractile mechanism. The results of Davidson (1970) showing the ability of reserpine to cause supersensitivity in spleen to a variety of unrelated agonists such as noradrenaline, acetylcholine, angiotensin and histamine tend to suggest the above hypothesis. Reserpine caused supersensitivity of spleen strips to noradrenaline in the present study. However, the relationship of ratio of slow to fast phase of the response to the concentration of noradrenaline was not significantly different from that obtained in tissues not treated with reserpine. This is clearly a pattern different from that obtained after cocaine or 6-hydroxydopamine. It is interesting to note that unlike these two agents, reserpine does not inhibit the uptake of noradrenaline by the sympathetic postganglionic neuronal membrane (Iversen, 1970).

Several workers (Carrier and Shibata, 1967; Garrett and Carrier, 1971) have suggested that alteration in the mode of utilisation of calcium for contraction may be the mechanism of supersensitivity due to reserpine. In the present study the potentiation of noradrenaline by reserpine appeared to be mediated by the same proportion of loosely and tightly bound calcium stores as the responses in untreated tissues. If the theory of increased utilisation of calcium stores in reserpine treated tissues is correct then both stores are probably equally involved.

Just as some potentiating agents such as cocaine and 6-hydroxydopamine altered the ratio of slow to fast phases of noradrenaline responses, blockade of alpha-adrenoceptors also had unexpected effects. Again, as stated earlier, it was expected that responses to noradrenaline which were inhibited due to the presence of a non-equilibrium blocking agent, phenoxybenzamine, would have the same proportion of slow to fast phase as control tissues if occlusion of receptors was the only process involved. Instead, comparable responses of spleen strips to noradrenaline after treatment with phenoxybenzamine had a larger slow phase than control tissues. It is possible that this was partly due to the fact that phenoxybenzamine, besides blocking the alpha-adrenoceptors also might have inhibited the uptake mechanism. This inhibition is now quite widely known to occur in many tissues (Iversen, 1967). Unlike cocaine and 6-hydroxydopamine, the difference of slow to fast phase ratios between phenoxybenzamine treated and control tissues was highest at higher concentrations of the agonist. With cocaine and 6-hydroxydopamine, the opposite was the case. Whether the ability of phenoxybenzamine to impair mobilisation of calcium for contraction (Somlyo and Somlyo, 1969; Somlyo et al.,

1969) is responsible for a depression of the fast phase (indicative of a decrease in release of tightly bound calcium) and the difference between these two different categories of drugs cannot be answered from the data presently available.

Splenic capsular smooth muscle - multiunit or single unit?

All three treatments mentioned above resulted in rhythmic contractions on addition either of noradrenaline or another alpha-adrenoceptor stimulant, phenylephrine. One of the common features of the three treatments is their ability to interfere with the functions of the sympathetic postganglionic nerve endings. This, however, does not seem to be responsible for the production of rhythmicity because tetrodotoxin, when used in concentrations high enough to block nerve conduction, did not abolish established rhythmic contractions. It is therefore most likely that either a direct effect of these procedures on some postsynaptic event or an indirect effect as a result of presynaptic changes may result in the initiation of rhythmicity and its propagation along the muscle cells.

The normal spleen strips showed neither spontaneous rhythmicity nor could this be evoked by stimulation with potassium or noradrenaline. These observations, along with a lack of 'after stretch contraction', suggested that the muscle cells in this tissue may be functioning in multiunit fashion under the prevailing experimental conditions. The critical proof for this can only come from electrophysiological studies.

Multiunit muscles are characterised by an abundance of nerve endings and lack of close contacts between adjacent muscle cells. On the other hand, single unit muscles which show the property of rhythmic contractions, e.g. small arterioles, show a larger number of

tight junctions or nexuses among adjacent cells (Rhodin, 1967). Histological examination of the spleen strips revealed that, although they behaved like multiunit muscles, adjacent muscle cells in them showed some interlocking of membranes with several areas of membrane fusion. A similar observation has also been made by Fillenz (1970). Thus the induction of rhythmicity in spleen strips treated with cocaine, 6-hydroxydopamine or reserpine did not appear as startling as it would otherwise have been. In the absence of electrophysiological evidence to support the conclusion that after treatment with reserpine, 6-hydroxydopamine or cocaine the splenic capsular smooth muscle behaved in a single unit fashion, the following indirect evidences were considered.

a) Rhythmic contraction of muscle indicates synchronous contractions of many cells of the preparation. Electrophysiological studies conducted in visceral smooth muscle (Bülbring, 1955; 1962) have shown that this involves generation of action potentials by a pacemaker region and propagation of a wave of activity along the entire strip of tissue. It is not unreasonable to conclude that a similar mechanism may also be operating in the spleen capsule.

b) Burnstock and Prosser (1960) reported on the ability of stretch to induce contraction and electrical changes in a variety of visceral smooth muscle preparations, namely rabbit bladder, guinea-pig taenia coli, cat small intestine and guinea-pig vas deferens. Some other preparations, for example, pig renal vein and carotid artery and cat nictitating membrane, did not respond to stretch. The muscle in the former group of preparations is known to be single unit in type. The absence of stretch induced contraction in the normal spleen and its appearance in the presence

of cocaine suggests that single unit properties can be induced by drugs. The occurrence of conduction of activity in the rhythmically active spleen preparations was also demonstrated indirectly by first inducing rhythmic contraction of spleen strips with cocaine and noradrenaline. The level of the bathing medium was then lowered so that half of the strip stayed out in contact with warm moist air. It has been seen earlier in the spleen (unpublished observation) and in the rabbit aorta (Kalsner and Nickerson, 1968) that on removal of the bathing medium the amount of agonist remaining in the extracellular space and biophase of the smooth muscle decreases due to the various inactivation mechanisms. This results in gradual relaxation of the muscle. As a working hypothesis we proposed that if rhythmic contractions were indeed due to synchronous activation of the entire muscle then reducing the area of contact of the tissue with the bathing medium would reduce the basal tension sustained by the muscle (due to progressive decrease in the agonist concentration of the part of the muscle exposed to air). However, the muscle exposed to air could still be activated electrically by the remaining portion of the muscle. If so, the magnitude of the rhythmic contraction should not change. For reasons mentioned earlier in the RESULTS section, disappearance of rhythmic contractions does not rule out the possibility of conducted activity but a persistence of contractions supports it strongly. This was found in some of our experiments, where exposure of half the strip to air resulted in an approximately 30% decrease in basal tension but no change in the amplitude of the rhythmic contractions.

The need for a critical level of depolarisation in response to an agonist in order to trigger the rhythmic contractions was suggested by

the dose-response relationship seen in our experiments. A small concentration of the agonist induced only a steady rise in tension, whereas higher concentrations produced rhythmicity in addition. However, still larger concentrations of the agonist produced a progressive increase in basal tension and frequency of rhythmic contractions and eventually the rhythmic contractions fused into a smooth mechanical response to the stimulant. Whether this increase in frequency represents a fractionation of the excitatory wave front or induction of pacemaker activity at multiple sites cannot be stated. We were puzzled at the inability of potassium to induce rhythmicity. In this respect the behaviour of the spleen was in sharp contrast with the vascular preparations studied by Johansson and Bohr (1966) and Biamino and Kruckenberg (1969) and suggests that depolarisation alone perhaps is not sufficient to induce rhythmicity in the spleen. An interesting effect of potassium was seen by Johansson and Bohr (1966) in the dog subcutaneous artery strips. In strips that had already contracted in response to noradrenaline or plasma, they found that addition of 15 - 30 mM of potassium chloride caused a decrease in tension and also a slowing of the frequency of rhythmic contractions. This was explained by the authors on the basis of a possible negative 'chronotropic' and 'dromotropic' effect of the ions on the pacemaker activity and intercellular conduction, respectively. The primary cause for these two effects was believed to be the depolarising action of potassium chloride. It is known in the heart (Vassale, 1965) that an increase in extracellular potassium concentration leads to an increase in potassium permeability which in turn reduces the steepness of the prepotentials and consequently the rate of firing of the automatic tissue. The effects of potassium on

the rhythmic contractions in the spleen appear to be more complex and varied. Increasing the external concentration of potassium abolished rhythmic contractions whenever present. In strips where the rhythmic contractions had fused, potassium caused a slight decrease in tension and unmasked slower rhythmic contractions. These responses resembled those obtained in the study of Johansson and Bohr (1966). However, after potassium had abolished established rhythmic contractions in the spleen when the agonist concentration was increased, rhythmicity was restored. When the alternate addition of potassium and agonist was continued, a stage came when further addition of potassium no longer decreased tension but instead caused an increase. The most likely explanation can be that the initial decrease in rhythmicity due to potassium resulted from hyperpolarisation of the muscle (cf. the explanation given by Johansson and Bohr). Addition of the agonist restored rhythmicity by lowering the membrane potential to a critical level for firing of action potentials. Higher concentration of potassium caused further contraction as a result of its depolarising action on the membrane. This effect is reminiscent of the observations in the experiments on the effect of potassium on the electrogenic sodium pump. A possible role of the sodium pump in the mediation of rhythmicity in the spleen was suggested by the abolition of rhythmicity and an increase in tension of the spleen strip when potassium was withdrawn from the medium. This finding is compatible with an increased oscillatory activity of an electrogenic sodium pump in the rhythmically contracting spleen which would tend to keep the membrane potential higher than usual. Removal of potassium, by decreasing the activity of the pump, would then cause depolarisation and a consequent increase in

tension. While this kind of explanation has previously been given for the mechanism of the slow wave in the intestine (Daniel and Chapman, 1963; Liu et al., 1969), it is also necessary to rule out the possibility that the effects of potassium seen in the spleen might be due to changes in ionic permeabilities rather than an effect on pump activity.

Lastly, the source of calcium responsible for rhythmic contractions was examined. Rhythmicity was always superimposed on the slow phase. Since the latter is dependent upon loosely bound calcium it is likely that the same source may contribute to the rhythmic contractions. On the other hand, this association between the two types of contraction may be a mere coincidence due to the brevity of the fast phase. Addition of 1 mM EGTA to the bathing medium of a rhythmically contracting spleen strip resulted in nearly complete suppression of the phasic changes in tension whereas the basal active tension did not show any deviation from its normal appearance. The above procedure was expected to reduce the free calcium concentration in the bathing medium by approximately 1 mM - a change which was earlier shown to have very little effect on the component of contraction produced by tightly bound calcium. Restoration of rhythmicity on adding 1 mM calcium chloride reinforced our conclusion that the rhythmic contractions were dependent on loosely bound calcium. Additional support was obtained by the greater ability of manganese chloride to decrease rhythmic contractions compared to zinc chloride.

These experiments on the rhythmic contractions in spleen strips support the contention of Somlyo and Somlyo (1968b) that smooth muscles are not inherently multiunit or single unit as was proposed by Bozler (1948) more than two decades ago. Instead an entire spectrum of behaviour

can be seen among different muscles and in the same muscle depending upon environmental and inborn factors. Nevertheless, the ability to induce single unit behaviour in a muscle that predominantly functions in a multiunit fashion makes the spleen a very attractive experimental model for studying the mechanism of automaticity in smooth muscle.

BIBLIOGRAPHY

Åblad, B. and Mellander, S. (1963). Comparative effects of hydralazine, sodium nitrite and acetylcholine on resistance and capacitance vessels and capillary filtration in skeletal muscle in cat. Acta Physiol. Scand. 58: 319-329.

Adrian, R.H. (1956). The effect of internal and external potassium concentration on the membrane potential of frog muscle. J. Physiol. (Lond). 151: 154-185.

Adrian, R.H. (1960). Potassium chloride movement and membrane potential of frog muscle. J. Physiol. (Lond). 151: 154-185.

Adrian, R.H. and Slayman, C.L. (1966). Membrane potential and conductance during transport of sodium, potassium and rubiduim in frog muscle. J. Physiol. (Lond). 184: 970-1014.

Axelsson, J. (1961). Dissociation of electrical and mechanical activity in smooth muscle. J. Physiol. (Lond). 158: 381-398.

Axelsson, J., Wahlström, B., Johansson, B. and Jonsson, O. (1967). Influence of the ionic environment on spontaneous electrical and mechanical activity of the rat portal vein. Circulation Res. 21: 609-618.

Barr, L., Headings, V.E. and Bohr, D.F. (1962). Potassium and the recovery of arterial smooth muscle after cold storage. J. Gen. Physiol. 46: 19-33.

Bauer, H., Goodford, P.J. and Hüter, J. (1965). The calcium content and ^{45}Calcium uptake of the smooth muscle of the guinea-pig taenia coli. J. Physiol. (Lond). 176: 163-179.

Bendall, J.R. (1961). A study of the kinetics of the fibrillar ATP-ase of rabbit skeletal muscle. Biochem. J. 81: 520.

Bendall, J.R. (1969). In: Muscles, molecules and movement. American Elsevier Publishing Co. Inc. New York.

Bertler, A., Carlsson, A. and Rosengren, E. (1958). A method for fluorimetric determination of adrenaline and noradrenaline in tissues. Acta physiol. Scand. 44: 273-292.

Bevan, J.A. and Osher, J.V. (1963). Effects of potassium on the resting length of vascular smooth muscle of the rabbit aorta and its response to l-norepinephrine. Circulation Res. 13: 346-351.

Beviz, A., Lundholm, L., Mohme-Lundholm, E. and Vamos, N. (1965). Hydrolysis of adenosine triphosphate and creatine phosphate in isometric contraction of vascular smooth muscle. Acta Physiol. Scand. 65: 268-272.

Beviz, A. and Mohme-Lundholm, E. (1964). The effect of adrenaline on hexosephosphate content of vascular smooth muscle. Acta Physiol. Scand. 65: 289-291.

Beviz, A. and Mohme-Lundholm, E. (1965). Influence of dihydroergotamine and adrenaline on the concentrations of glucose-6-phosphate, fructose-6-phosphate, adenosine triphosphate and creatine phosphate in bovine mesenteric artery. Acta Physiol. Scand. 65: 289-291.

Bianchi, C.P. (1968). Pharmacological actions on excitation-contraction coupling in striated muscle. Fedn.Proc. 27: 126-131.

Bickerton, R.K. (1963). The response of isolated strips of cat spleen to sympathomimetic drugs and their antagonists. J. Pharm. exp. Ther. 142: 99-110.

Bohr, D.F. (1963). Vascular smooth muscle: dual effect of calcium. Science. 139: 597-599.

Bohr, D.F. (1964a). Contraction of vascular smooth muscle. Can. Med. Assoc. J. 90: 174-179.

Bohr, D.F. (1964b). Electrolytes and smooth muscle contraction. Pharmac. Rev. 16: 85-111.

Bohr, D.F., Brodie, D.C. and Cheu, D.H. (1958). Effect of electrolytes on arterial muscle contraction. Circulation. 17: 746-749.

Bozler, E. (1948). Conduction, automaticity and tonus of visceral muscle. Experientia. 4: 213-218.

Brading, A.V. and Tomita, T. (1968). The action potential of the guinea-pig taenia-coli in low sodium solution. J. Physiol. (Lond). 197: 30-31P.

Brading, A.F., Bülbring, E. and Tomita, T. (1969). The effect of sodium and calcium on the action potential of the smooth muscle of the guinea-pig taenia coli. J. Physiol. (Lond). 200: 637-654.

Brecht, K., Konold, P. and Gebert, G. (1969). The effect of potassium, catecholamines and other vasoactive agents on isolated arterial segments of the muscular type. Physiologia Bohemoslov. 18: 15-21.

van Breemen, C. and Daniel, E.E. (1966). The influence of high potassium depolarization and acetylcholine on calcium exchange in the rat uterus. J. Gen. Physiol. 49: 1299-1317.

van Breemen, C., Daniel, E.E. and van Breemen, D. (1966). Calcium distribution and exchange in the rat uterus. J. Gen. Physiol. 79: 1265-1297.

Briggs, A.H. (1962). Calcium movements during potassium contracture in isolated rabbit aortic strips. Am. J. Physiol. 203:849-852.

Briggs, A.H. (1963). Characteristics of contraction in glycerinated uterine smooth muscle. Am. J. Physiol. 204:739-742.

Brodie, D.C., Bohr, D.F. and Smit, J. (1959). Dual contractile response of the aorta strip. Am. J. Physiol. 197: 241-246.

Bülbring, E. (1954). Membrane potentials of smooth muscle fibres of the taenia coli of the guinea-pig. J. Physiol. (Lond). 125: 302-315.

Bülbring, E. (1955). Correlation between membrane potential, spike discharge and tension in smooth muscle. J. Physiol. (Lond). 128: 200-221.

Bülbring, E. (1962). Electrical activity in intestinal smooth muscle. Physiol. Rev. 42 (Suppl. 5): 160-174.

Bülbring, E. and Kuriyama, H. (1963). Effects of changes in the external sodium and calcium concentrations on spontaneous electrical activity in smooth muscle of guinea-pig taenia coli. J. Physiol. (Lond). 166: 29-58.

Bülbring, E. and Tomita, T. (1968). The effect of catecholamines on the membrane resistance and spike generation in the smooth muscle of the guinea-pig taenia coli. J. Physiol. (Lond). 194:74-76P.

Bülbring, E. and Tomita, T. (1969a). Increase of membrane conductance by adrenaline in the smooth muscle of guinea-pig taenia coli. Proc. R. Soc. B. 172: 89-102.

Bülbring, E. and Tomita, T. (1969b). Suppression of spontaneous spike generation by catecholamines in the smooth muscle of the guinea-pig taenia coli. Proc. R. Soc. B. 172: 103-119.

Bülbring, E. and Tomita, T. (1969c). Effect of calcium, barium and manganese on the action of adrenaline in the smooth muscle of the guinea-pig taenia coli. Proc. R. Soc. B. 172: 121-136.

Bülbring, E., Burnstock, G. and Holman, M.E. (1958). Excitation and conduction in the smooth muscle of the isolated taenia coli of the guinea-pig. J. Physiol. (Lond). 142: 420-437.

Burnstock, G. and Prosser, C.L. (1960). Responses of smooth muscles to quick stretch; relation of stretch to conduction. Am. J. Physiol. 198: 921-925.

Burnstock, G., Holman, M.E. and Prosser, C.L. (1963). Electrophysiology of smooth muscle. Physiol. Rev. 43: 482-527.

Caldwell, P.C. (1968). Factors governing movement and distribution of inorganic ions in nerve and muscle. Physiol. Rev. 48: 1-64.

Carrier, O., Jr. and Shibata, S. (1967). A possible role for tissue calcium in reserpine supersensitivity. J. Pharm. exp. Ther. 155: 42-49.

Carmeliet, E.E. (1961). Chloride ions and the membrane potential of Purkinje fibres. J. Physiol. (Lond). 156: 375-388.

Carpenedo, F., Ferrari, M. and Furlanut, M. (1968). Effects of some spasmolytic agents on the lipid facilitated transport of calcium ions. J. Pharm. Pharmacol. 20: 733-734.

Carsten, M.E. (1967). Cardiac sarcotubular vesicles: effects of ions, ouabain and acetylstrophanthidin. Circulation Res. 20: 599.

Carsten, M.E. (1968). Tropomyosin from smooth muscle of the uterus. Biochemistry. 7: 960-967.

Carsten, M.E. (1969). Role of calcium by sarcoplasmic reticulum in the contraction and relaxation of uterine smooth muscle. J. Gen. Physiol. 53: 414-426.

Casteels, R. (1970). The relationship between the membrane potential and the ion distribution in smooth muscle cells. In: Smooth muscle. Ed.: E. Bülbring, A.F. Brading, A.W. Jones and T. Tomita. Edward Arnold (Pub) Ltd. London. pp 70-99.

Chapman, J.B. and Holman, M.E. (1968). Potassium contractures in the ureter of the guinea-pig. Aust. J. exp. Biol. med. Sci. 46: 503-514.

Chattopadhyay, D.P. (1961). Studies on the oxidative capacity of aortic tissue slices of rabbits. Ann. Biochim. exp. Med. (Calcutta) 22: 77-82.

Coe, J., Detar, R. and Bohr, D.F. (1968). Substrates and vascular smooth muscle contraction. Am. J. Physiol. 214: 245-250.

Cohen, C., Lowey, S. and Kucera, J. (1961). Structural studies on uterine myosin. J. Biol. Chem. 236: PC23-PC24.

Cross, S.B., Keynes, R.D. and Rybova, R. (1965). The coupling of sodium efflux and potassium influx in frog muscle. J. Physiol. (Lond). 181: 865-880.

Cuthbert, A.W. (1966). Electrical activity in mammalian veins. Bibl. Anat. 8: 11-15.

Cuthbert, A.W. and Sutter, M.C. (1965). The effect of drugs on the relation between action potentials discharge and tension in a mammalian vein. Br. J. Pharmac. Chemother. 25: 592-601.

Daemers-Lambert, C. (1964). Action du chlorure de potassium sur le metabolisme des esters phosphores et le tonus du muscle arteriel. Angiologie. 1: 249-274.

Daniel, E.E. (1963). On the roles of calcium, strontium and barium in contraction and excitability of rat uterine muscle. Archs. int. Pharmacodyn. Ther. 146: 298-349.

Daniel, E.E. (1964). Effect of drugs on contractions of vertebrate smooth muscle. A. Rev. Pharmac. 4: 189-222.

Daniel, E.E. (1965). Attempted synthesis of data regarding divalent ions in muscle function. In: Muscle. Ed.: W.M. Paul, E.E. Daniel, C.M. Kay and G. Monkton. Pergamon Press. London. pp 295-313.

Daniel, E.E. and Chapman, K.M. (1963). Electrical activity of the gastrointestinal tract as an indication of mechanical activity. Amer. J. dig. Dis. 8: 54-102.

Daniel, E.E. and Irwin, J. (1965). On the mechanism whereby EDTA, EGTA, DPTA, oxalate, desferrioxamine and 1,10 phenanthroline affect contractility of rat uterus. Can. J. Physiol. Pharmac. 43: 111-136.

Daniel, E.E. and Robinson, K. (1960a). The secretion of sodium and uptake of potassium by isolated uterine segments made sodium rich. J. Physiol. (Lond). 154: 421-449.

Daniel, E.E. and Robinson, K. (1960b). The relation of sodium secretion to metabolism in isolated sodium-rich uterine segments. J. Physiol. (Lond). 154: 445-460.

Davidson, W.J. (1970). Studies of supersensitivity in smooth muscle. M.Sc. Thesis. University of Manitoba.

Davidson, W.J. and Innes, I.R. (1970). Dissociation of potentiation of isoprenaline by cocaine from inhibition of uptake in cat spleen. Brit. J. Pharmac. Chemother. 39: 175-181.

Davies, R.E. (1963). A molecular theory of muscle contraction: calcium dependant contractions with hydrogen bond formation plus ATP-dependant extensions of part of the myosin-actin-cross-bridges. Nature. 199: 1068-1074.

Detar, R. and Bohr, D.F. (1968a). Oxygen and vascular smooth muscle contraction. Am. J. Physiol. 214: 241-244.

Detar, R. and Bohr, D.F. (1968b). Adaptation to hypoxia in vascular smooth muscle. Fedn. Proc. 27: 1416-1419.

Devine, C.E. and Somylo, A.P. (1962). Ultrastructure of vascular smooth muscle (VSM) studied with lanthanum. Fedn. Proc. 29: 455 Abs.

Dewey, M.M. and Barr, L. (1962). Intercellular connection between smooth muscle cells: the nexus. Science. 137: 670-672.

Dewey, M.M. and Barr, L. (1968). Structure of vertebrate intestinal smooth muscle. In: Handbook of Physiology, section 6. Alimentary canal, vol. IV. Ed.: B.C.F. Code. Pub. American Physiological Society. Washington. pp 1629-1654.

Diamond, J. and Marshall, J.M. (1969a). Smooth muscle relaxants: dissociation between resting membrane potential and resting tension in rat myometrium. J. Pharm. exp. Ther. 168: 13-20.

Diamond, J. and Marshall, J.M. (1969b). A comparison of the effects of various smooth muscle relaxants on the electrical and mechanical activity of rat uterus. J. Pharm. exp. Ther. 168: 21-30.

Dodd, W.A. and Daniel, E.E. (1960). Electrolytes and arterial muscle contractility. Circulation Res. 8: 451-463.

Durbin, R.P. and Jenkinson, D.H. (1961). The calcium dependence of tension development in depolarised smooth muscle. J. Physiol. (Lond). 157: 90-96.

Ebashi, S. (1963). Third component participating in the superprecipitation of natural actomyosin. Nature. 200: 1010.

Ebashi, S., Ebashi, F. and Kodama, A. (1967). Troponin as the Ca receptive protein in the contractile system. J. Biochem. (Tokyo). 62: 137-138.

Ebashi, S., Iwakura, H., Nakajima, H., Nakamura, R. and Choi, Y. (1966). New structural proteins from dog heart and chicken gizzard. Biochem. Z. 345: 201-211.

Edman, K.A.P. and Schild, H.O. (1962). The need for calcium in the contractile responses induced by acetylcholine and potassium in the rat uterus. J. Physiol. (Lond). 161: 424-441.

Euler, U.S. von and Lishajko, F. (1959). The estimation of catecholamines in urine. Acta physiol. scand. 45: 122-132.

Evans, D.H.L., Schild, H.O. and Thesleff, S. (1958). Effects of drugs on depolarised plain muscle. J. Physiol. (Lond). 143: 424-485.

Feinstein, M.B. (1966). Inhibition of contraction and calcium exchangeability in rat uterus by local anaesthetics. J. Pharm. exp. Ther. 152: 516-524.

Ferrari, M. and Carpenedo, F. (1968). On the mechanism of action of some myolytic agents on depolarised guinea-pig taenia coli. Archs. int. Pharmacodyn. Ther. 174: 223-232.

Fillenz, M. (1970). The innervation of the cat spleen. Proc. Roy. Soc. B. 174: 459-468.

Filo, R.S., Bohr, D.F. and Ruegg, J.C. (1965). Glycerinated skeletal and smooth muscle: calcium and magnesium dependence. Science. 147: 1581-1583.

Fleming, W.W. and Trendelenburg, U. (1961). The development of supersensitivity to norepinephrine after pretreatment with reserpine. J. Pharm. exp. Ther. 133: 41-51.

Frankenhaeuser, B. and Hodgkin, A.L. (1957). The action of calcium on the electrical properties of the squid axon. J. Physiol. (Lond). 137: 218-244.

Funaki, S. (1966). Electrical and mechanical activity of isolated smooth muscle from the portal vein of the rat. Bibl. Anat. 8: 5-10.

Furchgott, R.F. (1966). Metabolic factors that influence contractility of vascular smooth muscle. Bull. N.Y. Acad. Sci. 42: 996-1006.

Furchgott, R.F. and Wales, M.R. (1952). Utilisation of compounds of Krebs cycle for contraction energy by rabbit intestinal smooth muscle. Am. J. Physiol. 169: 326-336.

Garrett, R.L. and Carrier, O., Jr. (1971). Alteration of extracellular calcium dependence in vascular tissue by reserpine. Eur. J. Pharmacol. 13: 306-311.

Godfraind, T. and Kaba, A. (1969). Actions phasique et tonique de l'adrenaline sur un muscle lisse vasculaire et leur inhibition par des agents pharmacologiques. Archs. int. Pharmacodyn. 178: 488-491.

Golenhofen, K. and Petrányi, P. (1969). Spikes of smooth muscle in calcium-free solution (isolated taenia coli of the guinea-pig). Experientia. 25: 271-273.

Goodford, P.J. (1965a). The distribution of calcium in intestinal smooth muscle. In: Muscle. Ed.: W.M. Paul, E.E. Daniel, C.M. Kay and G. Monckton. Pergamon Press. London. pp 219-226.

Goodford, P.J. (1965b). The loss of radioactive ^{45}calcium from the smooth muscle of the guinea-pig taenia coli. J. Physiol. (Lond). 176: 180-190.

Goodford, P.J. (1967). The calcium content of the smooth muscle of the guinea-pig taenia coli. J. Physiol. (Lond). 192: 145-158.

Goodford, P.J. and Hermansen, K. (1961). Sodium and potassium movement in the unstriated muscle of the guinea-pig taenia coli. J. Physiol. (Lond). 158: 426-448.

Goodman, F.R. and Weiss, G.B. (1971). Dissociation by lanthanum of smooth muscle responses to potassium and acetylcholine. Am. J. Physiol. 220: 759-766.

Gordon, A.M., Huxley, A.F. and Julian, F.J. (1966). The variation in isometric tension with sarcomere length in vertebrate muscle fibres. J. Physiol. (Lond). 184: 170-192.

Greenberg, R. (1968). The role of calcium in supersensitivity induced by cocaine. Ph.D. Thesis. University of Manitoba.

Guyton, A.C., Ross, J.M., Carrier, Jr. O. and Walker, J.R. (1964). Evidence for tissue oxygen demand as the major factor causing autoregulation. Circulation Res. 15 (suppl.1): 60-68.

Hagiwara, S. and Naka, K. (1964). The initiation of spike potentials in barnacle muscle fibres under low intracellular Ca. J. Gen. Physiol. 48: 141-162.

Hagiwara, S. and Nakajima, S. (1966). Differences in Na and Ca spikes as examined by application of tetrodotoxin, procaine and manganese ions. J. Gen. Physiol. 49: 793-806.

Hagiwara, S. and Takahashi, K. (1967). Surface density of calcium ions and calcium spikes in the barnacle muscle fibre membrane. J. Gen. Physiol. 50: 583-601.

Hanson, J. and Huxley, H.E. (1955). The structural basis of contraction in striated muscle. Symp. Soc. exp. Biol. 9: 228-264.

Hattingberg, M. von., Kuschinsky, G. and Rahn, K.H. (1966). Der einfluss von pharmaka auf calcium gehalt und ^{45}calcium austausch der glatten muskulatur der taenia coli von meerschweinchen. Naunyn Schmiedeberg Arch Pharmakol. 253: 438-443.

Heilbrunn, L.V. and Wiercinski, F.J. (1947). The action of various cations on muscle protoplasm. J. Cell & Comp. Physiol. 29: 15-32.

Hinke, J.A.M. (1965). Calcium requirements for noradrenaline and high potassium ion contraction in arterial smooth muscle. In: Muscle. Ed.: E.E. Daniel, C.M. Kay and G. Monckton. Pergamon Press. London. pp 269-285.

Hiraoka, M., Yamagishi, S. and Sano, T. (1968). Role of calcium ions in the contraction of vascular smooth muscle. Am. J. Physiol. 214: 1084-1089.

Hodgkin, A.L. and Horowicz, P. (1959). The influence of potassium and chloride ions on the membrane potential of single muscle fibres. J. Physiol. (Lond). 148: 127-160.

Holman, M.E. (1957). The effect of changes in sodium chloride concentration on the smooth muscle of the guinea-pig's taenia coli. J. Physiol. (Lond). 136: 569-584.

Holman, M.E. (1958). Membrane potentials recorded with high resistance microelectrodes and the effects of changes in ionic environment on the electrical and mechanical activity of the smooth muscle of the taenia coli of the guinea-pig. J. Physiol. (Lond). 141: 464-488.

Holman, M.E. (1969). Electrophysiology of vascular smooth muscle. Ergebn. Physiol. 61: 137-177.

Holman, M.E. (1968). Introduction to electrophysiology of visceral smooth muscle. In: Handbook of Physiology, section 6. Alimentary Canal, vol IV. Ed.: C.F. Code. Pub. American Physiological Society. Washington, D.C. pp 1665-1708.

Holman, M.E., Kasby, C.B. and Suthers, M.B. (1968). The effect of increasing length on the electrical and mechanical activity of longitudinal smooth muscle of rabbit portal vein. Austr. J. exp. Biol. med. Sci. 46: p.29.

Holman, M.E., Kasby, C.B., Suthers, M.B. and Wilson, J.A.F. (1968). Some properties of the smooth muscle of rabbit portal veins. J. Physiol. (Lond). 196: 111-132.

Hudgins, P.M. (1969). Some drug effects on calcium movements in aortic strips. J. Pharm. exp. Ther. 170: 303-310.

Hudgins, P.M. and Fleming, W.W. (1966). A relatively nonspecific supersensitivity in aortic strips resulting from pretreatment with reserpine. J. Pharm. exp. Ther. 153: 70-80.

Hudgins, P.M. and Weiss, G.B. (1968). Differential effects of calcium removal upon vascular smooth muscle contraction induced by norepinephrine, histamine and potassium. J. Pharm. exp. Ther. 159: 91-97.

Hudgins, P.M. and Weiss, G.B. (1969). Characteristics of ^{45}Ca binding in vascular smooth muscle. Am. J. Physiol. 217: 1310-1315.

Hurwitz, L., Joiner, P.D. and von Hagen, S. (1967a). Mechanical responses of intestinal smooth muscle in a calcium-free medium. Proc. Soc. Exp. Biol. Med. 125: 518-522.

Hurwitz, L., Joiner, P.D. and von Hagen, S. (1967b). Calcium pools utilized for contraction in smooth muscle. Am. J. Physiol. 213: 1299-1304.

Huxley, A.F. (1957). Muscle structure and theories of contraction. Progr. Biophys. biophys. Chem. 7: 257-318.

Huxley, A.F. and Peachey, L.D. (1961). The maximum length for contraction in vertebrate striated muscle. J. Physiol. (Lond). 156: 150-165.

Huxley, H.E. (1967). Structural arrangements and contraction mechanism in striated muscle. Proc. Roy. Soc. B. 160: 442-448.

Imai, S. and Takeda, K. (1967a). Actions of calcium and certain multivalent cations on potassium contracture of guinea-pig's taenia coli. J. Physiol. (Lond). 190: 155-169.

Imai, S. and Takeda, K. (1967b). Effect of vasodilators upon the isolated taenia coli of the guinea-pig. J. Pharm. Exp. Ther. 156: 557-564.

Innes, I.R. (1960). Sensitization of the nictitating membrane to sympathomimetic amines by reserpine. Fedn. Proc. 19: 285.

Iversen, L.L. (1967). In: The uptake and storage of noradrenaline in sympathetic nerves. Cambridge University Press.

Johansson, B. and Bohr, D.F. (1966). Rhythmic activity in smooth muscle from small subcutaneous arteries. Am. J. Physiol. 210: 801-806.

Johansson, B., Jonsson, O., Axelsson, J. and Wahlstrom, B. (1967). Electrical and mechanical characteristics of vascular smooth muscle response to norepinephrine and isoproterenol. Circulation Res. 21: 619-633.

Kalsner, S. and Nickerson, M. (1968). A method for the study of mechanisms of drug disposition in smooth muscle. Canad. J. Physiol. Pharmac. 46: 719-730.

Kalsner, S., Nickerson, M. and Boyd, G. (1970). Selective blockade of potassium induced contractions of aortic strips by -diethyl-aminoethyldiphenylpropylacetate (SKF 525A). J. Pharm. exp. Ther. 174: 500-508.

Kao, C.Y. (1967). Ionic basis of electrical activity in uterine smooth muscle. In: Cellular Biology of the Uterus. Ed.: R.M. Wynn. Meridith Pub. Co. New York. pp 386-448.

Kao, C.Y. and Nishiyama, A. (1969). Ion concentrations and membrane potentials of myometrium during recovery from cold. Am. J. Physiol. 217: 525-531.

Karaki, H., Ikeda, M. and Urakawa, N. (1967). Effects of external calcium and some metabolic inhibitors on barium-induced tension changes in guinea-pig taenia coli. Jap. J. Pharmacol. 17: 603-612.

Kasuya, Y. and Goto, K. (1968). The mechanism of supersensitivity to norepinephrine induced by cocaine in rat isolated vas deferens. Eur. J. Pharmacol. 4: 355-362.

Keynes, R.D. and Lewis, P.R. (1951). The resting exchange of radioactive potassium in crab nerve. J. Physiol. (Lond). 113: 73-98.

van der Kloot, W.G. (1967). Potassium-stimulated respiration and intracellular calcium release in frog skeletal muscle. J. Physiol. (Lond). 191: 141-165.

Konold, P., Gebert, G. and Brecht, K. (1968). The effect of potassium on the tone of isolated arteries. Pflug. Arch. 301: 285-291.

Kosan, R.L. and Burton, A.C. (1966). Oxygen consumption of arterial smooth muscle as a function of active tone and passive stretch. Circulation Res. 18: 79-88.

Krejci, I. and Daniel, E.E. (1970a). Effect of contraction on movements of calcium 45 into and out of rat myometrium. Am. J. Physiol. 219: 256-262.

Krejci, I. and Daniel, E.E. (1970b). Effects of altered external calcium on fluxes of calcium 45 in rat myometrium. Am. J. Physiol. 219: 263-269.

Kroeger, E.A. (1970). Mechanism of action of hypoxia in airway smooth muscle: effect on energy and calcium metabolism. Ph.D. Thesis. University of Manitoba.

Kuriyama, H. (1963). The influence of potassium, sodium and chloride on the membrane potential of the smooth muscle of taenia coli. J. Physiol. (Lond). 166: 15-28.

Kuriyama, H., Osa, T. and Toida, N. (1966). Effect of tetrodotoxin on smooth muscle cells of the guinea-pig taenia coli. Brit. J. Pharmacol. 27: 366-376.

Larbi, E. and Zaimis, E. (1970). The effect of the chronic administration of clonidine (St 155) on vascular smooth muscle. The Br. J. Pharm. 39: 221P.

Leonard, E. (1957). Alteration of contractile response of artery strips by a potassium-free solution, cardiac glycosides and changes in stimulation frequency. Am. J. Physiol. 189: 185-190.

Liu, J., Prosser, C.L. and Job, D.D. (1969). Ionic dependence of slow waves and spikes in intestinal muscle. Am. J. Physiol. 217: 1542-1547.

Löffelholz, K. and Scholz, H. (1970). Inhibition of Mn^{++}-catalyzed autooxidation of adrenaline by ascorbic acid. Experientia. 26: 637-638.

Lowy, J. and Small, J.W. (1970). The organisation of myosin and actin in vertebrate smooth muscle. Nature. 227: 46-51.

Lullmann, H. and Siegfriedt, A. (1968). Über den Calcium-Gehalt und den ^{45}calcium-Austausch in Längsmuskulatur des Meerschweinchendünndarms. Pflügers Archiv. 300: 108-119.

Lundholm, L. and Mohme-Lundholm, E. (1960). The carbohydrate metabolism and tone of smooth muscle. Acta Pharmacol. Toxicol. 16: 374-388.

Lundholm, L. and Mohme-Lundholm, E. (1962). Studies on the effects of drugs upon lactic acid metabolism and contraction of vascular smooth muscle. Acta Physiol. Scand. 55: 45-63.

Lundholm, L. and Mohme-Lundholm, E. (1963). Dissociation of contraction and stimulation of lactic acid production in experiments on smooth muscle under anaerobic conditions. Acta Physiol. Scand. 57: 111-127.

Lütgau, H.C. and Niedergerke, R. (1958). The antagonism between Ca and Na ions on the frog heart. J. Physiol. (Lond). 143: 486-505.

Mailhot, R. (1970). Supersensitivity of cat spleen. Ph.D. Thesis. University of Manitoba.

Marechal, G. and Mommaerts, W.F.H.M. (1963). The metabolism of phosphocreatine during an isometric tetanus in the frog sartorius muscle. Biochim. Biophys. Acta. 70: 53-67.

Mueller, H. (1966). EGTA-sensitizing activity and molecular properties of tropomyosin prepared in presence of an SH-protecting agent. Biochem. Z. 345: 300-321.

Mullins, L.J. and Awad, M.Z. (1969). The control of the membrane potential of muscle fibers by the sodium pump. J. Gen. Physiol. 48: 761-775.

Nagasawa, J. (1965). Calcium movement in the smooth muscle of guinea-pig taenia coli. Tohuku. J. Exp. Med. 85: 72-82.

Nakajima, A. and Horn, L. (1967). Electrical activity of single vascular smooth muscle fibers. Am. J. Physiol. 213: 25-30.

Nayler, W.G., Price, J.M., Swann, J.B., McInnes, I., Race, D. and Lowe, T.E. (1968). Effect of the hypotensive drug ST 155 (Catapres) on the heart and peripheral circulation. J. Pharm. exp. Ther. 164: 45-59.

Needham, D.M. (1962). Contractile proteins in smooth muscle of the uterus. Physiol. Rev. 42 (Suppl. 5): 88-97.

Needham, D.M. and Shoenberg, C.F. (1964). Proteins of the contractile mechanism of mammalian smooth muscle and their possible location in the cell. Proc. Roy. Soc. B. 160: 517-524.

Needham, D.M. and Shoenberg, C.F. (1967). The biochemistry of the myometrium. In Cellular Biology of the Uterus. Ed. by R.M. Wynn, Meridith Pub. Co. New York. pp. 291-352.

Nemetchek-Gansler, H. (1966). Ultrastructure of the myometrium. In: Cellular Biology of the Uterus. (ed. R.M. Wynn). Meridith Pub. Co. New York. pp. 353-385.

Northover, B.J. (1968). The effects of drugs on the constriction of isolated depolarized blood vessels in response to calcium or barium. Brit. J. Pharmacol. 34: 417-428.

Paton, W.D.M. (1961). A theory of drug action based on the rate of drug-receptor combination. Proc. Roy. Soc. B. 154: 21-69.

Peachey, L.D. and Porter, K.R. (1959). Intracellular impulse conduction in muscle cells. Science. 129: 721-722.

Perry, S.V. and Grey, T.C. (1956). A study of the effects of substrate concentration and certain relaxing factors on the Mg-activated myofibrillar ATP-ase. Biochem. J. 64: 184-192.

Persoff, D.A. (1960). A comparison of methods for measuring efflux of labelled potassium from contracting rabbit atria. J. Physiol. (Lond). 152: 354-366.

Pfaffman, M., Urakawa, N. and Holland, W.C. (1965). Role of metabolism in K-induced tension changes in guinea-pig taenia coli. Am. J. Pharmacol. 208: 1203-1205.

Portzehl, H. (1952). Der Arbeitzyklus geordneter Aktomyosinsysteme. Z. Naturf. 7b: 1-10.

Rall, T.W. and Gilman, A.G. (1970). The role of cyclic AMP in the nervous system. Neurosciences Research Programme Bull. 8: 273-274.

Rang, H.P. and Ritchie, J.M. (1968). On the electrogenic sodium pump in mammalian non-myelinated nerve fibres and its activation by various external cations. J. Physiol. (Lond). 196: 183-221.

Rhodin, J.A.G. (1962). Fine structure of vascular walls in mammals, with special reference to smooth muscle components. Physiol. Rev. 42: 48-81.

Rhodin, J.A.G. (1967). The ultrastructure of mammalian arterioles and precapillary sphincters. J. Ultrastruct. Res. 18: 181-223.

Rice, R.V., Moses, J.A., McManus, G.M., Brady, A.C. and Blasik, L.M. (1970). The organisation of contractile filaments in a mammalian smooth muscle. J. Cell. Biol. 47: 183-196.

Ridgeway, E.B. and Ashley, C.C. (1967). Calcium transients in single muscle fibers. Biochem. Biophys. Res. Comm. 29: 229-234.

Riggs, D.S. (1970). In: A mathematical approach to physiological problems. The Williams and Wilkins Co., Baltimore, Md.

Ringer, S. (1880). Concerning the influence exerted by each of the constituents of the blood on the contraction of the ventricle. J. Physiol. (Lond). 3: 380-394.

Ringer, S. (1882). A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart. J. Physiol. (Lond). 4: 29-43.

Rubin, A.A., Zitowitz, L., Hausler, L.M. (1963a). Acute circulatory effects of diazoxide - an antihypertensive, nondiuretic benzothiadiazine. J. Pharm. exp. Ther. 136: 344.

Rubin, A.A., Zitowitz, L. and Hausler, L. (1963b). Acute circulatory effects of diazoxide and sodium nitrite. J. Pharm. exp. Ther. 140: 46 - 51.

Sbarra, A.J., Gilfillan, R.F. and Bardawil, W.A. (1960). The hexose monophosphate pathway in arterial tissue. Biochem. Biophys. Res. Commun. 3: 311-313.

Schatzmann, H.J. (1961). Calciumaufnahme und Abgabe am Darmmuskel des Meerschweinchens. Pflugers Arch. 274: 295-310.

Schild, H.O. (1967). The action of isoprenaline in the depolarised rat uterus. Brit. J. Pharmacol. 31: 578-592.

Schirmer, R.H. (1965). Die besonderheiten des contractilen proteins der arterien. Biochem. Z. 343: 269-282.

Shibata, S. and Briggs, A.H. (1966). The relationships between electrical and mechanical events in rabbit aortic strips. J. Pharm. exp. Ther. 153: 466-470.

Skou, J.C. (1965). Enzymatic basis for active transport of Na^+ and K^+ across cell membrane. Physiol. Rev. 45: 596-617.

Somlyo, A.V. and Somlyo, A.P. (1968a). Electromechanical and pharmomechanical coupling in vascular smooth muscle. J. Pharm. exp. Ther. 159: 129-145.

Somlyo, A.P. and Somlyo, A.V. (1968b). Vascular smooth muscle. I. Normal structure, pathology, biochemistry and biophysics. Pharmacol. Rev. 20: 197-272.

Somlyo, A.P. and Somlyo, A.V. (1969). Pharmacology of excitation-contraction coupling in vascular smooth muscle and in avian slow muscle. Fedn. Proc. 28: 1634-1642.

Somlyo, A.V., Haeusler, G. and Somlyo, A.P. (1970). Beta-adrenergic electrogenesis in vascular smooth muscle (VSM): Effect of theophylline (T), external potassium (K_o) and oligomycin (o). Fedn. Proc. 29: 2062 Abs.

Somlyo, A.P., Sandberg, R.L. and Somlyo, A.V. (1965). Vascular smooth muscle contraction as a function of stimulus frequency. In: Digest of the 6th International Conference on Medical, Electrical and Biological Engineering. Tokyo. pp 44-45.

Somlyo, A.V., Vinall, P. and Somlyo, A.P. (1969). Excitation-contraction coupling and electrical events in two types of vascular smooth muscle. Microvasc. Res. 1: 354-373.

Sparrow, M.P. and Simmonds, W.J. (1965). The relationship of the calcium content of smooth muscle to its contractility in response to different modes of stimulation. Biochim. Biophys. Acta. 109: 503-511.

Speden, R.N. (1964). Electrical activity of single smooth muscle cells of the mesenteric artery produced by splanchnic nerve stimulation in the guinea-pig. Nature. 154: 15-25.

Speden, R.N. (1967). Adrenergic transmission in small arteries. Nature. 216: 289-290.

Sperelakis, N. (1968). Ca^{45} and Sr^{89} movements with contraction of depolarised smooth muscle. Am. J. Physiol. 203: 860-866.

Sperelakis, N. and Tarr, M. (1965). Weak electrotonic interaction between neighbouring visceral smooth muscle cells. Am. J. Physiol. 208: 737-747.

Sperelakis, N., Schneider, M.F. and Harris, E.J. (1967). Decreased K^+ conductance produced by Ba^{++} in frog sartorius fibers. J. Gen. Physiol. 50: 1565-1583.

Steedman, W.M. (1966). Micro-electrode studies on mammalian vascular muscles. J. Physiol. 186: 382-400.

Stephens, N.L. and Wrogeman, K. (1970). Oxidative phosphorylation in smooth muscle. Proc. Can. Fed. Biol. Sci. 13: 89 Abs.

Su, C. and Bevan, J.A. (1965). The electrical response of pulmonary artery muscle to acetylcholine histamine and serotonin. Life Sci. 4: 1025-1029.

Su, C., Bevan, J. and Ursillo, R.C. (1964). Electrical quiescence of pulmonary artery smooth muscle during sympathomimetic stimulation. Circulation Res. 15: 20-27.

Szent-Györgyi, A. (1947). Chemistry of muscular contraction. Acad. Press. New York.

Szent-Györgyi, A. (1951). In: Muscular Contraction. Ed. 2, Academic Press, Inc. New York. pp. 144-145.

Tamai, T. and Kagiyama, S. (1968). Studies of cat heart muscle during recovery after prolonged hypothermia. Hyperpolarisation of cell membranes and its dependence on the sodium pump with electrogenic characteristics. Circulation Res. 22: 423-433.

Taylor, G.S., Paton, D.M. and Daniel, E.E. (1970). Characteristics of electrogenic sodium pumping in rat myometrium. J. Gen. Physiol. 56: 360-375.

Tomita, T. (1966). Membrane capacity and resistance of mammalian smooth muscle. J. Theor. Biol. 12: 216-217.

Tomita, T. (1966b). Electrical responses of smooth muscle to external stimulation in hypertonic solution. J. Physiol. (Lond). 183: 450-468.

Thomas, R.C. (1969). Membrane current and intracellular sodium changes in a snail neurone during extrusion of injected sodium. J. Physiol. (Lond). 201: 495-514.

Urakawa, N. and Holland, W.C. (1967). Ca^{45} uptake and tissue calcium in K-induced phasic and tonic contraction in taenia coli. Am. J. Physiol. 207: 873-876.

Urakawa, N., Karaki, H. and Ikeda, M. (1970). Effects of ouabain and metabolic inhibiting factors in Ca distribution during K-induced contracture in guinea-pig taenia coli. Jap. J. Pharmacol. 20: 360-366.

Van Der Kloot, W.G. (1967). Potassium-stimulated respiration and intracellular calcium release in frog skeletal muscle. J. Physiol. (Lond). 191: 141-165.

Vassale, M. (1965). Cardiac pacemaker potentials at different extra- and intracellular K concentrations. Am. J. Physiol. 208: 770-775.

Waugh, W.H. (1962). Adrenergic stimulation of depolarised arterial muscle. Circulation Res. 11: 264-276.

Waugh, W.H. (1964). Calcium and contraction of arterial smooth muscle. In: Muscle. Ed.: W.M. Paul, E.E. Daniel, C.M. Kay and G. Monckton. Pergamon Press. London. pp 253-266.

Weber, A. (1956). The ultracentrifugal separation of L-myosin and actin in actomyosin sol under the influence of ATP. Biochim. Biophys. acta. 19: 345-351.

Wertheimer, H.E. and Ben-Tor, V. (1962). Influence of diabetes on carbohydrate metabolism of aortic tissue. Diabetes. 11: 422-425.

West, T.C., Hadden, G. and Farah, A. (1951). Effect of anoxia in response of the isolated intestine to various drugs and enzyme inhibitors. Am. J. Physiol. 164: 565-572.

Winegrad, S. (1970). The intracellular site of calcium activation of contraction in frog skeletal muscle. J. Gen. Physiol. 55: 77-88.

Wohl, A.J., Hausler, L.M. and Roth, F.E. (1967). Studies on the mechanism of antihypertensive action of diazoxide: in vitro vascular pharmacodynamics. J. Pharm. exp. Ther. 158: 531-539.