# STUDIES OF THE ROLES OF CALCIUM IONS IN ANTERIOR MESENTERIC PORTAL VEIN

by

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B.Sc., University of British Columbia, 1968

A thesis submitted in partial fulfilment of

the requirements for the degree of

Master of Science

in the Department

of

Pharmacology

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April, 1971

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Date April 13, 1971

A preliminary report of part of this work was presented at the Canadian Federation of Biological Societies meetings held in Montreal during June, 1970.

## ABSTRACT

There are two opposing schools of thought concerning the source of calcium ions for the initiation and maintenance of contractions of smooth muscle. Bohr (1964) and Woodward et al. (1970) believe that the calcium for the initiation of contraction is released from bound intracellular stores, whereas Somlyo et al. (1969) believe that most of the activator calcium comes from the extracellular fluid. It was felt that the determination of the source(s) and sink(s) of calcium ions in arteriolar smooth muscle would be required for an understanding of the control of peripheral blood pressure, and so experiments to obtain this information were carried out using the rabbit anterior mesenteric portal vein as a model of arteriolar smooth muscle.

Spontaneous contractions of the vein stop within one minute after the addition of EGTA to the bath and can be returned by simply raising the extracellular calcium concentration. The addition of 1 mM $MnCl_2$, or raising the $MgCl_2$ concentration above 5 mM, or raising the $CaCl_2$ concentration above 10 mM all inhibit spontaneous activity; this inhibition can be rapidly reversed by the addition of appropriate amounts of EGTA.

The removal of free extracellular calcium either by adding EGTA or placing the vein in calcium-free solution inhibits the responses to all agonists within five minutes. If the calcium concentration is reduced from 2.5 mM to 0.1 mM, the responses to agonists are greatly decreased. If one sets the response in normal Kreb's solution to each concentration of agonist equal to 100 per cent, then the relative reduction of responses in low calcium solution is inversely proportional to both the potency and concentration of the agonist used.

If, however, one produces contractions by adding calcium to tissues bathed in calcium-free solution containing noradrenaline, then the curves of the relative response versus calcium concentrations are independent of the concentration of noradrenaline.

The addition of either EGTA or manganese to a tissue already contracted in response to any agonist produces a rapid relaxation to a decreased, but sustained tension. The degree of relaxation is proportional to the concentration of manganese or EGTA added.

The addition of manganese is also able to inhibit the initiation of responses to noradrenaline, KCl, serotonin, histamine and procaine. The inhibition by manganese of the responses to noradrenaline, KCl, and serotonin but not histamine and procaine can be reversed by increasing the extracellular calcium concentration.

The addition of $MnCl_2$ or $LaCl_3$ does not selectively inhibit a slow phase of the contraction to noradrenaline in the mesenteric portal vein as it does in aorta (Van Breeman, 1969).

The effect of adding $MnCl_2$ is similar to the effect of decreasing extracellular calcium, in that the relative inhibition of response is inversely proportional to the potency and concentration of agonist used.

The effect of altering pH is the same on contractions produced by each agonist tested; lowering the pH below 7.4 inhibits the responses, raising it above pH 7.4 potentiates the responses.

If the vein is placed in calcium-free solution containing EGTA for 10 minutes, the addition of $CaCl_2$ produces a contraction. The response to calcium is transient unless the final concentration of the added calcium is 5 mM or greater; at these higher calcium concentrations the

response is biphasic; an initial transient response is followed by a slow tonic response.

The addition of stock solutions to produce final concentrations of 5 mM $Mg^{++}$ or 0.5 mM $Mn^{++}$ or 0.1 mM $Ca^{++}$ in the bath after the EGTA treatment abolishes the transient responses to calcium but has little effect on the tonic portion of a contraction produced by 10 mM $Ca^{++}$.

When these EGTA experiments are carried out in low sodium solution (17% of normal or less) the response to 2.5 mM $Ca^{++}$ which is normally transient, becomes instead a sustained contraction.

It is concluded then that:

1. Calcium ions themselves are able to control the permeability of the membrane to calcium.
2. The initiation of responses to all agonists probably involves the release of membrane-bound calcium and the influx of extracellular calcium. Differences in efficacy are probably due to differences in ability to release the membrane-bound calcium.
3. A continued influx of calcium is required to maintain a contraction produced by any agonist.
4. Manganese competes with calcium at a membrane site to inhibit the initiation of contraction, and to relax a tissue which is already contracted.
5. Responses to all agonists are potentiated at a pH greater than 7.4, and decreased below pH 7.4.
6. The relaxation process in the mesenteric-portal vein seems to depend in some manner upon extracellular sodium ions.

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## ACKNOWLEDGEMENTS

The author thanks his supervisor, Dr. Morley C. Sutter, for his encouraging remarks and valuable criticism during the course of this work.

The technical assistance of Mrs. Judy Teiser is greatly appreciated.

The author also thanks Mrs. Judy Teiser and Miss Debbie Griswold for helping to prepare the figures, and Miss Gill Kearney for typing and proof-reading this thesis.

The author acknowledges his financial support by the Department of Pharmacology and the University of British Columbia.

# INTRODUCTION

One of nature's most fascinating phenomena must surely be the ability of some specialized striated muscles to undergo up to one thousand contraction-relaxation cycles in one second. When this is compared with the slow peristaltic movements produced by the contraction of most types of smooth muscle, it is not so surprising that smooth muscle research has only recently begun to receive very much attention.

This lack of attention is understandable but somewhat unjust, since although smooth muscle does not contract as rapidly as striated muscle, there is certainly a greater diversity both in the functions of contraction of smooth muscle, and in the ways in which contractions can be initiated. The contractile force generated by smooth muscle can be influenced by many endogenous compounds such as acetylcholine, adrenaline, histamine, serotonin, vasopressin, angiotensin, prostaglandins, etc. and also by electrical stimulation, whereas electrical excitation is the only known physiologically important means of initiating skeletal muscle contraction.

The functions of smooth muscle contraction range from propelling food through the gastro-intestinal tract, to the control of blood pressure and shunting of blood, to forcing a reluctant foetus out into the world.

Although smooth muscle research still lags behind, there is increasing evidence to show that differences between contractions of striated and smooth muscle may be due mainly to differences in the control of the excitation-contraction-relaxation cycle rather than differences in the molecular basis of contraction. With this in mind,

it may be useful to review the current concepts of striated muscle contraction and to compare this with the known facts of smooth muscle contraction.

## I CURRENT CONCEPTS OF STRIATED MUSCLE CONTRACTION

A striated muscle fiber or cell is composed of a large bundle of filaments or myofibrils running parallel to each other and perfectly aligned so that a cross section through the cell at any point would cut through the corresponding parts of each myofibril. It is this highly ordered nature which leads to the formation of dark and light bands, and hence the term striated muscle.

Using the electron microscope it is seen that the alternating dark and light bands are the result of an overlapping arrangement of thick and thin filaments. Carlsen, Knappeis and Buchtal (1961) showed that the degree of overlap decreased during passive stretch of the muscle and increased during contraction. This was the first evidence (using normal muscle) for the "sliding filament" hypothesis of H.E. Huxley and Hanson (1954) and A.F. Huxley (1956). This hypothesis held that co-ordinated interaction between the thick and thin filaments resulted in a sliding of the thin filaments along the thick filaments, resulting in shortening of the entire fiber.

Hanson and H.E. Huxley (1955) provided evidence for the biochemical composition of the thick and thin filaments. Using procedures which extracted myosin from the muscle they found that the thick filaments had disappeared while the thin filaments remained intact.

The energy required for contraction is provided by the hydrolysis of ATP. There is ATPase activity associated with myosin and it has been postulated that the filaments slide together by a series of cross bridging interactions each of which involves the hydrolysis of one molecule of ATP.

Although the sliding filament theory for contraction was quite well accepted, it was not until 1960 that the processes involved in the initiation and termination of the contraction began to be understood.

Marsh (1951) found a muscle extract which was able to prevent the superprecipitation of actomyosin caused by ATP. Ebashi (1961) showed that this relaxing factor could accumulate calcium ions in the presence of ATP and that various calcium chelating agents were also able to prevent actomyosin superprecipitation and to produce a relaxation of glycerinated muscle fibers.

Electron microscopic studies of the Marsh relaxing factor indicated that it was vesicular, and in fact contained triads which are characteristic of the sarcoplasmic reticulum (Ebashi and Lipman, 1962). "It is rather ironic that recognition of the essential role of calcium ion in contraction has resulted mainly from the investigation into the mechanism of relaxation" (Ebashi and Endo, 1968).

The discovery of the actual regulatory macromolecule with which calcium ions interact to initiate contraction is credited to Ebashi. He noted that relatively crude preparations of actomyosin required both calcium ions and ATP to produce superprecipitation. If, however, the preparation of actin was further purified, the requirement for calcium ions was lost (Ebashi, 1963). Furthermore, while the ATPase of unpurified

actomyosin was inhibited by EDTA, the ATPase of purified "synthetic" actomyosin was unaffected.

The contaminant of actin which endowed calcium sensitivity to the actomyosin was named native tropomyosin. Further purification and identification showed native tropomyosin to actually be composed of two proteins:

(1) Troponin, a globular protein with molecular weight 80,000 which binds four calcium ions per molecule. (Ebashi, S., Ebashi, F., and Kodana, 1967)

(2) Tropomyosin, a more filamentous protein with no affinity for calcium, but which binds strongly to actin. (Ebashi and Endo, 1968)

Endo et al (1966) used fluorescent antibodies to troponin and tropomyosin to show that these proteins were bound only to the thin filaments. It appears that troponin combines with tropomyosin to form native tropomyosin. The binding of native tropomyosin to actin produces an inhibitory effect on the interaction between myosin and actin and also inhibits actomyosin ATPase (Ebashi and Endo, 1968). The interaction of calcium with troponin reverses this inhibition.

The source of the calcium ions for this de-repression process, and the means by which it is released by an electrical impulse has been clarified by the combined techniques of histology, physiology and biochemistry.

An internal membrane structure in striated muscle had been discovered by histologists prior to World War I, but was dismissed as an artifact by physiologists until they realized that such a system might allow the passage of an electrical signal into the interior of

the cell and therefore decrease the time required for activation of the entire fiber.

This internal membrane structure consists of transverse tubules which are open to the extracellular space and wind around each of the individual fibrils, and the sarcoplasmic reticulum which forms an elongated sac parallel to the muscle fibers. The sarcoplasmic reticulum is not directly connected to the extracellular fluid, but it forms close junctions with the transverse tubules. (For a review of the morphology see Bianchi, 1968)

Although it has been known since the identification of the Marsh Factor that the sarcoplasmic reticulum can bind calcium, there is still very little evidence that the calcium bound by the sarcoplasmic reticulum, or contained within it, is the calcium which activates the contractile process. Most of this evidence comes from $^{45}Ca$ autoradiography studies of Winegrad (1965, 1968, 1970). Using this technique he has shown that in a resting muscle most of the exchangeable calcium is stored at the Z line in the terminal cisternae (those parts of the sarcoplasmic reticulum which are in close apposition to the transverse tubules). During a sustained contraction there is a decrease in the calcium found at the Z line, and a large increase in calcium associated with the thin filaments (according to Ebashi's theory the calcium actually is bound to troponin). Stopping the electrical stimulation results in a relaxation of the muscle and a movement of calcium from the thin filaments to the longitudinal part of the sarcoplasmic reticulum. This is followed by a slower movement of calcium back into the terminal cisternae.

The present hypothesis concerning skeletal muscle contraction can be summarized as follows: an action potential coming down the motor nerve initiates (via acetylcholine) an action potential in the sarcolemma. The depolarizing current is then carried inward by the transverse tubules (Huxley and Taylor, 1958). This depolarization of the transverse tubules in some manner influences the terminal cisternae, such that their affinity for calcium is reduced and free calcium is released. This calcium diffuses to its receptor sites on troponin which de-represses the actin-myosin interaction, allowing the ATPase to function, and the filaments to slide together. When the transverse tubules repolarize, the sarcoplasmic reticulum regains a higher affinity for calcium. The calcium is bound or taken up by the longitudinal elements of the sarcoplasmic reticulum and diffuses back to the terminal cisternae.

## II CURRENT CONCEPTS OF SMOOTH MUSCLE CONTRACTION

### A. Contractile Proteins of Smooth Muscle

Most researchers interested in the mechanisms of smooth muscle contractility have assumed that the molecular basis for smooth muscle contraction is similar to that in striated muscle, and have postulated that differences in the responses of the two muscle types are due to differences in the 'metabolism' of calcium. The evidence for this assumption is meagre and consists of comparisons of the properties of glycerinated fibers and isolated contractile proteins from the two types of muscle.

Filo, Ruegg and Bohr (1963) purified hog carotid artery actomyosin and showed superprecipitation in 0.5 mM ATP under conditions in which skeletal muscle also undergoes superprecipitation. Their preparation also showed the same changes in viscosity due to ATP addition as those described by Mommaerts (1948) for an actomyosin preparation from striated muscle. Filo's actomyosin preparation also exhibited calcium and magnesium-activated ATPase activity.

The total actomyosin content of the carotid artery is very low (about 10 mg/g tissue compared with 70 mg/g in skeletal muscle, Filo, Bohr, Ruegg, 1965). The other major difference between actomyosin from smooth or striated muscle is the condition necessary for its extraction. Smooth muscle actomyosin can be extracted by low ionic strength solution (0.05 M KCl) while extraction of actomyosin from skeletal muscle requires a higher ionic strength solution (0.6 M KCl).

Ebashi et al. (1966) showed that the amino acid composition of tropomyosin isolated from smooth muscle (chicken gizzard) or striated muscle were very similar. The smooth muscle however had a much higher tropomyosin: actomyosin ratio.

Filo et al. (1965) compared the responses of glycerinated hog carotid arteries with glycerinated psoas muscles prepared under similar conditions. The responses of the two muscles to calcium ions were identical: threshold response at $10^{-7}$ M calcium and maximum response at $10^{-6}$ M calcium. The arterial preparation required at least 3 mM magnesium to be present in order to produce a response to added calcium. The psoas preparation required less than 1 mM magnesium to maintain sensitivity to calcium, and also produced a contraction when magnesium

was added in the absence of calcium.

The greatest difference between smooth muscle and striated muscle, however, is the lack of an ordered structure of the contractile proteins in smooth muscle. Shoenberg (1966) using bovine arteries showed the presence of actomyosin in homogenates by viscosity and superprecipitation studies. Electron microscopy revealed only the presence of thin filaments, with characteristics similar to actin isolated from striated muscle. Shoenberg (1969) showed that myosin filaments could be prepared from chicken gizzard extracts in the presence of calcium and magnesium. She postulated that an increase in free calcium in vivo could cause myosin to aggregate into filaments and allow the actin and myosin to interact as in skeletal muscle. She also points out that none of the earlier X-ray diffraction studies, which showed a lack of ordered structure in smooth muscle (Elliot, 1964) had been done using isometrically contracting tissue.

## B. Calcium and Vascular Smooth Muscle

Since it is well known that calcium is required for vascular smooth muscle contraction, and since Ebashi has shown the presence of troponin in smooth muscle, it is reasonable to assume until further evidence is available that the calcium ions bind to troponin and de-repress the actin-myosin interaction as in skeletal muscle. The problem for understanding contraction of smooth muscle then, is to determine the sources and sinks of this calcium.

The intracellular tubular network which is so highly developed in striated muscle is nearly absent in vascular smooth muscle. However, the surface area of smooth muscle plasma membrane is greatly increased by numerous invaginations. These invaginations are usually described as spherical, about 450 Å in diameter (Mathews and Gardner, 1966) and have been shown to communicate with extracellular fluid with the use of lanthanum chloride (Devine and Somlyo, 1970) or ferritin (Sutter, unpublished results). Recent electron microscope studies of rabbit anterior mesenteric vein using serial longitudinal sections (Sutter,unpublished results) have shown these vesicles to be about 400 Å in diameter and up to 2000 Å in length. Almost all of the vesicles have an opening to the extracellular fluid visible in one or more of the sections. Rhodin (1962) calculated that these invaginations increase the membrane surface area by twenty five per cent. When one also considers the small diameter of smooth muscle cells, resulting in a large surface area to volume ratio, and a small diffusion distance, it seems possible that the smooth muscle plasma membrane with its invaginations may perform the same role as does the sarcoplasmic reticulum in skeletal muscle.

Thus it can be postulated that electrical impulses or drugs could interact with the plasma membrane, releasing bound calcium into the cytoplasm and producing a contraction. The opposing theory of smooth muscle activation proposes that drugs or electrical impulses alter the permeability of the plasma membrane to calcium, and that the calcium ions which trigger the contraction come from the extracellular fluid.

Somlyo and Somlyo (1968b) distinguish between two types of vascular smooth muscle: phasic muscles in which action potentials are produced in response to drugs or electrical stimulation, (anterior mesenteric vein, mesenteric arterioles), and tonic muscles which respond with graded depolarizations (aorta, pulmonary artery). These would correspond to Bozler's (1948) single-unit (phasic) and multi-unit (tonic) classification of smooth muscle types. The differences between spike generating mesenteric veins and arteries and the gradedly responsive aorta include not only differences in electrical and contractile responses to drugs, but also differences in the effects of calcium depletion. The response of rabbit aorta to noradrenaline persists for an hour in calcium-free medium (Nash, Luchka and Jhamandas, 1966, Severson and Sutter, 1969), whereas rabbit anterior mesenteric veins continue to respond for only five to ten minutes after calcium withdrawal (Severson and Sutter, 1969). Somlyo and Somlyo (1968b) suggest that this difference is due to a greater calcium permeability in the aorta.

## III ANTERIOR MESENTERIC PORTAL VEIN AS A MODEL OF ARTERIOLAR SMOOTH MUSCLE

Although a great amount of the research done on vascular smooth muscle has been done using aortic strips, it is well established that small arteries and arterioles are the vessels which control peripheral vascular resistance and which would most likely be implicated in hypertension.

For this reason, the emphasis in this laboratory has been placed on the investigation of spike producing vascular smooth muscle, and in particular the anterior mesenteric portal vein.

Steedman (1966) used micro-electrodes to measure spike potentials of arterioles *in vivo* in the rat mesentery. The average membrane potential was 39 millivolts with action potentials of up to 35 millivolts. She observed rhythmic slow wave depolarizations with spike potentials occuring at the peaks of the slow waves. Local administration of adrenaline, noradrenaline or vasopressin,and stimulation of the splanchnic nerves all produced an increased amplitude of slow waves and increased frequency of action potentials.

The anterior mesenteric portal vein exhibits essentially these same features both *in vitro* and *in situ*. Johansson and Ljung (1967) measured *in situ* responses of rabbit and cat portal veins to splanchnic nerve stimulation and to adrenergic amines. The veins contracted spontaneously about 6-8 times per minute. Application of noradrenaline either locally or intravenously produced an increase in portal vein tension. Splanchnic nerve stimulation also produced an increase in tension, an effect which could be inhibited by pretreatment with guanethidine (which should deplete catecholamine stores from nerve endings). Guanethidine had no effect on either spontaneous contractions or tension increases produced by noradrenaline; thus the contractions must be initiated by pacemaker cells in the smooth muscle layers.

In vitro studies on the anterior mesenteric vein were first carried out by Cuthbert and Sutter (1964). They showed spontaneous contractions accompanied in one case by spike-like action potentials.

Sutter (1965) did a more complete study of the effects of drugs on this preparation. Spontaneous activity was invariably present in both longitudinal and spiral preparations of the anterior mesenteric vein. Noradrenaline and adrenaline both produced strong contractions with a threshold of $10^{-8}$ g/ml and maximum response at $10^{-5}$ g/ml. These contractions were blocked by phentolamine or dihydroergotamine.

Isopropylnoradrenaline, a predominantly beta adrenergic agonist, inhibited spontaneous contractions at low doses ($10^{-7}$ g/ml) and produced an initial inhibition of spontaneous activity followed by a contraction at higher doses ($10^{-5}$ g/ml).

The isolated anterior mesenteric veins contracted in response to the addition of acetylcholine, serotonin, or angiotensin. The acetylcholine response was inhibited by atropine but the response to serotonin could not be blocked by either methysergide or morphine.

Following these initial observations which indicated that the isolated anterior mesenteric portal vein would provide a model system for studying an arteriolar-like tissue, several investigators have tried to correlate changes in contractile activity produced by drugs or altered ionic composition with changes in membrane potential.

The spontaneous activity of the portal vein in normal Kreb's solution is characterized by rhythmical phasic contractions, each of which is slightly preceded by a burst of action potentials (Cuthbert and Sutter, 1965, Axelsson et al., 1967, Holman et al., 1968). If the calcium concentration in the bath is not altered, there is a good correlation between spontaneous electrical and mechanical activities under most experimental conditions.

Holman et al. (1968) showed that spontaneous activity ceases below 28° C and that raising the temperature in the range of 28°-38° C resulted in parallel increases in the frequency of action potentials and associated contractions. Axelsson et al. (1967) showed an initial increase in both burst frequency and contraction in potassium-free solution, followed by a co-ordinated decrease in both. Substances which inhibit spontaneous contractions of the vein also inhibit action potentials. This effect was shown by Cuthbert and Sutter (1965) using theophylline and isopropylnoradrenaline, and by Rhodes and Sutter (1969) using diazoxide.

It appears, therefore, to be impossible to dissociate spontaneous electrical activity and tension changes if the calcium concentration in the bathing medium is not altered. Axelsson et al. (1967) showed, however, that upon increasing the potassium concentration in calcium-free medium, spontaneous action potentials could be recorded without any accompanying changes in tension.

The correlation between changes in electrical activity and tension due to the addition of drugs is not so good, however. Cuthbert and Sutter (1965) found that application of noradrenaline to vein strips produced an initial increase in spike activity correlated with an increase in tension. However, after a few minutes, the electrical activity returned to normal while the tension remained elevated. Even more striking is the fact that noradrenaline and other agonists can still produce a contraction when the vein is in a depolarized state, produced either by a potassium rich solution (Edman and Schild, 1962) or by ouabain (Mathews and Sutter, 1967). Somlyo and Somlyo (1968a)

have coined the term pharmaco-mechanical coupling for contractions produced by drugs, which do not require the additional step of membrane depolarization.

Their theory of pharmaco-mechanical coupling may be summarized as follows:

(i) The interaction of drug with membrane produces an increase in calcium permeability, perhaps due to the release of membrane bound calcium.

(ii) The increased influx of calcium will produce both a depolarization and contraction under normal conditions, but if the cell is already depolarized the increased influx of calcium still produces a contraction.

Thus Somlyo and Somlyo believe that excitatory drugs produce parallel changes in electrical and mechanical activities in smooth muscle, rather than the series depolarization-contraction linkage found in skeletal and cardiac muscles. This interesting hypothesis will be delved into more deeply in the Discussion.

## Statement of the Problem

In order to understand the molecular basis for hypertension, it will be necessary to discover the means by which arteriolar resistance is controlled, i.e. how is the tension produced by arteriolar smooth muscle regulated?

We are using the isolated anterior mesenteric portal vein as a model system since *in vivo* it has many physiological and pharmacological properties similar to arterioles (see Introduction, Anterior Mesenteric Portal Vein . . .), and in this regard can provide far more relevant data than can the thoracic aorta. Experiments were carried out to try to determine the source(s) of calcium ions required for contractions of the anterior mesenteric portal vein to various agonists, and the means by which membrane permeability to calcium is controlled. The experiments were of four main designs:

1. The effects of varying the extracellular calcium on both the time course and magnitude of contractions produced by various agonists.

Although it is well established that no contractions can be elicited in calcium-free solution (in the anterior mesenteric vein), there is little quantitative information relating contractile tension to extracellular calcium concentration. The relative importance of extracellular calcium for the initiation of contractions produced by various agonists is also not known.

Contractions of the mesenteric portal vein produced by most agonists are not phasic, and indeed may last for more than an hour.

Does the calcium which initiates the contraction remain in the cytoplasm to allow the maintenance of contraction or is there a continuous turnover of calcium ions from source to sink? The effect of lowering the extracellular calcium on the sustained phase of the contraction was also tested. If there is no turnover of calcium ions during this phase then removing the extracellular calcium should have no effect.

2. The effects of adding manganese chloride (or lanthanum chloride) to the bathing medium on the spontaneous activity and the responses to agonists.

If indeed the influx of calcium ions is necessary for the initiation and/or maintenance of contraction of the vein, then the addition of an inhibitor of calcium influx should inhibit the initiation of the contractile response and/or relax a vein which is already contracting.

Sabatini-Smith and Holland (1969) showed that 0.1 mM manganese reduced the uptake of $^{45}Ca^{++}$ by isolated rabbit atria to 25% of control. Hagiwara and Nakijimo (1966) and Bulbring and Tomita (1968) showed that manganese could block action potentials in barnacle muscle and guinea pig taenia coli respectively. Calcium ions are believed to be the chief current carrying species for the action potentials in both these muscles.

Van Breemen (1969) showed that lanthanum chloride blocked the movement of $^{45}Ca^{++}$ across an artificial phospholipid-cholesterol membrane and also across the squid giant axon (Van Breemen and de Weer, 1970).

Thus the use of manganese or lanthanum as an inhibitor of calcium influx might also help to show the importance of extracellular calcium for the initiation and/or maintenance of contractions.

3. The effects of removing some of the bound calcium using EGTA.

Frankenhaeuser and Hodgkin (1957) showed that increasing calcium concentration decreased sodium permeability of the squid giant axon and shifted the curve of rate of rise of action potential versus membrane potential such that a greater depolarization was required to produce the same rate of rise. They referred to this as a stabilizing effect of calcium. Many people have suggested a similar role for calcium in smooth muscle, and Somlyo and Somlyo (1968) have even suggested (without evidence) that calcium may control its own permeability.

It may be possible to show the existence of this "stabilizer calcium" by treating the tissues with EGTA; the permeability characteristics of the membrane should be greatly changed if indeed stabilizer calcium has been removed. This change in membrane permeability could be shown by the addition of various divalent cations. If the addition of calcium for example, produced a different effect after EGTA treatment then it would without EGTA treatment, then it is probable that the removal of bound calcium with EGTA has directly or indirectly "destabilized" the membrane.

4. The effects of altering pH and sodium concentration.

Although it has been shown that lowering the extracellular sodium concentration from 143 mM to 25 mM has very little effect

on the amount of tension produced by noradrenaline or KCl (Biamino and Johansson, 1970; Sutter, unpublished results), it was thought that lowering the sodium concentration might have an effect on the responses of calcium depleted tissues (see #3 above). An interaction between sodium and calcium fluxes was first reported by Baker et al. (1969) with squid giant axon and later by Glitsch et al. (1970) with guinea-pig atria. Both the influx and efflux of calcium appeared to be linked with a movement of sodium ions in the opposite direction.

If the influx of calcium in the vein is indeed carrier-mediated (as postulated by Glitsch for the guinea-pig atria), then decreasing the pH should decrease the affinity of the carrier for calcium and hence inhibit contractile responses if calcium influx is necessary for contractions. The experiments with altered pH were done strictly as a preliminary study since it is impossible from this simple approach to determine the site(s) which is (are) affected by altering the pH.

# METHODS AND MATERIALS

## I. MATERIALS

### A. Tissue Preparation

New Zealand white rabbits of either sex and weighing from 2.0 to 3.5 kg. were killed by a blow to the back of the neck. The abdominal wall was cut open, and after removing as much connective tissue as possible, the anterior mesenteric portal vein was removed from the point of entry into the liver, to the branching of the anterior mesenteric vein. The vein was placed in a petri dish containing modified Kreb's solution (see section I. B) and any remaining connective tissue was dissected free. For organ bath experiments the vein was bisected longitudinally, but for sucrose gap experiments the vein was simply cut longitudinally to form a flat sheet.

### B. Physiological Salt Solutions

Since many of the experiments involved the addition of $MnCl_2$ or extra $CaCl_2$ which would precipitate as insoluble phosphates in ordinary Kreb's solution, the following solution lacking phosphate was routinely used.

(1) Modified Kreb's Solutions

NaCl, 118 mM; KCl, 4.7 mM; $CaCl_2$, 2.6 mM; $MgCl_2$, 1.1 mM; $NaHCO_3$, 25 mM; glucose, 5.6 mM.

The solution was bubbled with 95% $O_2$-5% $CO_2$ and adjusted to pH 7.4 with HCl.

(2) Bicarbonate-free Kreb's Solution

(This solution was used in experiments with $LaCl_3$ to prevent $La_2(CO_3)_2$ precipitation). Same composition as modified Kreb's solution except that the 25 mM $NaHCO_3$ was replaced with 25 mM Tris(hydroxymethyl)aminomethane.

The solution was bubbled with 100% $O_2$ and adjusted to pH 7.4 with HCl.

(3) 16% Sodium Solution

Same composition as modified Kreb's except the 118 mM NaCl was replaced by 118 mM Tris-chloride.

The solution was bubbled with 95% $O_2$-5% $CO_2$ and adjusted to pH 7.4 with HCl.

(4) Sodium-free Kreb's solution

Same composition as modified Kreb's except the 118 mM NaCl and 25 mM $NaHCO_3$ were replaced with 143 mM Tris-chloride.

The solution was bubbled with 100% $O_2$ and adjusted to pH 7.4 with HCl.

The physiological salt solutions were made up in large quantities without glucose and then the glucose (1.0 g/l) was added on the day of the experiment.

C. Drug Preparations

Stock solutions of drugs were prepared using distilled water or by using procedures outlined in the Merck Index of Chemicals and Drugs.

Drugs were obtained as follows:

1. L-Noradrenaline-D-bitartrate monohydrate, Mann Research Laboratories.
2. Phentolamine, Ciba.
3. Ouabain, Mann Research Laboratories.
4. Serotonin creatinine sulphate monohydrate, Mann Research Laboratories.
5. Procaine Hydrochloride, K and K Laboratories.
6. Tetrodotoxin, Sankyo.
7. EGTA (ether glycol bis-amino tetraacetic acid), Geigy, Sigma.
8. EDTA (ethylene diamine tetraacetic acid), Fisher Scientific.

All drug concentrations are expressed in terms of the final concentration of free base.

D. Apparatus

1. Organ Bath

The apparatus is shown in Figure 1. It is a standard 18 ml muscle bath obtained from Glass Appliances Ltd., Aberdeen Scotland. The warming coil also has an 18 ml capacity so that a complete change of bathing fluid can be effected without altering temperature. The temperature of the bathing fluid was maintained at $37^{o}$ C by a continual circulation of warm water through the water jacket from a thermostatically controlled water pump. Aeration was accomplished by passing a small diameter polyethylene tube through the bottom drain tube and into the bottom of the central compartment. The end of the tubing was crushed with pliers and then pierced with a needle to produce a fine flow of bubbles over the tissue.

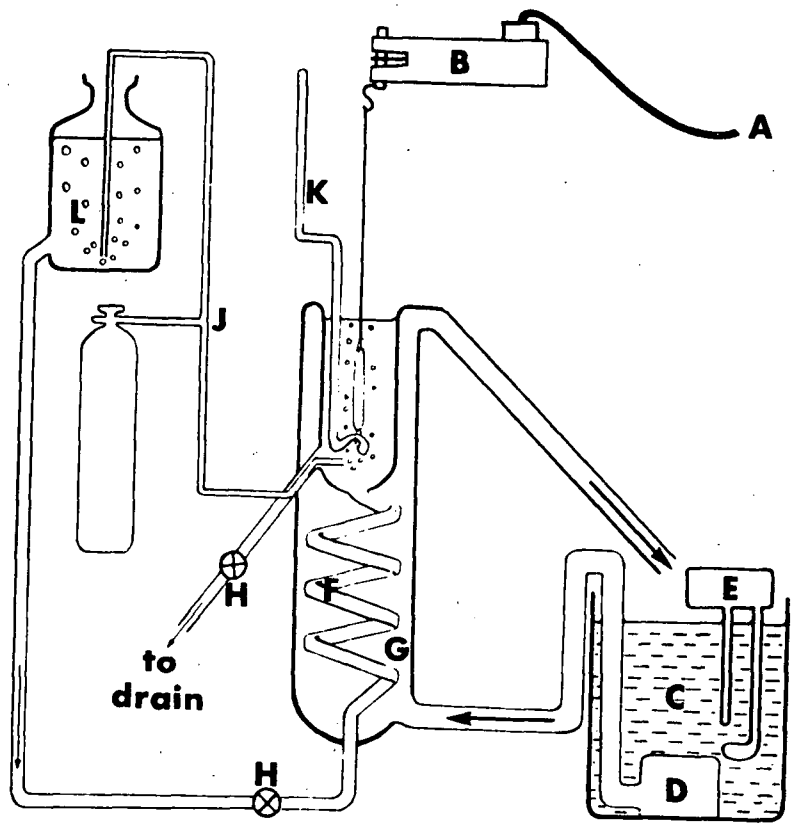


FIGURE 1

Organ Bath Apparatus

A Grass Model 7 Polygraph

B Grass Model FT 03C Force Transducer

C Water reservoir

D Water pump

E Heater

F Pre-warming coil

G Water jacket

H Stopcocks

J Aeration apparatus

K Support rod for tissues

L Bathing solution

The tissue was anchored at the bottom by a loop of surgical thread passed through the tissue and over a glass hook. The other end was connected to a Grass FT03C force displacement transducer which in turn was connected to a Grass Model 7 Polygraph. The isometric contractions produced by veins in four separate organ baths were measured simultaneously by this method. Tension on the tissue could be altered by raising or lowering the force transducer using a rack and pinion. Resting tension was maintained at 500 mg. throughout the experiment by this method.

Drugs were added to the bath in volumes of 0.18 ml or 0.36 ml using a 1 ml tuberculin syringe with a #22 needle. Drugs were usually washed out by emptying the bath from the lower drain tube and refilling; this technique however produced an artifact in the tension record which could be eliminated by using an overflow washing technique. The tissue remained viable for at least eight hours when kept at $37^{o}$ C in well oxygenated modified Kreb's solution.

2. Sucrose Gap Apparatus

This apparatus consisted of a horizontal three-chambered bath made of lucite plastic, and allowed measurement of electrical activity and tension simultaneously. The tissue was attached by a loop of thread to a hook in the right chamber, then passed through the center chamber and then attached via a lucite plastic lever in the left chamber to a Grass FT03C force displacement transducer. The diffusion of ions between chambers was prevented by using rubber membranes placed on either side of the central chamber. 300 mM sucrose solution

(previously demineralized with Rexyn I-300 (Fisher Scientific) flowed slowly through the center compartment which kept the outer baths electrically isolated from each other. The portion of tissue in the right compartment was depolarized by crushing with forceps or by bathing it in 100 mM $K_2SO_4$ solution. The silver-silver chloride recording electrodes were made from 0.006 inch diameter silver wire, encased except for an 0.5 cm tip within a glass tube sealed with Pyseal cement (Fisher Scientific). The exposed tip was chlorided electrolytically using 100 mM KCl. The electrodes were fixed in place, one in each of the outer compartments, and were adjusted so that the electrode tips made direct contact with the tissue. The electrodes and the force transducer were connected to a Grass Model 7 Polygraph.

If the ionic composition of the left chamber was altered, a change in electrical potential between the electrodes could be measured which was observed even in the absence of living tissue. This change in electrical potential is due to an alteration of the chemical activity of the ions involved in the silver-silver chloride electrode. To avoid this non-specific change in potential, the apparatus was designed so that any experimental compounds ($MnCl_2$ for example) could be added to both outer compartments simultaneously.

The tissue was maintained at 37° C by water circulating through tubing wrapped around the bath, or in a later design through channels which were an integral part of the apparatus. Both outer compartments were bubbled with 95% $O_2$-5% $CO_2$ which also served to promote mixing of solutions.

## II METHODS

### A. Experiments in Normal Kreb's Solution

Non cumulative dose response curves were obtained from noradrenaline ($10^{-9}$ to $10^{-5}$ g/ml), serotonin ($10^{-8}$ to $10^{-4}$ g/ml), procaine ($10^{-5}$ to $5 \times 10^{-3}$ g/ml) and for KCl (20-80 mM final concentration). The usual procedure involved starting with the threshold dose, allowing the tension to reach maximum, then washing and allowing at least five minutes after return to basal tension before challenging with the next higher dose. Occasionally the order of dosing was randomized to show that the response depended only on the concentration of drug, not on the order in which the dose-response curve was obtained.

### B. Experiments to study the interaction of extracellular calcium concentration and the responses to agonists.

Similar non-cumulative dose response curves were obtained using Kreb's solution with the calcium concentration varied from less than $10^{-5}$ M to $2 \times 10^{-2}$ M. The lowest calcium concentration was achieved by adding $10^{-4}$ M EGTA (ether glycol bis-amino tetraacetic acid) to the Kreb's solution (zero calcium Kreb's). The tissues were equilibrated for 30 minutes before testing, and then challenged with two test doses of agonist before the actual dose-response curve was obtained.

A second type of experiment was done to study the importance of extracellular calcium. First the tissues were soaked in calcium-free

solution until no contractions could be elicited, then one of the following procedures was followed:

(1) Calcium chloride solution was added to each bath (final concentration 0.1 mM to 10.0 mM) and the tissues were allowed to equilibrate for 5 minutes. The excitatory agent to be studied was then added and the tension produced was recorded.

(2) The excitatory agent was added to the tissues while they were still in calcium-free solution. Calcium chloride solution was then added to initiate a contraction.

These two procedures were followed with noradrenaline and KCl using several calcium concentrations to determine if the order of addition had any effect on the tension produced.

C. Experiments using Manganese chloride or Lanthanum chloride

The protocol for obtaining dose-response curves in the presence of manganese or lanthanum was as follows:

(1) control response without inhibitor ($Mn^{++}$ or $La^{+++}$)

(2) response to same dose of agonist after equilibrating for three minutes in the presence of manganese or lanthanum

(3) second control response to show reversibility

This same procedure was followed with each concentration of agonist. The experiments using Lanthanum chloride required bicarbonate free Kreb's solution to prevent Lanthanum carbonate precipitation.

A second type of experiment using Manganese involved producing a contraction to an agonist in normal Kreb's solution, then adding

manganese (0.5-2.0 mM final concentration) and measuring the rate of relaxation. Experiments using manganese were routinely performed in the presence of $10^{-4}$ M ascorbic acid to prevent manganese catalyzed oxidation of noradrenaline to noradrenachrome.

D. Experiments involving chelation of bound calcium with EGTA

The tissues were bathed in zero calcium Kreb's solution containing 1.0 mM EGTA for 10 minutes. After 10 minutes the tissues were washed twice with zero calcium Kreb's solution (containing 0.1 mM EGTA). The effects of adding various divalent cations to the calcium depleted tissues were then tested. Following this, the cycle of bathing in 1.0 mM EGTA and washing with zero calcium Kreb's solution was repeated before testing another cation solution.

The effect of varying the concentration of EGTA in the solutions was tested and it was found that raising the concentration from 1.0 to 5.0 mM had no effect on the responses to cations. It was also found that varying the EGTA in the washing solution from 0 to 1.0 mM had no effect on the responses. For this reason the standard procedure as given above used 1.0 mM EGTA for 10 minutes followed by two washing with zero calcium solution containing 0.1 mM EGTA.

In the experiments which tested the inhibitory effect of manganese or lanthanum, these inhibitors were added after the washings with zero calcium solution; the tissues were allowed to equilibrate with the inhibitor for three minutes before adding the test solution of calcium or strontium.

E. Experiments involving altered pH

The tissues were equilibrated in the appropriate solution for 10 minutes before adding the agonist. The pH of the bathing solution was checked (if pH was the variable) by placing a micro pH electrode directly into the bath after the agonist had been added. Each agonist was tested several times with similar bathing solution until a consistent contraction was produced.

F. Experiments involving zero sodium solution

The tissues were equilibrated for two hours in zero sodium Kreb's before responses to agonists were tested.

# RESULTS

## I. EFFECTS OF ALTERING THE EXTERNAL CALCIUM CONCENTRATION

The maintenance of spontaneous activity in the rabbit portal vein is very dependent upon extracellular calcium. Within five minutes of exposure to calcium-free solution no further spontaneous tension changes occur, and this correlates with a marked decrease in electrical activity of the membrane as measured by the sucrose gap method. (Similar results were found by Cuthbert and Sutter, 1965.)

Spontaneous activity can be halted even more rapidly by the addition of the calcium chelating agent EGTA to the bathing medium. Figure 2 shows the result of adding sufficient EGTA to chelate all of the extracellular calcium. Spontaneous activity ceased within two minutes, but could be restored to normal by the addition of excess calcium (2(A)). Figure 2(B) shows that the addition of excess $MgCl_2$ did not restore spontaneous activity, and therefore it is the specific chelation of calcium ions by the EGTA which has stopped spontaneous activity. The addition of $SrCl_2$ (2(C)) produced an increase in basal tension with superimposed spontaneous activity.

Results similar to those shown in Figure 2 can be obtained by exposing the tissues to calcium-free solution without EGTA for 5 minutes and then adding the various divalent cations. However, under these conditions the return of spontaneous activity after readdition of calcium takes 10-15 minutes, whereas the addition of strontium causes an immediate return of activity similar to that

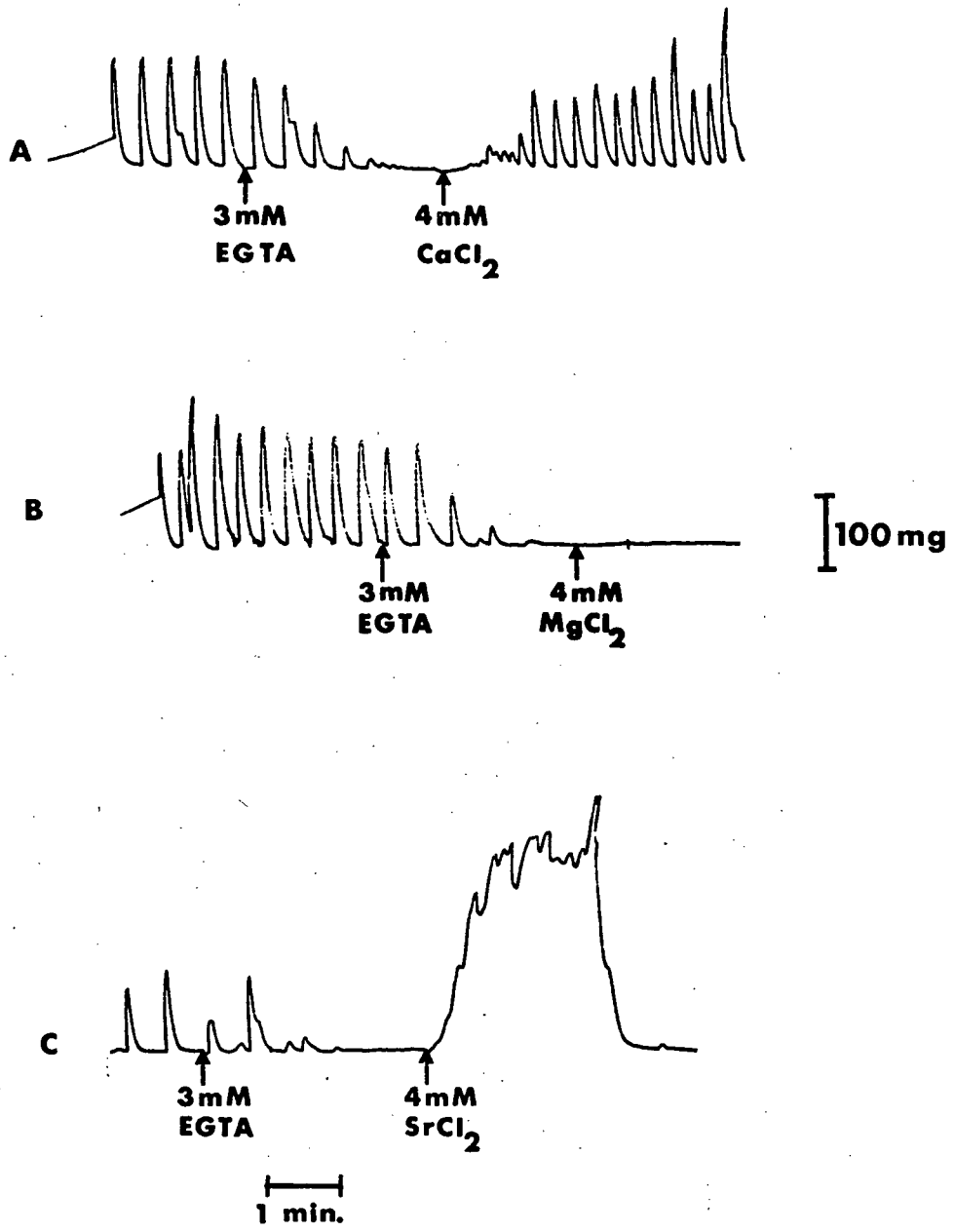


FIGURE 2

The effect of EGTA upon spontaneous activity of the anterior mesenteric portal vein.

Concentration of free calcium in the solutions before addition of EGTA was 2.5 mM

A. Readdition of $CaCl_2$ reversed the effect of EGTA and allowed return of normal spontaneous activity.
B. The addition of $MgCl_2$ had no effect.
C. Addition of $SrCl_2$ produced a large contraction.

shown in Figure 2(C).

In order to try to determine the importance of bound calcium for drug induced contraction, the effects of lowering the external calcium concentration on responses to agonists were studied.

After fifteen minutes in calcium-free solution none of the excitatory agents tested (noradrenaline, serotonin, histamine, procaine, potassium chloride) were able to produce a contraction even when given in what would normally be supramaximal doses.

The effect of lowering the calcium concentration of the bathing medium upon the dose response curves to noradrenaline is shown in Figure 3. The response to noradrenaline is limited by the extracellular calcium concentration up to 2.5 mM $Ca^{++}$. If the calcium concentration is raised to 10 mM or greater (not illustrated) then the responses to noradrenaline are actually decreased relative to the response in 2.5 mM calcium. The dose response curves to all agonists tested were affected in the same general manner by alteration of the external calcium concentration.

It appears therefore that the smooth muscle cells of the portal vein are very dependent on extracellular or rapidly exchangeable bound calcium for the initiation of a contraction elicited in any manner.

Figure 4 shows the effect of lowering external calcium on the time course of the responses to $10^{-5}$ g/ml noradrenaline. In all cases the tissues were bathed in calcium free medium until no response to noradrenaline could be obtained. The dashed lines represent the responses to $10^{-5}$ g/ml noradrenaline obtained after 5 minutes

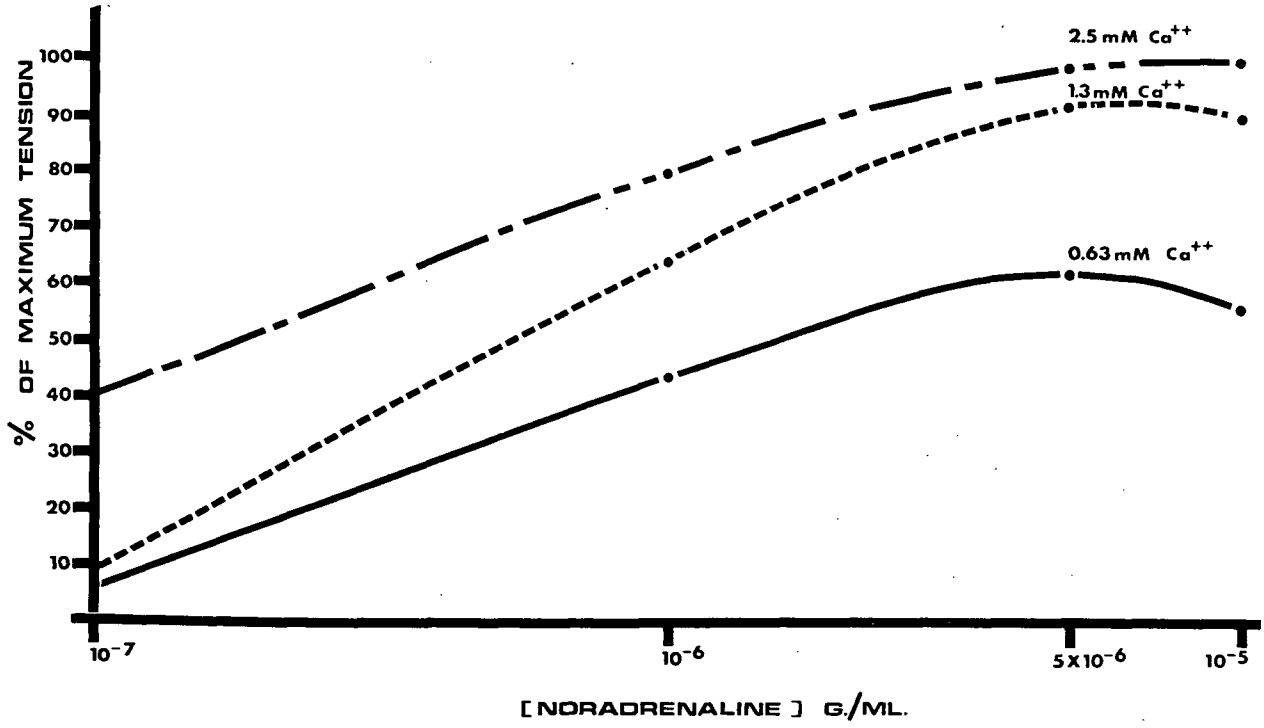


FIGURE 3

Effects of lowering the calcium concentration in the Kreb's solution upon the dose response curve to noradrenaline.

The response of each vein strip to $10^{-5}$ g/ml noradrenaline in 2.5 mM $Ca^{++}$ was set equal to 100 per cent. The strips were equilibrated for 30 minutes in each solution and then challenged with $10^{-6}$ g/ml noradrenaline until a constant contraction was produced before the dose response curve was obtained.

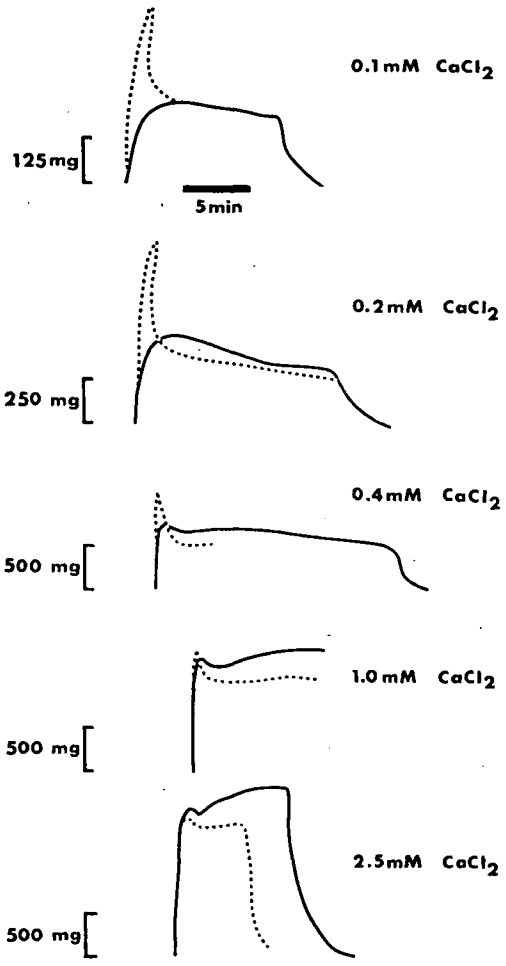


FIGURE 4

Time course of contractions to $10^{-5}$ g/ml noradrenaline with various extracellular calcium concentrations.

Each vein strip was washed in calcium-free medium until no contraction was obtained to noradrenaline. The dotted traces represent responses to noradrenaline after 5 minutes equilibration with Kreb's solution containing the calcium concentration indicated. The solid traces represent the responses obtained by first adding noradrenaline to calcium-free solution and then suddenly increasing the calcium concentration by adding concentrated $CaCl_2$ solution to the bath via a syringe.

equilibration in bathing medium containing calcium concentrations as indicated. The solid lines represent the responses obtained by adding calcium to tissues bathed in calcium-free solution with $10^{-5}$ g/ml noradrenaline already in the bathing medium. The only difference between these two procedures is the sequence in which the calcium and noradrenaline were added.

The greatest effect of calcium pre-incubation was seen at very low calcium concentrations (0.1 mM and 0.2 mM). At these concentrations of calcium, addition of $10^{-5}$ g/ml noradrenaline produced a biphasic response; a phasic response of short duration, followed by a tonic contraction of decreased magnitude. Without pre-incubation with calcium, the phasic response was totally eliminated at these low calcium concentrations. Increasing the external calcium concentration had a greater effect on the tonic response than on the phasic response and at concentrations greater than 1 mM $Ca^{++}$, the tonic response was larger than the initial response. With increased calcium concentration, the differential effect of pre-incubation with calcium also disappeared.

Figure 5 shows the effects of lowering calcium upon the responses to $10^{-7}$ g/ml noradrenaline. The tissues had been washed with calcium-free solution until no response was produced by $10^{-6}$ g/ml noradrenaline. The dashed lines again represent the responses to noradrenaline after 5 minutes pre-incubation with the calcium concentration as indicated, and the solid lines represent the responses obtained by adding the noradrenaline before the calcium. With this low dose of noradrenaline there was no definite two phase response, and there was little difference

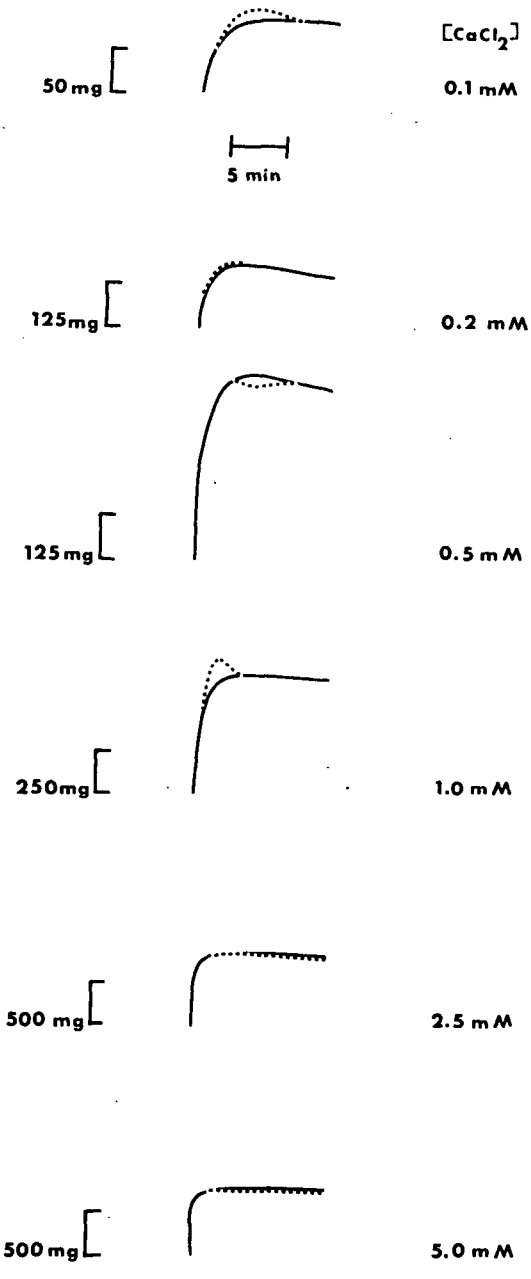


FIGURE 5

Time course of contractions to $10^{-7}$ g/ml noradrenaline with various extracellular calcium concentrations. Experimental details as in Figure 4.

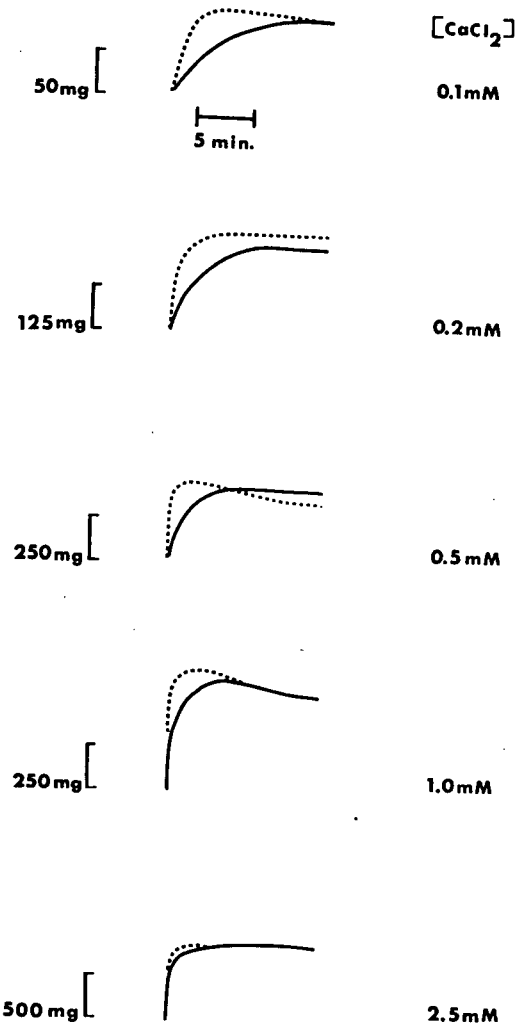


FIGURE 6

Time course of contractions to 80 mM KCl with various extracellular calcium concentrations. Experimental details as in Figure 4.

in the responses whether the tissues are pre-incubated with calcium or not.

The results from similar experiments using 80 mM KCl as the agent producing the contractile response are shown in Figure 6. Although the responses to 80 mM KCl cannot be easily separated into phasic and tonic components, the 5 minute pre-incubation with calcium before addition of KCl resulted in a greater rate of tension production, (even though the same final tension could be produced if the KCl solution is added before the calcium). Very similar results were obtained using a lower concentration of KCl (40 mM) although of course the tension produced was much smaller.

Figures 7(A) and 7(B) show the effects of altering extracellular calcium on both the phasic and tonic responses to $10^{-5}$ g/ml noradrenaline. Each strip was placed in calcium-free medium until no response to noradrenaline could be measured, and then incubated for five minutes with the indicated calcium concentration before testing with noradrenaline. Each point represents the average response, and the bars represent the standard deviations of eight vein strips. The response of each vein in 1.0 mM $Ca^{++}$ was considered to be 100 per cent for the purpose of these graphs. The phasic response (7(A)) is proportional to the logarithm of the calcium concentration (up to 1.0 mM $Ca^{++}$) whereas the tonic response (7(B)) is linearly proportional to calcium concentration within this same range. The tonic response becomes equal to magnitude to the phasic response when the extracellular calcium concentration is between 0.8 mM and 1.0 mM. When the calcium concentration is raised further

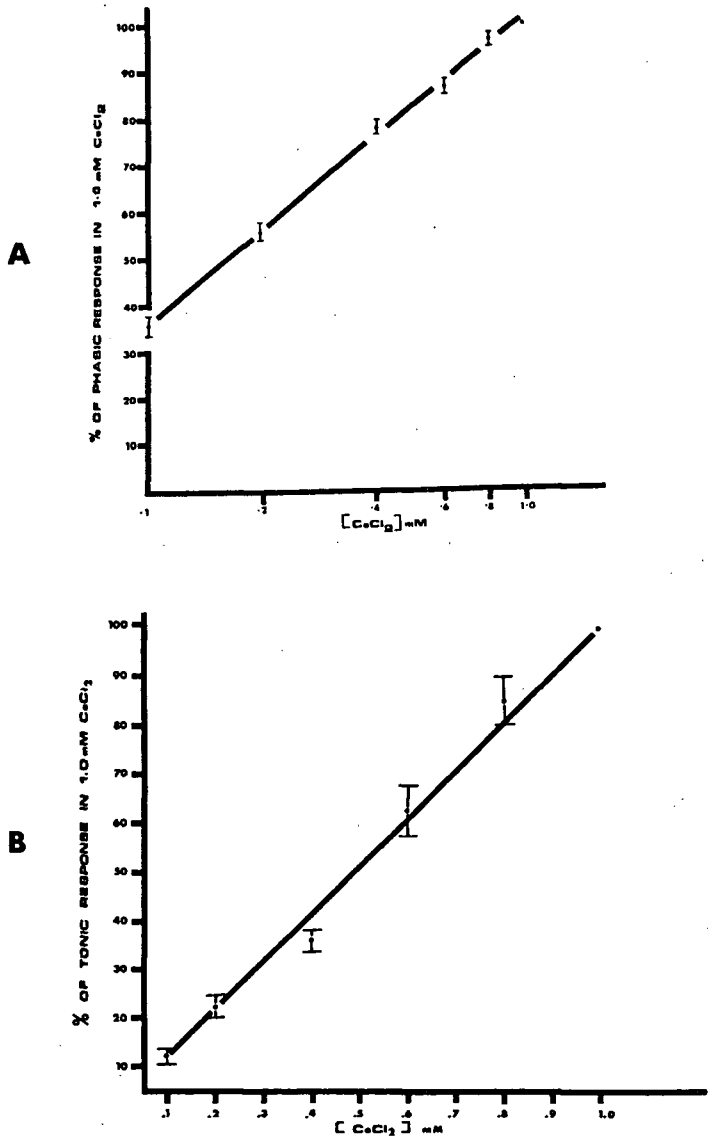


FIGURE 7

Effects of lowering extracellular calcium on the two phases of a contraction to $10^{-5}$ g/ml noradrenaline.

The strips were washed in calcium-free solution and then equilibrated with a given calcium concentration for 5 minutes before testing with noradrenaline.

A Effect of low calcium on the phasic contraction

B Effect on the tonic portion of the biphasic contraction (tension remaining 15 minutes after addition of noradrenaline)

The vertical bars indicate the standard errors of the mean (8 vein strips).

it is difficult to separate the response to noradrenaline into two distinct phases.

The effects of lowering external calcium on the phasic responses to various doses of noradrenaline are shown in Figure 8. The tissues were equilibrated for 5 minutes in each of the solutions containing a particular amount of calcium before testing with noradrenaline. The maximum response obtained at each dose of noradrenaline was set equal to 100 per cent, therefore the curves represent relative effects of lowering external calcium on the response to each dose of noradrenaline. When the external calcium concentration was less than 0.5 mM, the responses to low doses of noradrenaline were greatly reduced, whereas the responses to high doses of noradrenaline were less affected.

In the experiments shown in Figure 8, the addition of noradrenaline initiated the responses. However, if the contractile response produced by noradrenaline is due simply to increased permeability to calcium, then it should not matter in which order the noradrenaline and calcium are added, i.e. whether the addition of noradrenaline or calcium actually initiates the contraction.

Figure 9 shows however that if noradrenaline was added to calcium-free solution and then calcium was added later, the differential effect of decreasing extracellular calcium on the responses to various doses of noradrenaline (as seen in Figure 8) did not occur (under these conditions). The curves of response versus calcium concentration overlap for doses of noradrenaline from $10^{-7}$ g/ml to $10^{-5}$ g/ml. Thus the order of calcium and

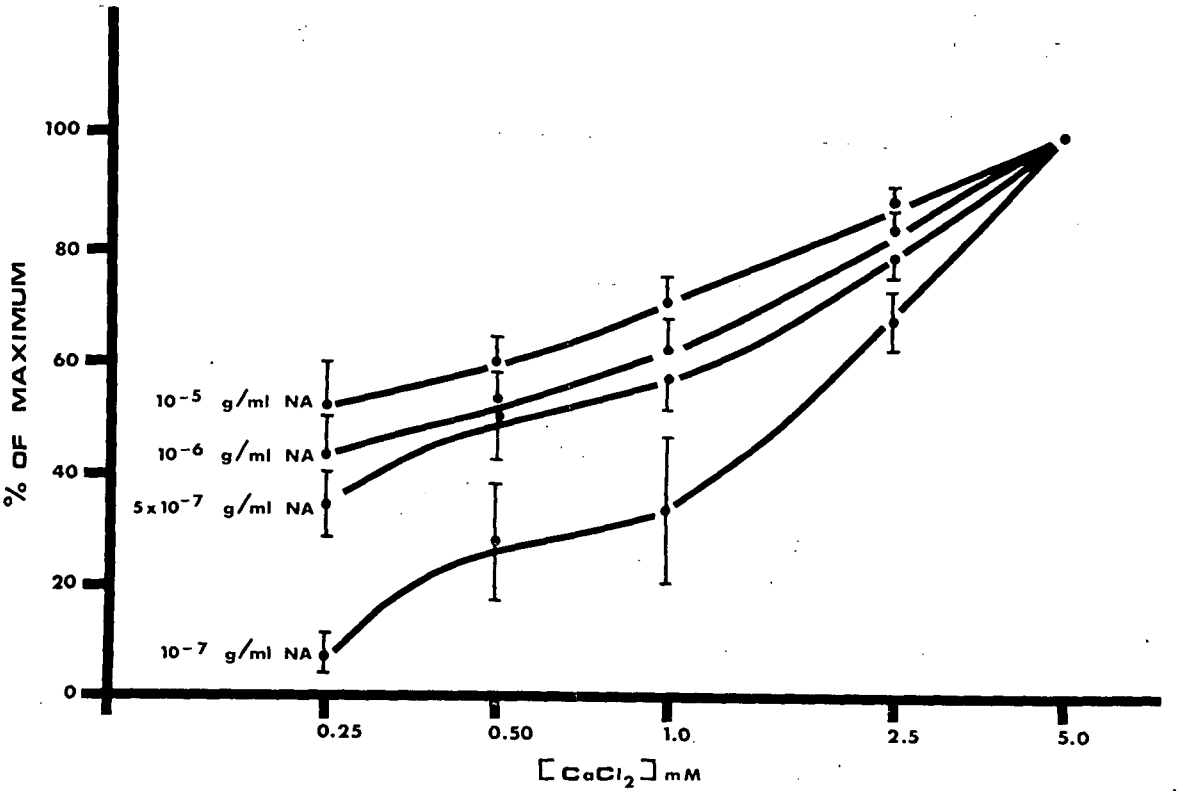


FIGURE 8

Effect of varying extracellular calcium concentration on the phasic responses to various doses of noradrenaline.

Each strip was washed in calcium-free solution until no further contraction could be elicited by noradrenaline. $CaCl_2$ solution was added to the bath to give a final concentration of $Ca^{++}$ as indicated. After 5 minutes equilibration the strip was tested with one of the doses of noradrenaline. The strip was then washed in calcium-free medium and re-equilibrated with calcium before testing with the next dose of noradrenaline.

Each point represents the mean of 12 vein strips (6 animals). The vertical bars represent the standard errors of the means.

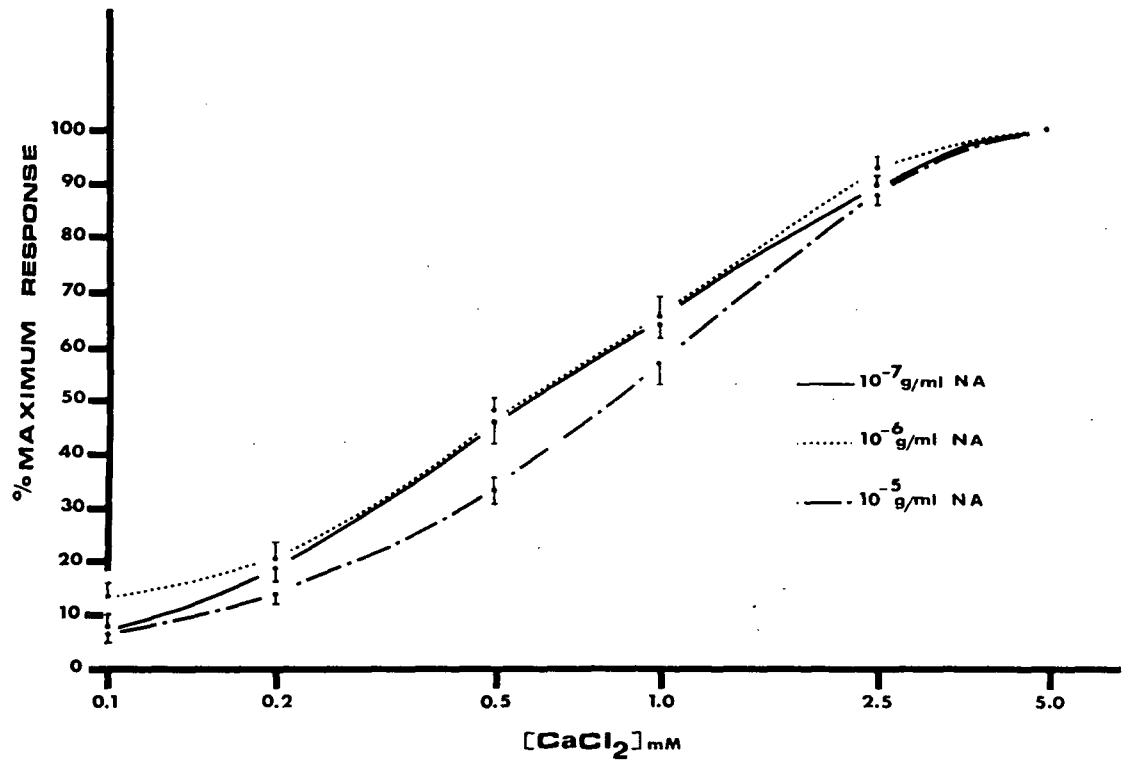


FIGURE 9

Effects of varying extracellular calcium on noradrenaline contractions when the tissues were not pre-equilibrated with calcium.

The tissues were washed in calcium-free solution until no contraction could be elicited by noradrenaline. Noradrenaline was then added. The contractions were produced by the addition of $CaCl_2$ solution to achieve a final calcium concentration as indicated.

noradrenaline addition is important, and therefore a simple increase in calcium permeability cannot be total responsible for the initiation of a contraction produced by noradrenaline.

Additional effects (upon noradrenaline responses) of altering the concentration of calcium in the bathing medium are shown in Figures 10 and 11. Figure 10(A) shows the typical response to $10^{-5}$ g/ml noradrenaline in 2.5 mM $Ca^{++}$. This concentration of noradrenaline produced a maximum response. When the calcium concentration was raised to 12.5 mM (10(B)), the response to noradrenaline was reduced a small but consistent amount. If however the calcium concentration was raised after the addition of noradrenaline, a supra-maximal response was obtained. These results are consistent with the effects of raised calcium concentration upon spontaneous activity, i.e. when the calcium concentration was greater than 7.5 mM, spontaneous activity was abolished. This effect could be reversed by chelating some of the calcium with EGTA, or by returning to Kreb's solution containing 2.5 mM $Ca^{++}$.

The supramaximal response obtained by raising the extracellular calcium concentration during the tonic portion of a noradrenaline contraction could also be reversed by simply chelating the extra calcium with EGTA (Figure 11).

Although the responses to all agonists are rapidly abolished in calcium-free solution, one might be able to learn something of the utilization of different calcium pools by examining the shapes of response versus calcium concentration curves for various agonists. Figure 12 compares the relative responses to $10^{-6}$ g/ml noradrenaline

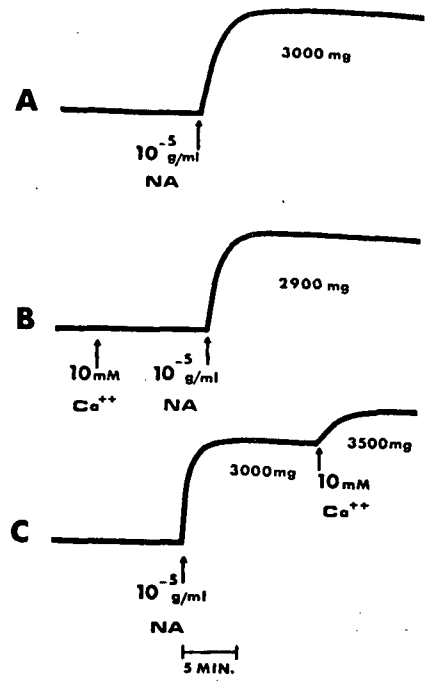


FIGURE 10

Effects of excess calcium on contractions to $10^{-5}$ g/ml noradrenaline.

A Contraction produced by $10^{-5}$ g/ml noradrenaline in normal Kreb's solution. (2.5 mM $Ca^{++}$)

B Slight depressant effect of raising the calcium concentration to 12.5 mM before adding noradrenaline.

C Supramaximal response produced by increasing the calcium concentration after the strip has reached peak tension in normal Kreb's solution.

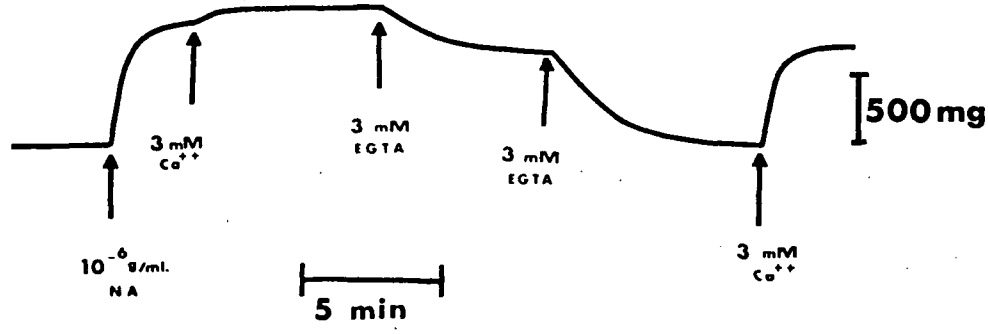


FIGURE 11

Effects of altering the extracellular calcium concentration on the tonic portion of a maximum response to noradrenaline.

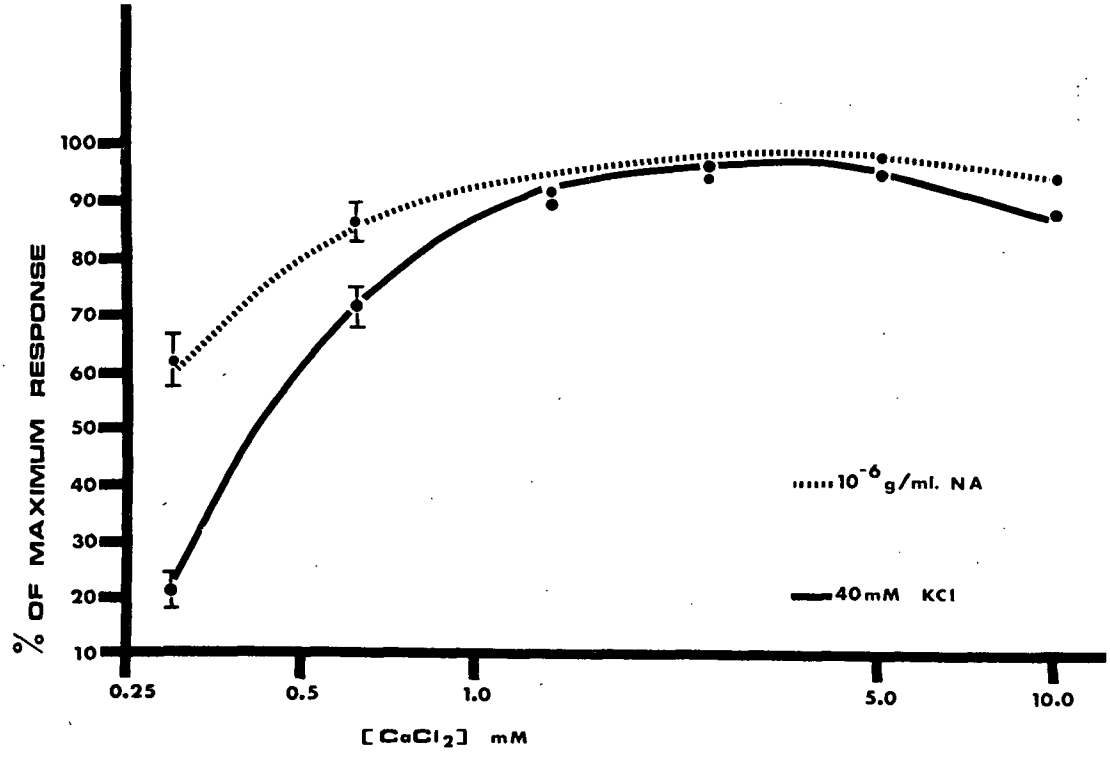


FIGURE 12

Comparison of the effects of lowering extracellular calcium concentration on the responses to $10^{-6}$ g/ml noradrenaline and 40 mM KCl.

The maximum response produced by each agonist is set equal to 100 per cent.

and 40 mM KCl with varying extracellular calcium. The maximum response obtained with each agonist was set equal to 100 per cent. Thus when the extracellular calcium concentration was below 0.5 mM, the response to 40 mM KCl is significantly reduced relative to the response to $10^{-6}$ g/ml noradrenaline. However, these particular concentrations of noradrenaline and KCl do not produce equal contractions in normal Kreb's solution (2.5 mM $Ca^{++}$). For this reason similar experiments were carried out using various concentrations of both excitatory agents (Figure 13). This figure shows that the differential effect of lowering calcium on the responses to $10^{-6}$ g/ml noradrenaline and 40 mM KCl was related to the relative potencies of the drug concentrations used rather than differences in the mechanism of action of the two agents. In fact the relative dependency on extracellular calcium shown in Figure 13 has exactly the same rank order as the maximum contractions produced by the various drug concentrations; i.e. the greater the maximum contraction produced by a given concentration of drug, the less effect lowering external calcium had on the responses to that particular concentration of drug. This parallel between drug potency, and dependence upon extracellular calcium, also includes responses to serotonin and procaine.

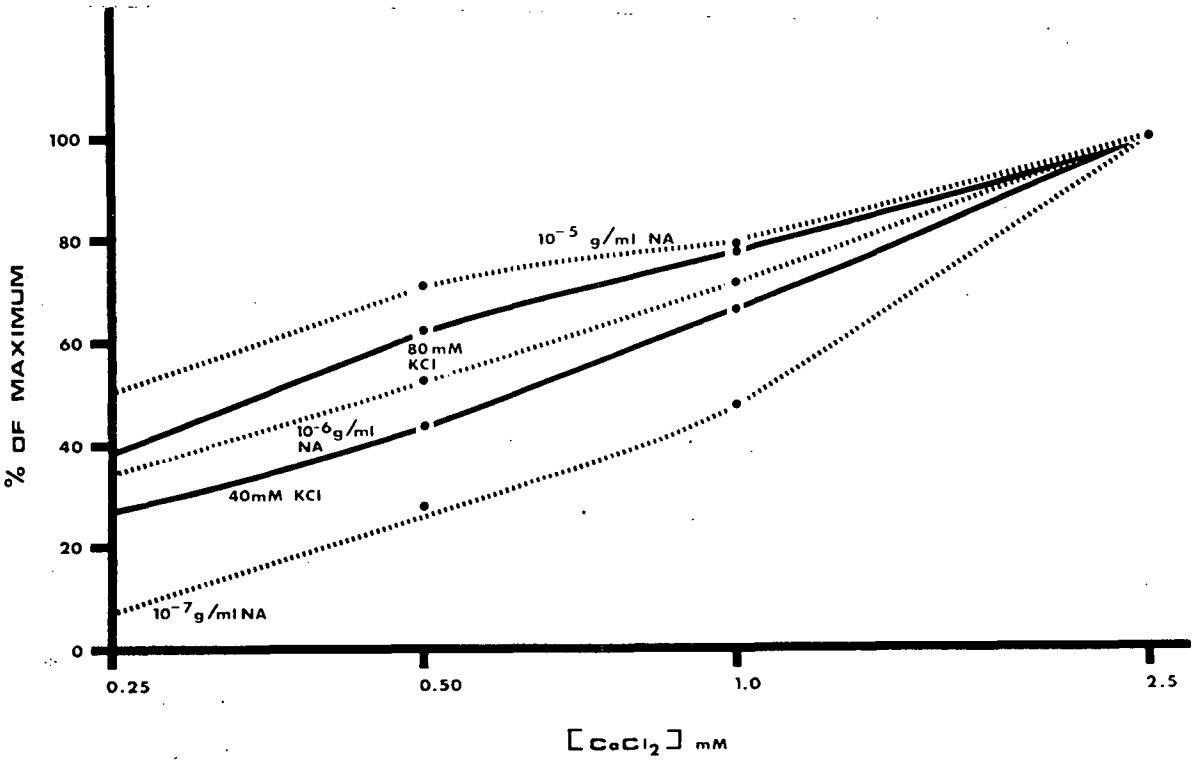


FIGURE 13

Effects of altering external calcium concentration on the responses to various doses of noradrenaline and KCl.

Maximum response to each agonist concentration is set equal to 100per cent.

Tissues were equilibrated for 30 minutes in Kreb's solution with the indicated calcium concentration before the responses to the various agonists were tested.

## II. EFFECTS OF ADDING MANGANESE CHLORIDE TO THE KREB'S SOLUTION

The second series of experiments designed to differentiate between the various sources of "activator calcium" involved the use of manganese.

Manganese chloride has been shown to be an effective inhibitor of transmembrane calcium ion flux in several different tissues. Manganese inhibits action potentials in both barnacle muscle (Hagiwara and Naka, 1964) and guinea-pig taenia coli (Bulbring and Tomita, 1968). The current carrying species for the action potential in these tissues is believed to be calcium ions. Manganese (0.1 mM) has also been shown to inhibit $^{45}Ca^{++}$ uptake in rabbit atria (Sabatini-Smith and Holland, 1969).

The addition of 0.5 mM $Mn^{++}$ to normal Kreb's solution totally inhibited spontaneous activity of rabbit anterior mesenteric vein (Figure 14). This inhibition could be reversed by adding EDTA which has a very high affinity for manganese (Martell, 1964), but could not be reversed by raising the extracellular concentration of calcium. The addition of $MnCl_2$ blocked both the spontaneous contractions and the associated membrane potential changes as measured by a sucrose gap apparatus (Figure 15). The delayed inhibitory action of manganese in the sucrose gap apparatus (cf. Figure 14) may be due to poor mixing which was necessary to reduce electrical artifacts produced by excess bubbling.

This ability of manganese to decrease membrane excitability and inhibit spontaneous activity is also shared by magnesium and calcium

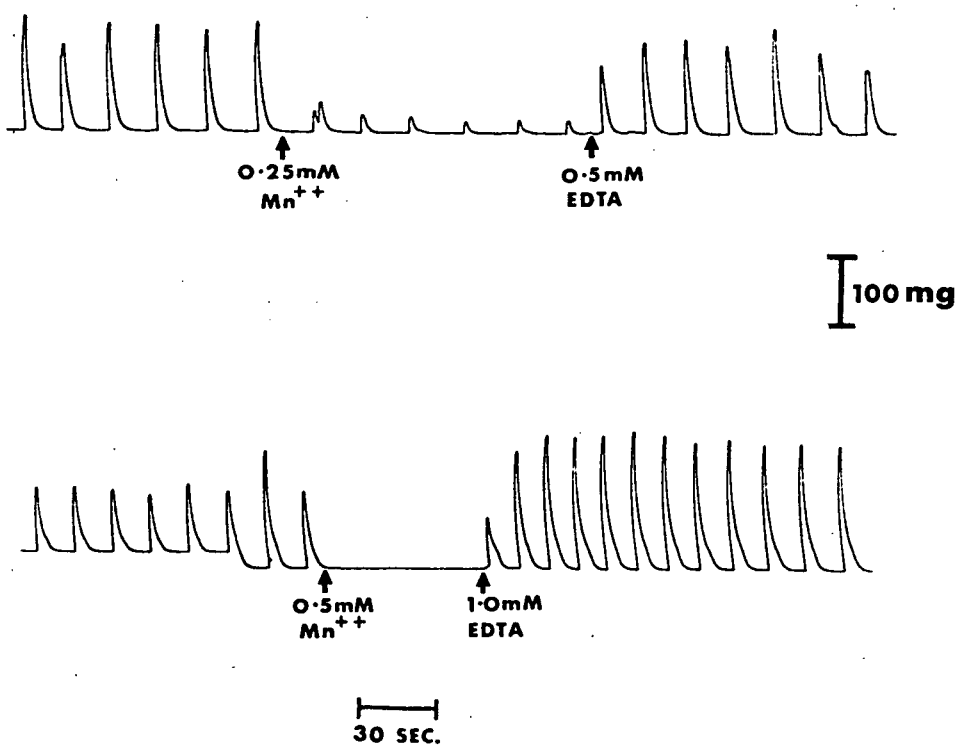


FIGURE 14

Effects of manganese upon spontaneous activity.

The tissues were bathed in normal Kreb's solution containing 2.5 mM $Ca^{++}$. The inhibitory effect of manganese was completely reversed by EDTA which has a higher affinity for manganese than for calcium or magnesium.

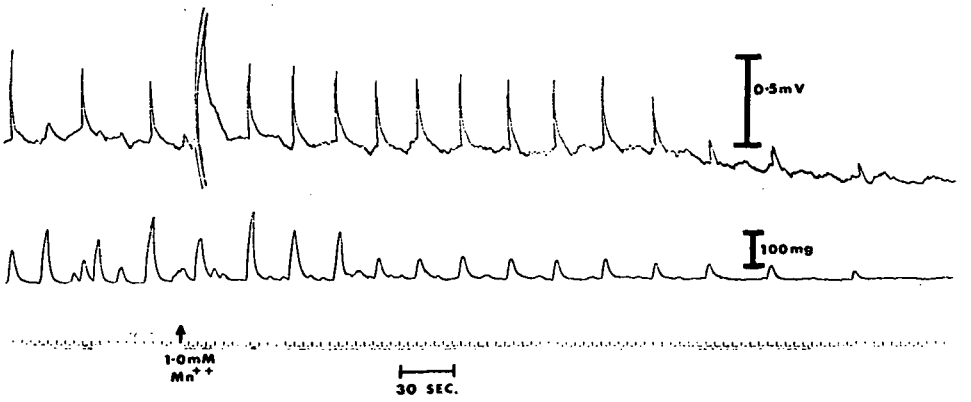


FIGURE 15

The effects of manganese on spontaneous electrical and mechanical activity of the mesenteric vein.

The tissue was placed in a sucrose gap apparatus with which electrical activity and tension can be measured simultaneously. The extended time period for complete inhibition of spontaneous activity may have been due in part to poor mixing in the bath.

ions. Increasing the magnesium concentration to 5 mM or the calcium concentration to 10 mM had the same effect as the addition of 0.5 mM Manganese to normal Kreb's.

The addition of manganese to normal Kreb's solution inhibited the responses produced by every agonist tested (noradrenaline, adrenaline, serotonin, histamine, procaine, and potassium chloride). Manganese produced a shift in the noradrenaline dose response curve as shown in Figure 16. The inhibition was not truly competitive since it was non-surmountable and yet it was not strictly non-competitive either, since the dose of noradrenaline required for 50 per cent maximum response was shifted from $2.0 \times 10^{-7}$ g/ml to $3.5 \times 10^{-7}$ g/ml by the addition of manganese.

The effect of manganese on ouabain treated veins was also tested. After 90 minutes in $10^{-5}$ M ouabain, responses to noradrenaline were slightly potentiated. The addition of 1 mM $Mn^{++}$ produced the same relative inhibition of the responses to $10^{-7}$ g/ml noradrenaline and $10^{-6}$ g/ml noradrenaline as it did in normal Kreb's solution.

The addition of manganese had a much greater inhibitory effect on contractions produced by low doses of noradrenaline (Figure 17). Manganese also had a much greater effect on responses to low doses of noradrenaline when the responses were obtained as in Figure 9. i.e. the tissues were bathed in calcium-free solution with 0.1 mM ascorbic acid added; noradrenaline was added and then a contraction was produced by the addition of calcium to achieve a final concentration of 2.5 mM. 1 mM $MnCl_2$ was added before the noradrenaline during the experimental responses. It is important to note that

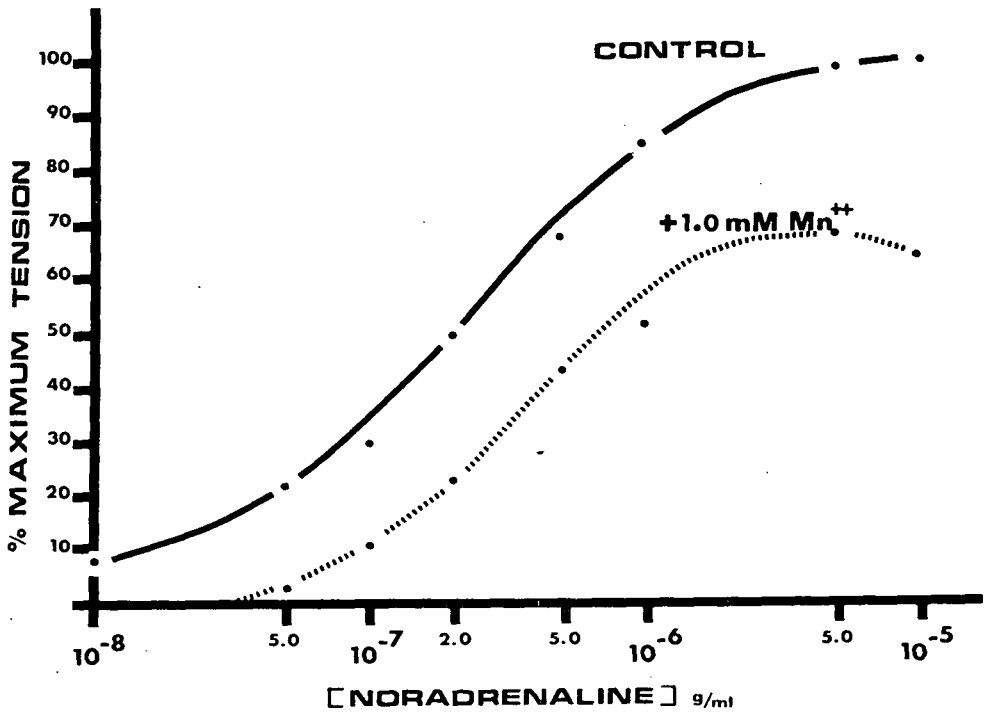


FIGURE 16

The effect of 1 mM $Mn^{++}$ on the dose-response curve to noradrenaline.

The tissues were challenged with noradrenaline of a given dose, the peak tension was recorded and then the tissues were washed. Manganese chloride solution was added to the baths to give a final concentration of 1.0 mM. After one minute the tissues were again challenged with the same dose of noradrenaline. The tissues were then washed twice to remove the manganese and the above procedure was followed with each succeeding dose of noradrenaline. Although not shown in this figure, it has been well established that two washings after treatment with manganese will allow complete recovery of response to noradrenaline (95%-105% of the pre-manganese controls).

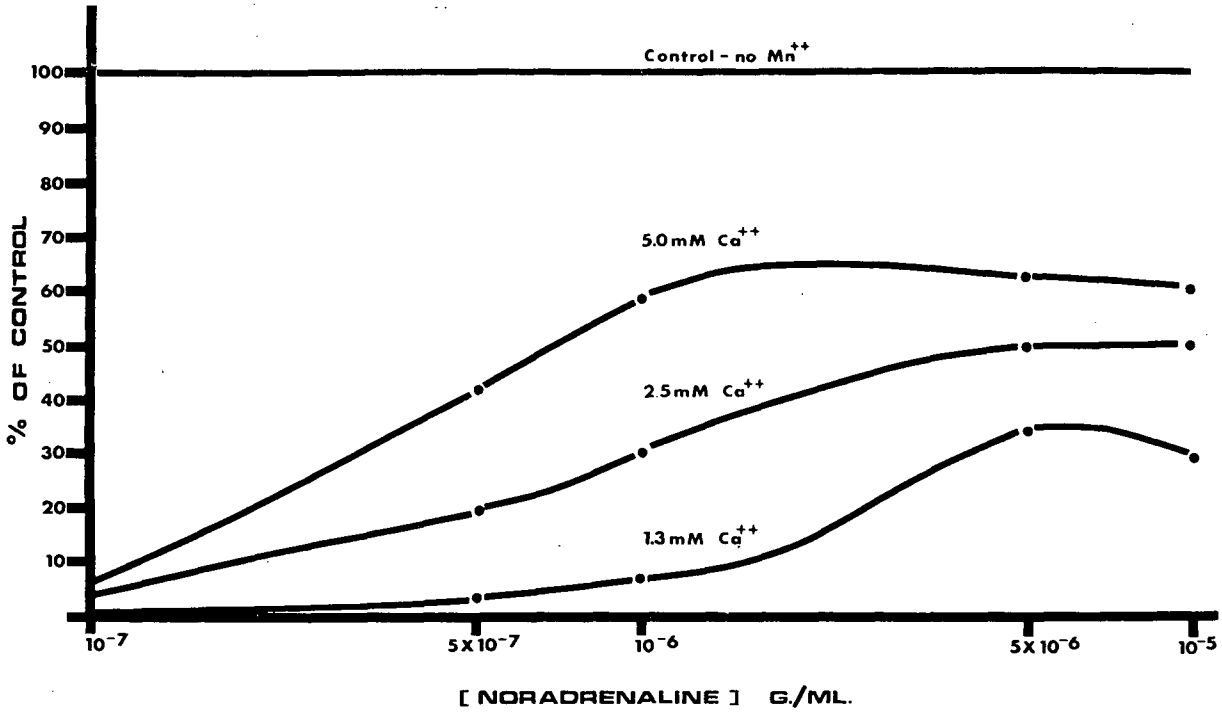


FIGURE 17

Relative inhibitory effect of 1 mM $Mn^{++}$ on the responses to various doses of noradrenaline.

The experimental procedure was the same as in Figure 16 except that the tissues were equilibrated in Kreb's solution containing the various concentrations of calcium before any response is obtained. The control response for each concentration of calcium and noradrenaline was set equal to 100 per cent. The inhibitory effect of manganese was much greater for low doses of noradrenaline. 0.1 mM ascorbic acid was also present in all bathing solutions.

ascorbic acid was used in these experiments. If it is not added the manganese rapidly catalyzes the conversion of noradrenaline to noradrenochrome and hence lowers the effective concentration of drug.

The addition of manganese did not have differential inhibitory effects on the biphasic response to $10^{-6}$ g/ml noradrenaline. The phasic and tonic components of the contraction were reduced equally in the presence of 1mM $MnCl_2$.

A similar experiment was performed using lanthanum chloride as the inhibitor instead of manganese. Lanthanum is a much more potent inhibitor of noradrenaline contractions than manganese; 0.5 mM $LaCl_3$ is equipotent as 5.0 mM $MnCl_2$. However the effect of lanthanum was not reversible. Washing the tissues for one hour in normal Kreb's solution only partially restored the response to noradrenaline. In contrast, inhibition by manganese is completely reversed by a brief 5 minute washing in normal Kreb's. The addition of 0.5 mM $LaCl_3$ to the bath, like manganese, had no differential inhibitory effect on the two phases of the response to $10^{-6}$ g/ml noradrenaline.

The addition of manganese to a vein strip during the tonic phase of a response to noradrenaline produced an immediate relaxation to a decreased, but sustained tension (Figure 18). The degree of relaxation was proportional to the logarithm of the concentration of manganese added (Figure 19). The relaxation produced by the manganese could be reversed by the addition of EDTA to bind the manganese, (Figure 18) or partially reversed by increasing the calcium concentration (not illustrated).

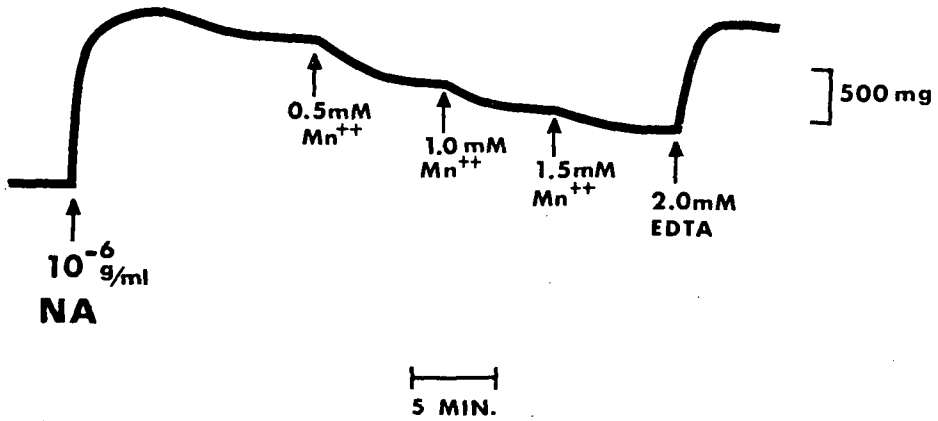


FIGURE 18

Relaxation of the response to $10^{-6}$ g/ml noradrenaline by the addition of manganese (relaxation versus time).

The tissue was bathed in normal Kreb's solution (2.5 mM $Ca^{++}$) with 1 mM ascorbic acid added to prevent the oxidation of noradrenaline to noradrenochrome. The relaxation produced by manganese could be reversed by EDTA as shown or by raising the extracellular calcium concentration to 10 mM

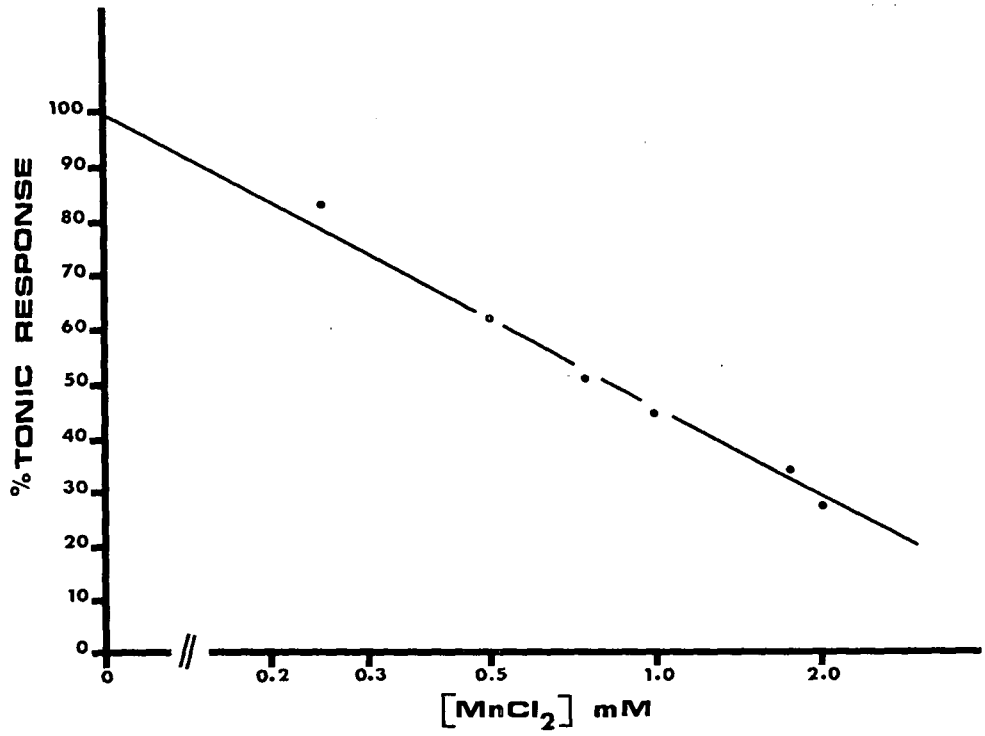


FIGURE 19

Relaxation of responses to $10^{-6}$ g/ml noradrenaline by the addition of manganese (relaxation versus manganese concentration).

The tissues were bathed in normal Kreb's to which 1 mM ascorbic acid was added. The same relaxation was produced by a given concentration of manganese whether this concentration was achieved with one large addition or in several steps with smaller additions.

This interaction between manganese and calcium ions and the effect of this interaction on the inhibition of noradrenaline responses by manganese is shown in Figures 20 and 21. The control response with each calcium concentration was set equal to 100 per cent so that the values shown in the two figures represent the relative inhibitory effects of manganese at various calcium concentration. Thus in Figure 20 it can be seen that for any given concentration of manganese, the inhibitory effect can be decreased by raising the extracellular calcium concentration. The same data is replotted in Figure 21 using the reciprocals for both axes.

If the curves are extrapolated (dotted lines) they all intersect the axis at approximately the same point (0.01 or 100 per cent of the control). This would indicate that the interaction between manganese and calcium is indeed competitive, or at least follows competitive kinetics.

The competitive nature of the interaction between manganese and calcium is shown again in Figures 22 and 23 in which the excitatory agent is 40 mM KCl. In Figure 23 as in Figure 21, if the curves are extrapolated to the ordinate they appear to intersect at 0.01. Theoretically it should be possible to completely prevent the inhibitory action of manganese by sufficiently increasing the extracellular calcium concentration. However when the calcium concentration is raised above 10 mM, the excess calcium itself becomes inhibitory (see Figure 10) and so the inhibitory effect of manganese is potentiated rather than reduced.

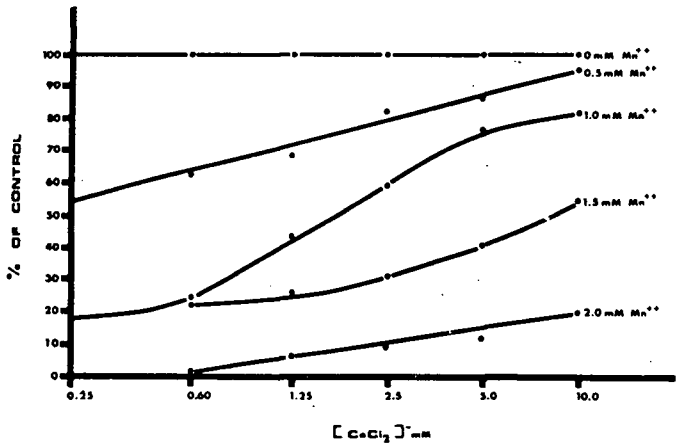


FIGURE 20

Effect of increasing extracellular calcium on the inhibition of responses to $10^{-6}$ g/ml noradrenaline by 1 mM $MnCl_2$.

Each strip was equilibrated for 20 minutes in Kreb's solution containing calcium concentration as indicated. It was then challenged with $10^{-6}$ g/ml noradrenaline several times until a consistant response was obtained. Manganese solution was added to give a final concentration from 0.5 mM to 2.0 mM as shown in the figures. Any given tissue was only tested with one particular manganese concentration. The calcium concentration in the Kreb's was increased, and after equilibrating for 20 minutes, the procedure was repeated. As seen from the top curve (0 mM $Mn^{++}$) the control response was always set equal to 100 per cent.

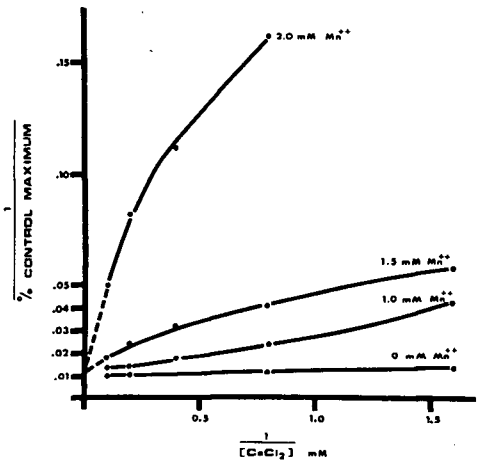


FIGURE 21

Interaction between calcium and manganese and its effect on responses to $10^{-6}$ g/ml noradrenaline.

Same data as in Figure 20 replotted as inverses to show the competitive nature of the calcium-manganese interaction.

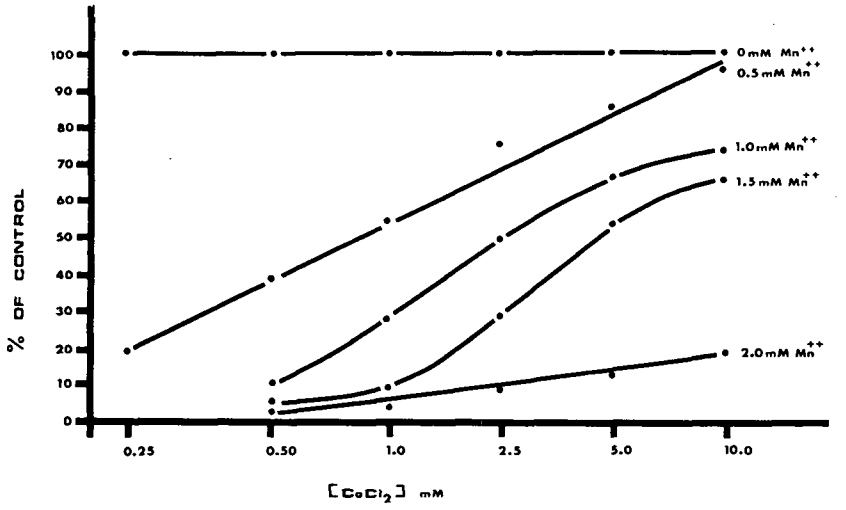


FIGURE 22

Effect of increasing extracellular calcium on the inhibition of responses to 40 mM KCl by 1 mM $MnCl_2$.

The experimental procedure was the same as for Figure 20 except that 40 mM KCl rather than $10^{-6}$ g/ml noradrenaline was used to produce a contraction.

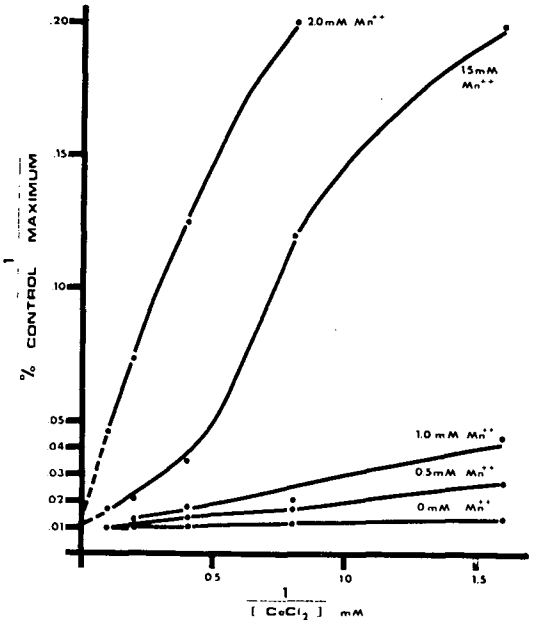


FIGURE 23

Interaction between calcium and manganese and its effect on responses to 40 mM KCl.

Same data as in Figure 22 replotted as inverses.

The relative inhibitory effect of 1 mM $Mn^{++}$ on contractions produced by various concentrations of KCl was also tested. The results were similar to those found for increasing doses of noradrenaline; the contractions produced by 20 mM KCl were inhibited to a much greater degree than those produced by 80 mM KCl.

To complete the study of the effects of manganese on the mesenteric vein, Figure 24 shows the inhibition of responses to serotonin and procaine by 1 mM $Mn^{++}$. The control response for each drug at each calcium concentration is set equal to 100 per cent. The addition of manganese completely inhibited the response to procaine and this inhibition could not be reversed by increasing the calcium concentration. The inhibition of serotonin responses was greater for the low dose of serotonin as with noradrenaline and KCl, and again increasing extracellular calcium lessened the inhibition.

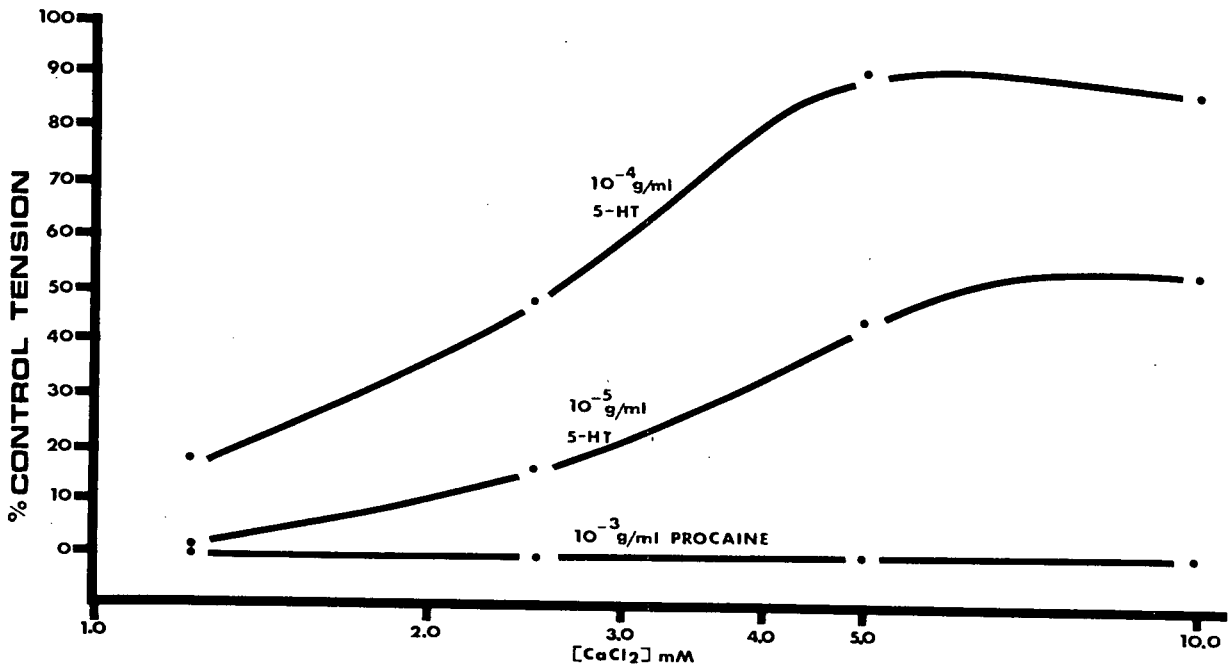


FIGURE 24

Effect of 1 mM $Mn^{++}$ on the responses to 5-hydroxytryptamine and procaine.

Control responses without manganese were set equal to 100 per cent.

## III. EFFECTS OF EGTA TREATMENT ON THE ANTERIOR MESENTERIC VEIN

As mentioned previously, placing the tissues in calcium-free medium (no added EGTA), results within ten minutes in the inability to respond to any excitatory agent. Readdition of calcium immediately reverses this inhibition.

The addition of 1 mM EGTA to the calcium-free Kreb's solution results in a faster inhibition of responses (less than 5 minutes). After the EGTA is removed, readdition of calcium produces a contraction. This was unexpected since no contraction was produced by the readdition of calcium to tissues bathed in calcium-free solution without EGTA. Figure 25 shows the responses obtained by readdition of calcium to tissues bathed for 10 minutes in calcium-free Kreb's solution to which 1 mM EGTA was added. After the 10 minute equilibration the tissues were washed twice with calcium-free solution containing 0.1 mM EGTA to remove excess chelating agent, and then concentrated $CaCl_2$ solution was added to the bath to produce a final calcium concentration as shown.

The contractile response to the readdition of calcium was phasic unless the final calcium concentration was greater than 5 mM, in which case the response was biphasic. There was a rapid phasic response lasting about one minute, followed by a prolonged tonic contraction. The magnitude of the phasic contraction was proportional to the logarithm of the calcium concentration (Figure 26).

The addition of $SrCl_2$ after EGTA treatment produced a contraction followed by the immediate onset of spontaneous activity (similar to Figure 2(C)). The addition of either 5 mM $MnCl_2$ or 5 mM $MgCl_2$

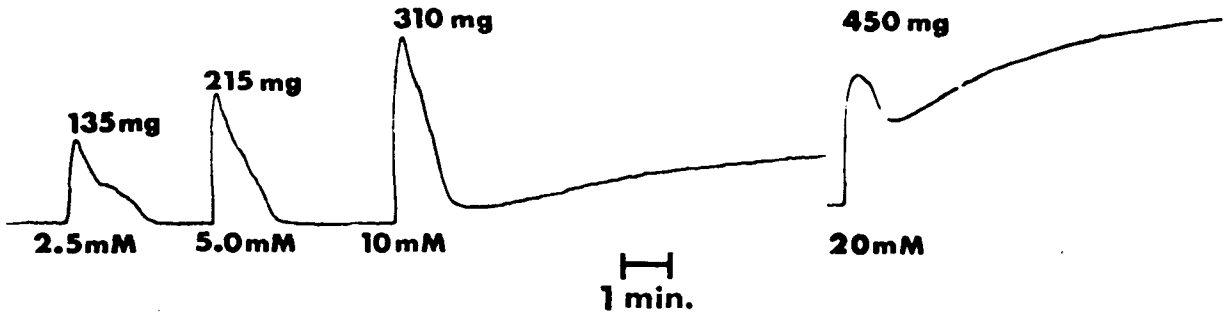


FIGURE 25

Responses to $CaCl_2$ after calcium depletion with EGTA (tension versus time).

Tissues were equilibrated in zero calcium Kreb's. 1 mM EGTA was added to each bath and left in for 10 minutes. After washing out the excess EGTA, $CaCl_2$ was added to give final calcium concentrations as shown.

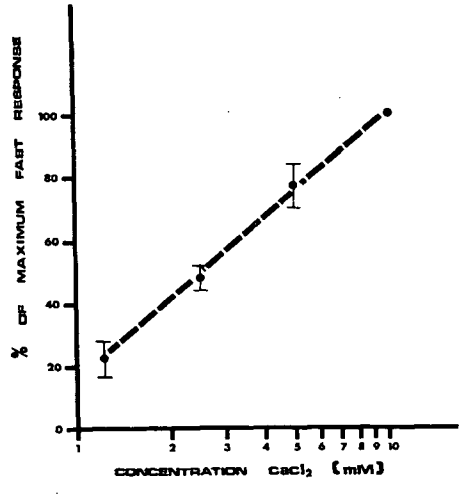


FIGURE 26

Responses to $CaCl_2$ after EGTA treatment (tension versus calcium concentration).

The bars represent the standard errors of the mean (12 vein strips). The magnitude of the phasic contraction produced is proportional to the logarithm of the calcium concentration.

had no direct effect, and in fact both ions had an inhibitory effect on the response to $CaCl_2$.

Figure 27 shows the effect of adding 1 mM $MnCl_2$ to the baths after the tissues have been subjected to the EGTA treatment. The top tracing (27(A)) is the control response to 10 mM $CaCl_2$. The addition of 1 mM $MnCl_2$ (27(B)) completely blocked the phasic contraction, but had no effect on the tonic component. This inhibitory effect of manganese was completely reversible (27(C)). The addition of 1 mM $MnCl_2$ completely blocked the responses to low concentrations of $CaCl_2$.

The addition of $MgCl_2$ was also able to block the phasic response to $CaCl_2$, but 5 mM $MgCl_2$ was required to produce an inhibition equivalent to 0.5 mM $MnCl_2$.

The addition of a small amount of calcium (0.1 mM final free calcium) to the bathing medium after calcium depletion with EGTA had a result similar to the addition of 1 mM $MnCl_2$ (Figure 28(B)): the phasic component of the biphasic response is almost completely abolished.

Lüttgau and Niedergerke (1958) showed that the tension produced by a frog heart was directly proportional to the calcium concentration and inversely proportional to the square of the sodium concentration. If this competition also occurs in the smooth muscle of the portal vein, then decreasing the extracellular sodium concentration should have an effect on these responses to calcium after EGTA treatment.

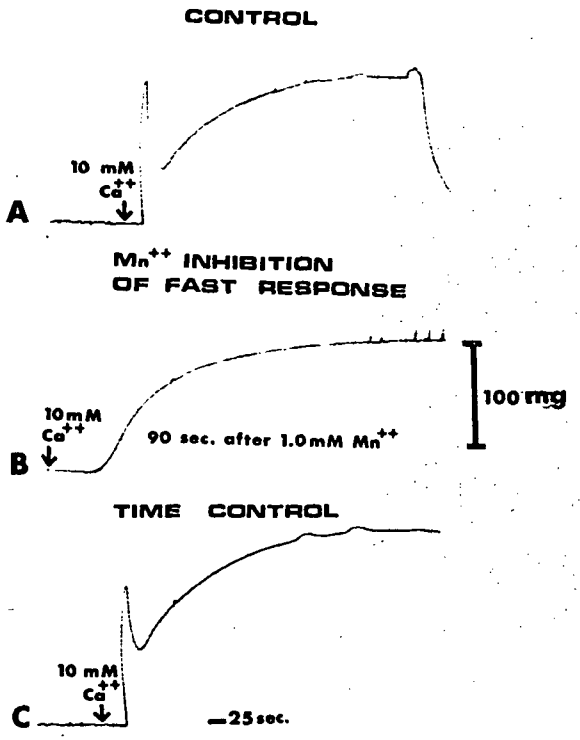


FIGURE 27

Effects of 1 mM $MnCl_2$ on the responses to $CaCl_2$ after calcium depletion by EGTA.

Tissues were depleted of calcium by bathing for ten minutes with calcium-free Kreb's containing 1 mM EGTA. The EGTA was then washed out and calcium was added to give a final concentration of 10 mM. The addition of 1 mM $Mn^{++}$ (27B) abolished the phasic component of the response.

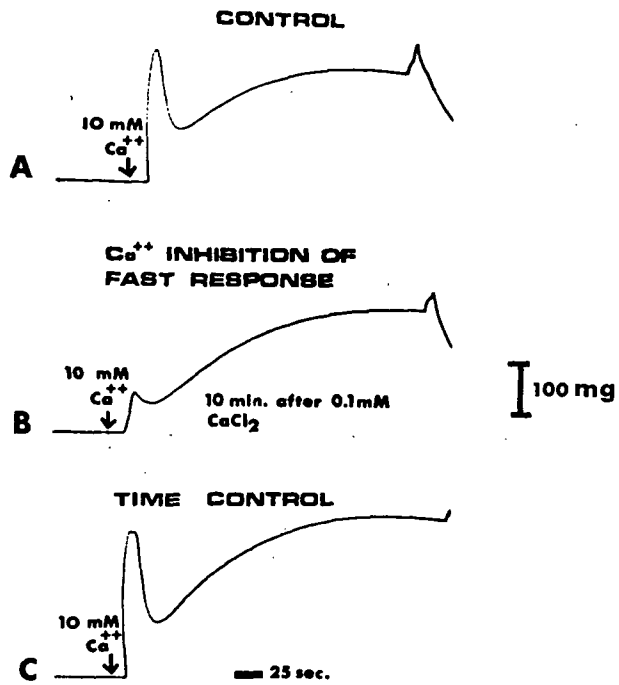


FIGURE 28

Effects of 0.1 mM $CaCl_2$ on the responses to 10 mM $CaCl_2$ after depletion of calcium by EGTA.

Same experimental design as in Figure 27. The addition of a low concentration of calcium partially inhibited the phasic component of the response.

If the sodium concentration was decreased to 25 mM or less, the response to a low concentration of calcium (2.5 mM or less) after EGTA treatment was tonic rather than the normal transient contraction shown in Figure 25. Thus lowering the external sodium concentration had little effect on the initiation of contraction, but greatly extended the time course of contraction.

The response to 10 mM $CaCl_2$ in zero sodium solution is shown in Figure 29. The response in zero sodium (29(B)) was similar to the control response (29(A)) except that spontaneous activity has been superimposed on the normal response. Figure 29(C)) shows a second control in normal calcium free Kreb's to show the reversibility of the effects of low sodium.

The effects of altering the pH on these calcium contractions was also examined. Figure 30 shows the responses to 10 mM $CaCl_2$ after EGTA treatment under conditions of varying pH. Below pH 7.4 the slow tonic component is abolished. As the pH is raised above 7.4, both phases of the contraction are increased with a slightly greater relative increase of the tonic phase.

To see if the effect of pH was specific to these calcium contractions or a generalized phenomenon, the effect of altering pH on the responses to noradrenaline and KCl was also tested. (Figure 31) It appears that this effect of increased tension produced at high pH is a generalized phenomenon and probably related in some way to calcium metabolism, or to the interaction between actin and myosin.

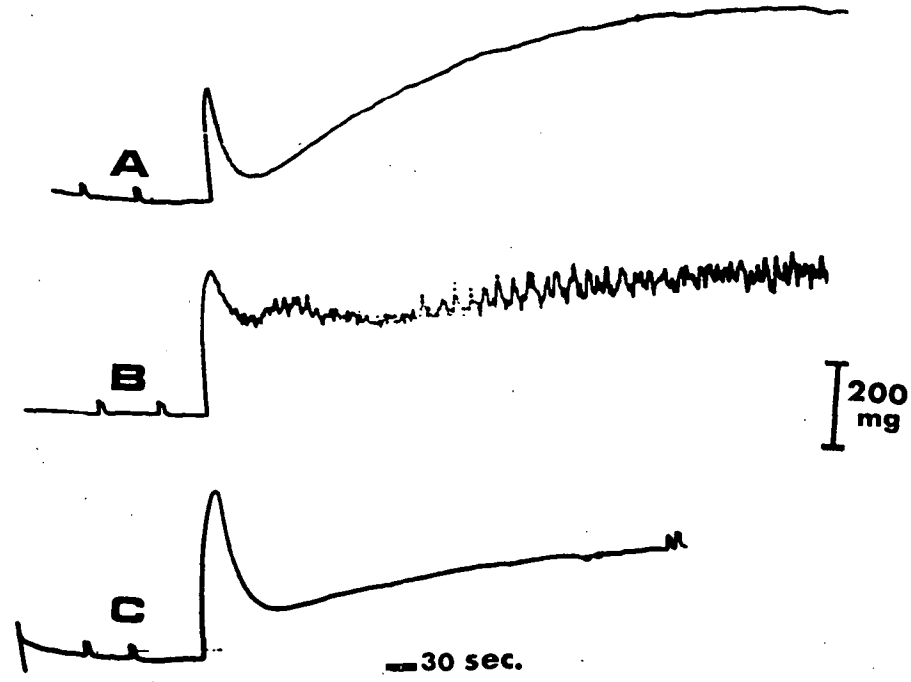


FIGURE 29

Effect of zero sodium solution on calcium responses after EGTA treatment.

A Control response to 10 mM $CaCl_2$ after 10 minutes in calcium-free solution with 1 mM EGTA (with 143 mM $Na^+$).

B Response to 10 mM $CaCl_2$ after 30 minutes in sodium-free Kreb's solution with 1 mM EGTA added for the last 10 minutes.

C Control response in calcium-free Kreb's solution (with 143 mM $Na^+$) to show reversibility of effects of sodium-free solution.

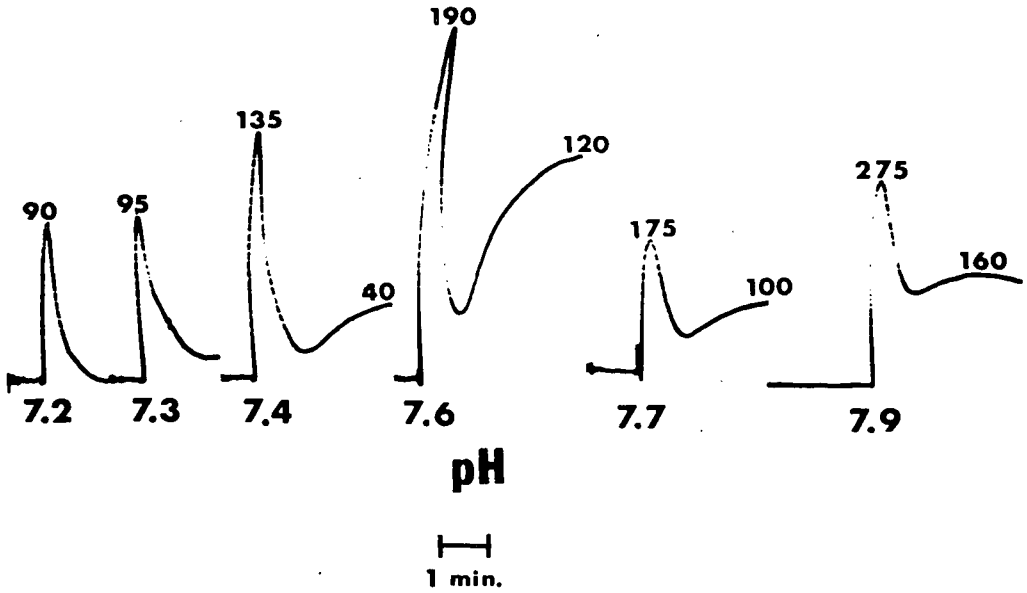


FIGURE 30

Effects of altering pH on the calcium responses after EGTA treatment.

The tissues were bathed in calcium-free, bicarbonate-free Kreb's solution with 25 mM Tris, and aerated with 100% $O_2$. The pH of the solution was adjusted with HCl and the pH was checked again by putting a micro pH electrode directly into the organ baths. The tissues were bathed for 10 minutes at this pH with 2 mM EGTA added. The tissues were washed with Kreb's solution of the same pH and responses were then elicited by the addition of $CaCl_2$ (final bath concentration: 10 mM). The small numbers on top give the tension produced (milligrams).

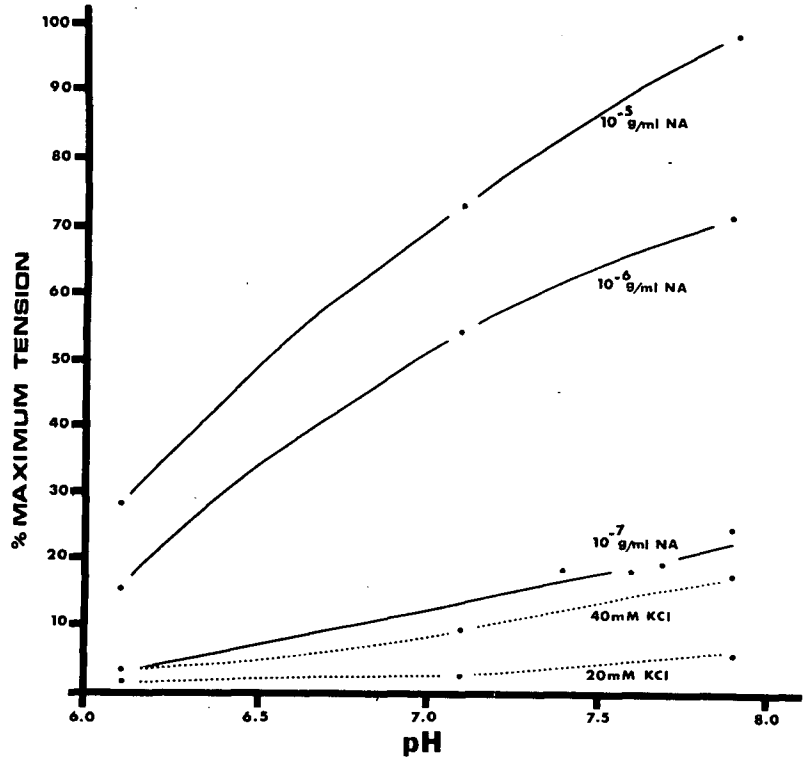


FIGURE 31

Effect of pH on responses to noradrenaline and KCl.

Tissues were equilibrated for 10 minutes in bicarbonate-free Kreb's solution with a given pH and then tested with each concentration of agonist several times until the response became constant.

# DISCUSSION

In this discussion we would like to show how our new evidence can be used in conjunction with previous work to elucidate the roles of calcium in regulating contractions of the rabbit anterior mesenteric portal vein. The aims of this discussion are to show that:

(a) extracellular or loosely bound calcium are the only sources of activator calcium for contractions induced by any stimulus;

(b) a continuous influx of calcium is required to maintain a contraction;

(c) calcium permeability is controlled by membrane bound calcium.

The discussion will be arranged under four major headings:

(1) Calcium and spontaneous activity.

(2) Calcium and the initiation of response to agonists.

(3) Calcium and tonic contractions.

(4) Control of calcium influx.

## I. Calcium and Spontaneous Activity

Calcium ions appear to be the major current carrying species for action potentials in the mesenteric portal vein. The evidence for this comes from two types of experiments:

(a) lowering extracellular sodium (the other ion most likely to

carry the current), or using an inhibitor of sodium influx have little effect on spontaneous activity.

(b) lowering extracellular calcium, or blocking calcium influx, totally inhibits spontaneous electrical and mechanical activities.

The experimental details are as follows.

The addition of tetrodotoxin had no effect on the spontaneous activity of the vein (Holman et al., 1968). Action potentials and phasic contractions of rat portal vein could be obtained for several hours in sodium free, sucrose substituted solution (Biamino and Johansson, 1970). This shows either that sodium is not necessary for action potentials, or at least that other ions are capable of substituting for sodium under these conditions.

Moreover, we have found that spontaneous activity is rapidly abolished by adding EGTA, and can be returned to normal by raising the extracellular calcium concentration. Manganese which has been shown to block calcium mediated action potentials in barnacle muscle, (Hagiwara and Nakajimo, 1966) abolishes spontaneous activity of the mesenteric portal vein. The inhibition with manganese is rapidly reversed by EDTA (which does not cross cell membranes), showing that this action of manganese most likely is on the cell membrane.

Another criterion often used (Hagiwara and Naka, 1964; Bulbring and Tomita, 1968) to show the existence of calcium mediated action potentials is the ability of other divalent cations to sustain action potentials in the absence of calcium.

Strontium chloride is able to support spontaneous electrical and mechanical activities in calcium-free solution (see also Severson and Sutter, 1969). This is unlikely to be due to release of bound calcium since we have shown that spontaneous activity can still be evoked if strontium is added to tissues which have soaked in calcium-free solution with 1.0 mM EGTA for more than an hour.

Thus it appears that calcium ions can carry a significant portion of the depolarizing current during an action potential. Would the amount of calcium carrying the depolarizing current raise the intracellular calcium concentration sufficiently to activate the contractile machinery?

This question can be answered by determining the actual transfer of charge which would occur during an action potential. The charge transferred equals the capacitance of the membrane multiplied by the amplitude of the action potential. Estimates for the capacitance of smooth muscle membrane (taenia coli) range from 1.5 uF/$cm^2$ (Tomita, 1970) to 3.0 uF/$cm^2$ (Tomita, 1966). The action potential in rabbit anterior mesenteric vein is about 19 mV (Cuthbert et al., 1965). A reasonable estimate for the cell diameter would be from 3 to 5 u (Burnstock, 1970).

An action potential of 19 mV would require a charge transfer of:

$Q = CV$ (charge = capacitance x voltage)

$= (1.5 \text{ to } 3.0 \text{ uF}) \times 19 \text{ mV}$

$= (2.9 \text{ to } 5.8) \times 10^{-8}$ coulombs/$cm^2$ of membrane

$= (1.5 \text{ to } 3.0) \times 10^{-13}$ moles $Ca^{++}$/$cm^2$ of membrane

We now have to determine the volume and surface area of the average cell. However, smooth muscle cells are not uniform in shape, and so two extreme cell shapes will be considered, on the assumption that the actual cells would have intermediate volumes and surface areas.

(a) Assume the cell is a cylinder:

Volume = $\pi r^2 l$ where r is the radius, l is the length

Surface area = $2\pi rl$ (underestimate since micropinocytotic vesicles would increase surface area)

If we assume all of the charge is carried by calcium ions, then the change in intracellular calcium concentration during an action potential would be:

$$\text{Rise in intracellular calcium} = \frac{(\text{influx per unit area}) \times \text{surface area}}{\text{volume}}$$

$$= \frac{(\text{influx per unit area}) \times 2\pi rl}{\pi r^2 l}$$

$$= \frac{(\text{influx per unit area}) \times 2}{r}$$

$$= \frac{(1.5 \text{ to } 3.0) \times 10^{-13}) \times 2 \text{ moles } Ca^{++}/cm^2}{(1.5 \text{ to } 2.5) \times 10^{-4} \text{ cm}}$$

$$= 0.6 \times 10^{-6} \text{ M to } 2.0 \times 10^{-6} \text{ M } Ca^{++}$$

(b) Assume the cell is a double cone:

Volume = $2 \times \frac{(\pi r^2 l)}{3}$

Surface area = $2 \times (\pi rl)$

Making calculations similar to those above:

Rise in intracellular calcium concentration = $0.9 \times 10^{-6}$ M to $3.0 \times 10^{-6}$ M.

These two shapes, the cylinder and double cone, give the outside limits for the actual cell shape, and so even if one ignores the presence of vesicles, a single action potential could raise the intracellular calcium concentration to at least $6 \times 10^{-7}$ M. Would this concentration of calcium produce a contraction?

Since the binding constant of troponin for calcium ions is $6 \times 10^{5}$ $M^{-1}$, (Ebashi et al. 1967) a calcium concentration of $1.6 \times 10^{-6}$ M should half-saturate the troponin binding sites (and presumably give a half-maximal contraction). Using the skinned fiber preparation from frog skeletal muscle (better preparation than glycerinated fibers since the native tropomyosin is readily denatured by glycerination), Hellam and Podolsky (1966) showed a calcium threshold of $10^{-7}$ M with the maximum tension achieved with $7 \times 10^{-6}$ M $Ca^{++}$.

Using either of the above criteria, it would appear that if a sufficient portion of the depolarizing current is carried by calcium ions, that a single action potential (actually a multi-spike complex in the vein) would raise the intracellular calcium concentration sufficiently to account for the accompanying contraction.

## II. Calcium and the Initiation of Contractions to Agonists

Previous evidence showing the importance of extracellular or loosely bound calcium for contractions of the mesenteric portal vein comes from two types of experiments. Firstly, contractions to all types of agonists are rapidly abolished in calcium-free solution, (Cuthbert and Sutter, 1965, Severson and Sutter, 1969, Biamino and Johansson, 1970). Secondly, Somlyo et al. (1969) have shown a good correlation between the maximum depolarization produced by a drug (excluding a depolarizing solution of KCl) and the maximum tension achieved. Somlyo then assumes that the degree of depolarization can act as an index of calcium permeability, and if this is correct the maximum tension achieved by a drug is proportional to the increase in calcium permeability. In the absence of direct data to show the relative contribution of individual ionic species producing the depolarization, one can really only speculate that the variations in maximum depolarization are due to variations in ionic permeability including calcium permeability, which in turn leads to variations in maximum tension produced.

We have also found that decreasing the extracellular calcium concentration rapidly and markedly decreased the responses to noradrenaline, adrenaline, KCl, serotonin, histamine, acetylcholine, and procaine. Since by definition tightly bound intracellular stores of calcium would not be so rapidly depleted, there are three possible hypotheses for these results.

(1) Somlyo's theory is correct; the activator calcium for all contractions comes from the extracellular fluid or loosely bound surface stores.

(2) The majority of the activator calcium is released from intracellular sites, but the influx of a small amount of calcium is necessary to release this bound calcium.

(3) The presence of membrane bound calcium is required for contraction but no actual calcium influx is required.

The third hypothesis could be tested by measuring the rate of calcium exchange in the absence and presence of drugs. Although this has not been done using the mesenteric vein, the results from other types of smooth muscle show that the rate of calcium exchange does increase during contractions, and hence this third hypothesis is not likely correct (Briggs and Melvin, 1961, Van Breemen and McNaughton, 1970). The problem then is to differentiate between the first two hypotheses. What does each hypothesis imply about the nature of excitation-contraction coupling?

1. Activator calcium comes from the extracellular fluid.

This hypothesis implies that drugs can produce graded increases in calcium permeability in response to increased drug concentration. Each individual cell would therefore contract in a graded manner to increased drug concentration.

2. Activator calcium comes from bound intracellular stores and is released by the influx of a small amount of calcium.

This hypothesis implies the presence of an intracellular bound calcium store, but does not imply that drugs can produce graded

increases in calcium permeability. What is the function of the calcium influx in this hypothesis?

Under normal conditions, this influx would produce a depolarization and increase the intracellular calcium level. It is unlikely that the depolarization per se is necessary for excitation-contraction coupling since mesenteric veins which are fully depolarized by KCl (Somlyo and Somlyo, 1968a) or ouabain (Mathews and Sutter, 1967) still contract in response to noradrenaline. This means that it is the rise in intracellular calcium itself which is important for excitation-contraction coupling. This "calcium induced release of bound calcium" phenomenon has recently been shown by Endo et al. (1970) to occur in skinned skeletal muscle fibers, thus raising this hypothesis for the initiation of contractions, from mere speculation to a real possibility. The amount of calcium required to induce further calcium release was $10^{-6}$ M, which as they say was "usually below the threshold for contraction". Now assuming a rise in free intracellular calcium in mesenteric portal vein can release bound calcium as proposed by Endo for skeletal muscle, at some critical intracellular calcium concentration this phenomenon would become self-perpetuating and each individual cell should fire in an all-or-none manner.

How do the data known about the mesenteric vein, both from the present experiments and previous workers, fit with these two hypotheses? First is there in fact a graded increase in calcium permeability with different drugs or increasing doses of drugs? (As would be required by hypothesis number one)

If one examines the frequency-distribution of membrane potentials of rabbit mesenteric vein, a normal frequency distribution is observed both in the absence and in the presence of drugs (Somlyo et al., 1969). When noradrenaline (10 ug/ml) or serotonin (20 ug/ml) was added there was a general shift towards lower membrane potentials and no bimodal distribution (indicating all-or-none depolarization) was seen. Noradrenaline produced a greater depolarization and also a greater contractile response than did serotonin. This means that smooth muscle cells have a normal statistical distribution with regard to electrical responses to drugs, and also that the ionic permeability of each individual muscle cell can be altered in a graded manner by different drugs. Presumably this would include a graded increase in the permeability to calcium. If this evidence does indeed demonstrate graded increases in calcium permeability, the criterion necessary for the first hypothesis is satisfied, but this does not rule out hypothesis number two. What evidence is there for a bound intracellular calcium store as required by the second hypothesis?

First, smooth muscle cells have a poorly developed sarcotubular system when compared with skeletal or cardiac muscle (Burnstock, 1970) and thus have less available intracellular calcium if one assumes that the tubules are the primary intracellular calcium storage sites in smooth muscle.

Second, in other types of smooth muscle which are believed to utilize intracellular calcium stores for contractions, biphasic responses are obtained in response to agonists (Bohr, 1964 using

rabbit aorta; Woodward, Bose and Innes, 1970 using cat spleen capsule). However, unless the calcium concentration is very low, we found that the tension produced by the mesenteric portal vein in response to agonists increases monotonically with time.

These two pieces of data both argue against the release of intracellular bound calcium during contraction of mesenteric portal vein. There is one further bit of evidence which argues against the second hypothesis.

The second hypothesis implies that each cell should fire in all-or-none manner. The graphs of tissue response versus calcium concentration (figures 8, 9, 12, 13) must then reflect the statistical variation of individual cells with regard to calcium permeability, or the statistical variation of critical intracellular calcium concentration required to initiate the all-or-none contraction of each cell. Figure 9 shows that the statistical variation is very large; in fact it ranges over at least a one hundred fold change in extracellular calcium concentration. Such a broad variation among cells from the same tissue would seem to be very unusual and leads one to suspect that individual cells are not contracting in an all-or-none manner, and that the second hypothesis is therefore not likely correct.

Another major argument in smooth muscle research has been whether or not different agonists mobilize calcium from different sources. Hudgins and Weiss (1968) showed that procaine would relax rabbit aorta contracted in response to noradrenaline but not to 25 mM KCl. Van Breemen (1970) showed that lanthanum chloride had a greater inhibitory effect on contractions to KCl

than on noradrenaline induced contractions, again using rabbit thoracic aorta. Severson and Sutter (1969) showed that after placing the aorta in calcium free solution, responses to KCl were lost more quickly than those to noradrenaline. This result was also found by Hudgins and Weiss using equipotent doses of KCl and noradrenaline.

The experiments with procaine prove very little; procaine certainly resembles noradrenaline more closely than it does KCl, and so it is hardly surprising that it has more of an effect on noradrenaline contractions. The experiments with $LaCl_3$ and calcium-free solutions could be explained simply if KCl and noradrenaline have different effects on calcium permeability. To my knowledge no one has compared the effects of low calcium on KCl and noradrenaline induced contractions under equilibrium conditions using rabbit aorta.

These arguments cast some doubts that different agonists mobilize calcium from different sources in the aorta. Experiments using the mesenteric portal vein are more clear cut in showing that different agonists do not utilize different sources of activator calcium.

Severson and Sutter (1969) showed that there was little difference in the rates at which KCl and noradrenaline responses were lost after exposure to calcium-free solution. We have shown in Figure 13 that equipotent doses of KCl and noradrenaline are equally affected by lowering the extracellular calcium concentration. Experiments using serotonin showed that responses to equipotent doses of this agonist were also equally affected by low extracellular calcium.

We also found that manganese chloride had an equal inhibitory effect on contractions to equipotent doses of noradrenaline, adrenaline, serotonin, histamine, KCl, and procaine.

These experiments would indicate that it is unlikely that different calcium sources are mobilized in the mesenteric portal vein for the contractions to various excitatory agents. It appears that contractions produced by all agonists are initiated by an increased calcium influx, and so differences in efficacy are explained by differences in the ability of drugs to increase calcium permeability.

III. Calcium and sustained contractions

We have evidence that once the contraction has been initiated by this influx of calcium, a continuous influx of extracellular calcium is still necessary to maintain a tonic contraction. Figure 11 shows that the removal of extracellular calcium with EGTA results in a prompt relaxation to a lower tension. Also Figure 7(B) shows that the tonic tension is directly proportional to extracellular calcium concentration in the range from 0.1 to 1.0 mM. This likely means that there is a continual calcium removal process and that during a sustained contraction the intracellular concentration is the result of a balance between calcium influx and removal.

We have also shown that the addition of manganese to a vein contracted by noradrenaline (Figure 18) KCl, or serotonin produces a relaxation. This cannot be an effect of manganese on the contractile

proteins since EDTA rapidly reverses the inhibition (Figure 18). This data supports the hypothesis that a continued influx of calcium is required to maintain tension. The relaxation caused by addition of manganese can be partially reversed by raising extracellular calcium, showing that the relaxation is probably due to a competition with calcium at the cell membrane.

Thus it appears that calcium ions for both the initiation and maintenance of contractions in the mesenteric portal vein come from the extracellular fluid. How then is the membrane permeability to calcium controlled?

## IV. Control of calcium influx

The experiments involving calcium depletion with EGTA (Section III of Results) reveal several interesting facts about control of calcium permeability. The addition of calcium chloride has no effect on tissues bathed in normal Kreb's solution (containing 2.5 mM calcium). Bathing the tissues in calcium-free solution (no added EGTA) inhibits responses to all agonists, but the readdition of calcium still produces almost no response on its own. However, when the tissues are bathed for ten minutes in calcium-free solution containing 1 mM EGTA, the readdition of calcium produces a contraction.

Thus the chelation of some bound calcium (presumably bound to the membrane) has made the membrane more permeable to added calcium. This function of membrane bound calcium in the vein is probably analogous to the stabilizing action of calcium described by Frankenhaeuser and Hodgkin (1957) for the squid giant axon.

Further evidence that calcium controls its own permeability is shown in Figure 28. The addition of a small amount of calcium (0.1 mM final concentration) after EGTA treatment inhibits responses to large doses of calcium. The addition of either manganese or excess magnesium was also able to inhibit the response to calcium.

This ability of calcium to control its own influx was predicted by Somlyo and Somlyo (1968b). He believes that agonists initiate contractions by releasing membrane bound calcium thus increasing calcium permeability. We have found evidence that agonists do indeed release membrane bound calcium (Figures 4 to 7). The transient portion of the biphasic response to added noradrenaline with 0.1 mM calcium in the bathing solution (Figure 4) must be due to the release of loosely bound calcium into the cytoplasm. If the transient response was due to simple influx of calcium (or release of intracellular calcium), then the same response should have been obtained by adding 0.1 mM calcium to tissues bathed in calcium free medium containing $10^{-5}$ g/ml noradrenaline. Further evidence that the transient response is due to release of membrane bound calcium comes from analysis of Figure 7.

Figures 7(A) and 7(B) show quantitatively the effects of reduced extracellular calcium concentration on the two phases of the response to $10^{-5}$ g/ml noradrenaline. When the extracellular calcium concentration is 1.0 mM, the phasic and tonic responses are approximately equal in magnitude. If Figure 7(A) were replotted with a linear calcium scale, the curve would resemble

a rectangular hyperbola, indicative of a saturation phenomenon. This cannot be due to saturation of calcium binding sites on troponin since the tonic response (Figure 7(B)) is still increasing linearly when the extracellular calcium concentration is 1.0 mM. It must therefore represent a saturation of membrane binding sites for calcium.

If the release of membrane bound calcium leads to increased calcium permeability, then drug efficacy can be explained by differences in the ability of drugs to release this bound calcium. This idea is consistent with our observation that as the concentration of agonist is decreased, the transient component of the contraction in low calcium (due to release of membrane bound calcium) was greatly decreased. For example, the transient response in low calcium is not produced by the addition of $10^{-7}$ g/ml noradrenaline (Figure 5) or by 40 mM KCl.

As mentioned in the discussion of spontaneous activity, manganese appears to act on the cell membrane to prevent the influx of calcium. Manganese is also able to inhibit the initiation of contraction to all agonists.

The addition of 1 mM $MnCl_2$ to the Kreb's solution produced a non-surmountable inhibitory shift of the noradrenaline dose response curve (Figure 16) and also of the dose response curves to KCl and serotonin. Manganese also inhibited the responses to noradrenaline after the tissues had been in a solution containing $10^{-4}$ M ouabain for 90 minutes. Thus manganese does not inhibit the contractile responses solely by

inhibiting action potentials, since this ouabain treatment renders the cell incapable of generating action potentials (Mathews and Sutter, 1967).

The inhibitory effects of manganese are consistent with Somlyo's theory that excitatory drugs have some primary effect on the membrane which leads to parallel changes in tension and electrical activity. Manganese interferes with this primary effect, which is presumably the release of membrane bound calcium.

The competitive nature of the interaction between manganese and calcium can be shown for contractions produced by either noradrenaline (Figure 20) or KCl (Figure 22). The ability of calcium to reverse the inhibitory effects of manganese is not always observed however. Raising the extracellular calcium will not reverse the manganese induced inhibition of spontaneous activity or inhibition of contraction to procaine or low doses of noradrenaline ($10^{-8}$ g/ml). This is expected since we have shown that raising the calcium concentration can itself inhibit spontaneous activity and the response to procaine. Thus manganese appears to act as "super-calcium" to lessen the calcium permeability change produced by agonists and in this manner stabilize the membrane.

V. Effects of altering sodium concentration or pH

These last two types of experiments are really only preliminary observation. Studying the interactions between calcium ions and either hydrogen ions or sodium ions may provide more information about the nature of calcium binding sites and the importance of extracellular calcium for both the initiation and maintenance of contractions.

The potentiation of contractile responses to all types of stimuli (Figure 31) is interesting. At least four possible explanations can be put forward for further experimentation:

(1) an effect on the actin-myosin interaction independent of calcium ions;

(2) an increased affinity of troponin for calcium ions;

(3) an increased influx of calcium;

(4) a decreased efflux of calcium.

The fact that lowering the extracellular sodium concentration changes a normally transient contraction (response to 2.5 mM $Ca^{++}$ after EGTA treatment) into a sustained contraction, is also interesting and should be further studied with regard to a sodium-calcium exchange mechanism as described by Glitsch et al. (1970) in guinea-pig atria. It is possible that this exchange may be important for the relaxation process in the mesenteric vein. Very recent experiments indicate that this is indeed true. Although small contractions may be produced by noradrenaline, adrenaline, KCl, serotonin, and acetylcholine after 90 minutes in zero sodium solution (Tris substitution), relaxation does not occur after washing out the drugs with zero sodium solution. Washing the tissues with normal Kreb's solution produces an immediate relaxation.

## SUMMARY

The rabbit anterior mesenteric portal vein which we are using as a model for arteriolar smooth muscle contracts in response to a wide variety of agonists. The response to each agonist appears to be initiated by the release of membrane bound calcium which produces an increased calcium permeability. We feel that the activator calcium for the contractile process comes solely from the extracellular fluid or loosely bound membrane stores. The efficacy of a particular drug is related to its ability to release this membrane bound calcium which is controlling calcium influx. Manganese acts on the cell membrane and inhibits the normal increase in calcium permeability produced by drugs.

Since we have shown that tonic contractions of the vein require a continuous influx of calcium, there probably is some continuous calcium removal process. Preliminary experiments indicate that extracellular sodium somehow is somehow involved, since tissues in zero sodium solution do not relax after removal of the drug.

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