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A POSSIBLE MECHANISM FOR THE CONTROL OF VASCULAR SMOOTH MUSCLE INTRACELLULAR CALCIUM CONCENTRATION

by
Charles Louis Seidel

A dissertation submitted in partial fulfillment
of the requirements for the degree of
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ABSTRACT

A POSSIBLE MECHANISM FOR THE CONTROL OF VASCULAR SMOOTH MUSCLE INTRACELLULAR CALCIUM CONCENTRATION

by
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Chairman: David F. Bohr

As in skeletal muscle the calcium ion is thought to be necessary for the activation of vascular smooth muscle contractile protein. However, unlike skeletal muscle vascular smooth muscle does not appear to possess an intracellular calcium sequestering system and therefore, the mechanism by which vascular smooth muscle reduces its intracellular calcium concentration below that needed to activate the contractile proteins is unknown. The purpose of my thesis is to determine a possible mechanism by which the vascular smooth muscle reduces its intracellular calcium concentration.

Many investigators have suggested that the cell membrane regulates the rate at which calcium enters and/or leaves the cell. By using radioactively labeled calcium we have determined the effects of sodium-free medium, low temperature and vasoactive agents on the uptake and release of calcium from rabbit thoracic aorta as well as the effects of varying extracellular calcium concentration on calcium

efflux. From this information we have proposed a possible mechanism by which the intracellular calcium concentration is reduced and have described the effects of norepinephrine and isoproterenol on this mechanism.

There appear to be two pools of calcium which exchange with extracellular calcium: an initial rapid pool, the rate constant of which increases with increasing extracellular calcium and a pool which is lost slowly, the rate constant of which is depressed by increasing extracellular calcium. Sodium-free medium increases the radioactive calcium content and decreases the rate constant for the washout of calcium from both pools. Low temperature ($2^{\circ}C$) and norepinephrine (10^{-6} g/cc) cause an increase in the radioactive calcium content and decrease the rate constants for washout into either calcium or calcium-free media. Isoproterenol (10^{-8} g/cc) causes a decrease in radioactive calcium content but has no effect on the rate constants for washout.

It is concluded that there is a calcium-calcium exchange system in vascular smooth muscle which is influenced by sodium since in the absence of sodium calcium influx is greater than efflux. Low temperature also reduces efflux so that it is less than influx however, the depression of efflux is not great enough to suggest that it is a metabolic process. Some of the energy for calcium efflux may be derived from the movement of extracellular calcium down its electrochemical gradient into the cell. It is proposed that the vasoconstrictor norepinephrine initiates tension by decreasing calcium efflux without affecting calcium influx,

thereby causing an increase in intracellular calcium. The data suggests that the vasodilator isoproterenol depresses calcium influx without changing calcium efflux with the net effect being a decrease in calcium content and therefore, deactivation of the contractile proteins.

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INTRODUCTION

Knowledge concerning the workings of vascular smooth muscle can be obtained from several avenues of approach. The process of excitation-contraction coupling, that is, the event or sequence of events linking membrane excitation to contractile protein activation is the avenue that I have pursued.

One soon realizes that the study of excitation-contraction coupling consists of at least four subtopics. One subtopic includes identification of the contractile regulator substance. The second involves identification of the mechanism by which the regulator substance initiates contraction. Another includes the study of the conditions necessary for release of the regulator substance. The fourth includes a description of the mechanism by which the intracellular level of the regulator substance is reduced. The latter subtopic is the one that I have investigated.

Excitation-contraction coupling has been most extensively studied in skeletal muscle. Several recent reviews (11, 34, 35) summarize the current thinking and indicate that the calcium ion is the regulator, for it is released during excitation (23) and allows activation of the contractile proteins. From skeletal muscle homogenates a fraction has been isolated which consists of membrane bound vesicles.

These vesicles have been shown to sequester calcium at very rapid rates (9, 27) and are able to reduce the extravesicular calcium to very low levels (9). The vesicles are believed to be fragments of the skeletal muscle sarcoplasmic reticulum (31).

The cause of calcium release from the reticulum and its role once released is still poorly understood. The work of Ebashi (11) has contributed much to the understanding of calcium's action. He has identified a protein complex, troponin-tropomyosin, which inhibits the adenosine triphosphatase activity of actomyosin under conditions of low calcium concentration. In the presence of calcium this inhibition is blocked. How calcium prevents this inhibition or how troponin-tropomyosin inhibits actomyosin adenosine triphosphatase is still unknown.

Even less is known about the cause of calcium release from the sarcoplasmic reticulum. Since the transverse tubules are closely aligned with the lateral sacs of the sarcoplasmic reticulum it has been suggested (8, 10, 28) that depolarization of the transverse tubules causes a permeability change in the sarcoplasmic reticulum increasing the release of calcium from the reticulum.

In smooth muscle as in skeletal muscle calcium is the substance that initiates contraction (14). The protein complex troponin-tropomyosin also is present in smooth muscle (26) and is the site of calcium action. However, in smooth muscle it is not known how the level of intracellular calcium is reduced or how an increase in the level of intracellular

calcium is initiated by excitation. These are the problems to which I have addressed myself.

Smooth muscle does not possess the well developed sarcoplasmic reticulum present in skeletal muscle but vesicles have been observed along the inner surface of the membrane (32). These vesicles are able to hydrolyze adenosine di- and triphosphates (33); however, it is not known what role this characteristic plays in the function of the vesicles, for the function of the vesicles has not been determined.

Measurement of total tissue calcium indicates that smooth muscle calcium concentration is similar in all types of smooth muscle (1, 28, 39) ranging between two and three millimoles per kilogram wet weight. If it is assumed that 10^{-7}M of calcium is the threshold amount needed to activate the contractile proteins (15), the magnitude of total tissue calcium indicates that there is an excess of calcium associated with the smooth muscle cell. This means that the smooth muscle must possess some way of controlling its intracellular ionic calcium concentration so that it does not remain tonically contracted.

There are at least five ways in which intracellular ionic calcium could be prevented from surpassing the threshold necessary to activate the contractile proteins. 1) The cell membrane could be impermeable to calcium; 2) the electrical gradient across the cell membrane could prevent intracellular calcium concentration from increasing; 3) there could be a one to one exchange between intracellular and extracellular calcium so that there was no net calcium uptake;

4) calcium could be bound and not available to the contractile proteins; 5) there could be a carrier mediated calcium efflux system. Each of these represents a control mechanism by which the smooth muscle cell could maintain free intracellular ionic calcium below that necessary to activate the contractile proteins and represents a possible site of action of contractile agents.

Van Breeman et. al. (39) determined that extracellular radioactive calcium exchanged with all but 0.5mmoles/Kg wet weight of the total uterine calcium within two hours. Goodford (15) observed that extracellular radioactive calcium completely exchanged with the total tissue calcium of taenia coli within ten minutes and that the amount of radioactive calcium remained constant for as long as two hours. In both uterine and intestinal smooth muscle extracellular radioactive calcium distributed in a volume larger than the extracellular space. If one assumes that the distribution in excess of that attributable to the extracellular space is not due solely to extracellular binding, then these findings suggest that extracellular radioactive calcium is capable of exchanging with intracellular calcium. This implies that the membrane is not impermeable to calcium and therefore, membrane impermeability is not reponsible for preventing activation of the contractile proteins.

The measured resting membrane potential of smooth muscle ranges between 40 and 70mV inside negative (2, 15, 39). Van Breeman (39) calculated the intracellular calcium concentration expected if the resting membrane potential

was 50mV and the extracellular calcium concentration was 1.5mM. His calculations indicated that the concentration would have to be 67mM. Since the total uterine calcium concentration is 2.25mmoles/Kg wet weight it is obvious that the electrical gradient favors a further increase in total calcium. Therefore, the membrane potential is not responsible for maintaining ionic intracellular calcium below that necessary for activation of the contractile proteins and in fact there must be some system which prevents calcium from reaching electrochemical equilibrium. The system which prevents calcium from reaching electrochemical equilibrium may also be the system by which the cell reduces the intracellular ionic calcium concentration.

Such a system may be a one to one transmembrane calcium exchange. If, for every calcium ion that moves into the cell one moves out, there will be no net increase in intracellular calcium. However, such a system does not seem to exist in smooth muscle. Van Breeman et. al. (39) observed that uteri accumulated calcium while in a high calcium medium and lost calcium while in a low calcium medium. Hurwitz (21) also found that the calcium content increased in strips of intestinal smooth muscle as the length of exposure to 36mM extracellular calcium increased. One would not expect to find an increase in intracellular calcium as extracellular calcium increased if a one to one exchange system existed (assuming that these changes in total calcium represent transmembrane calcium fluxes).

The work of Hurwitz (21, 22) is especially interesting

for it suggests another mechanism by which intracellular calcium could be prevented from surpassing the threshold concentration necessary to activate the contractile proteins. He observed that exposure of longitudinal strips of ileal muscle to 36mM calcium caused an increase in total tissue calcium, but very little increase in tension. This suggests that extracellular calcium is able to enter the cell without being able to gain access to the contractile proteins. Hurwitz suggested that this calcium was bound intracellularly.

Bound calcium and the process of calcium binding have many meanings. To some, bound calcium is calcium adhering to cellular structures such as the cell membrane or intracellular organelles by a passive physical-chemical process. However, to others, bound calcium represents calcium sequestered within specific structures with the process of binding requiring energy and/or a mediated transport system.

Observation of the rate of tension decline in a calcium-free medium has suggested that there are two bound calcium pools, one which is loosely bound and another which is tightly bound (12, 13, 17, 18, 37). Tension induced by depolarization, either by high extracellular potassium or direct electrical stimulation, is rapidly lost in a calcium-free medium, while the tension induced by cholinergic or adrenergic agents is slowly lost. Depolarization is thought to release loosely bound calcium, while adrenergic and cholinergic agents release tightly bound calcium. The mechanism by which calcium is bound is not indicated by these observations, but they suggest that the amount of calcium

bound in either of these two pools is influenced to a different degree by extracellular calcium.

Van Breeman et. al. (39) have suggested that binding in uteri is energy dependent. They base this opinion on the observation that the calcium binding constant determined in metabolically poisoned uteri is not large enough to lower intracellular calcium below that necessary to activate the contractile proteins. It is suggested that this binding could be to vesicles found in uteri and/or the inside of the cell membrane.

In contrast to the work of Van Breeman is the work of Holland et. al. (29, 30, 38) which suggests that energy is necessary to prevent calcium binding in taenia coli. It was observed that during a potassium induced contracture total calcium and calcium influx increased. The contractile response was blocked by dinitrophenol, but the increase in total calcium was not blocked. The authors suggested that energy was needed to keep the calcium released by potassium in an exchangeable form so that it was available to the contractile proteins. In the presence of dinitrophenol, sufficient energy to maintain calcium in an exchangeable form would not be available and tension would not develop.

As described previously Hurwitz has suggested that calcium is bound within ileal smooth muscle (21, 22). He has suggested that this binding involves some type of saturable transport system (22) which is pictured as regulating the release of calcium from the binding site. It is also thought that this transport system can be induced to release

more calcium in the presence of acetylcholine.

In addition to transport systems that regulate the release of calcium from intracellular bound stores, transmembrane calcium transport systems have also been suggested.

Pfaffman et. al. (30) observed in taenia coli that ouabain, unlike dinitrophenol, blocked both the increased tension and increased calcium content produced by high extracellular potassium. The authors suggested that sodium efflux was linked to calcium influx and that when ouabain blocked sodium efflux, calcium influx also was blocked preventing an increase in total tissue calcium and therefore, potassium induced tonic contracture.

Bauer et. al. (1) have suggested the existence of an energy requiring extrusion system in taenia coli. They observed that exposing taenia coli to $4^{\circ}C$ temperature or to a glucose-free oxygen-free medium increased the calcium content. They interpreted these findings as suggesting that there was an energy requiring calcium efflux pump. However, in a later paper Goodford (16) observed that calcium efflux was unaffected by glucose-free oxygen-free medium or dinitrophenol, but was depressed by $4^{\circ}C$. He suggested that calcium efflux was a physical process and explained the increased content found by Bauer et. al. as being due to an increase in calcium influx without a change in calcium efflux. Goodford did not rule out the existence of active calcium extrusion, but did not feel that it was the rate limiting step in calcium efflux.

Van Breeman et. al. (39) observed similar changes in

calcium fluxes in poisoned uteri. It was determined that uteri, poisoned with iodoacetate, increased their calcium content without a change in calcium efflux. This is similar to the finding of Bauer et. al. (1) and Goodford (16) in taenia coli. However, Van Breeman interpreted these findings as indicating that there was an energy requiring process which prevented extracellular calcium from crossing the cell membrane. Since this system prevented calcium from coming into the cell, it was called an exclusion system to differentiate it from an extrusion system which would be involved with calcium efflux. Blockage of such an exclusion system by iodoacetate would explain the increased calcium influx seen by Van Breeman et. al.

Very few experiments have been performed to describe the mechanim of calcium handling in vascular smooth muscle. It has been observed that epinephrine increases the radioactive calcium content of vascular smooth muscle (5) and that norepinephrine decreases radoactive calcium efflux (19). Epinephrine induced tension may be due to an increase in calcium influx resulting from the inhibition of a calcium exclusion system. Norepinephrine induced tension on the other hand may be due to a decrease in calcium efflux resulting from inhibition of a calcium extrusion system.

From his study of coronary arteries Bohr (3) suggested that alpha receptor activation causes an increase in ionized calcium associated with the contractile proteins while beta receptor activation causes a reduction in ionized calcium. It is possible that such changes in ionized calcium could

be initiated by changes in activity of transmembrane calcium transport system.

There is also evidence to suggest that the efflux of sodium is linked to the influx of calcium. When vascular smooth muscle was exposed to ouabain spontaneous tension developed (4). The increased tension was interpreted as indicating an increase in intracellular calcium. If intracellular sodium and extracellular calcium are linked in an exchange system, an increase in intracellular sodium produced by ouabain would increase the activity of the exchange system and lead to an increase in intracellular calcium and therefore, an increase in tension. In support of this hypothesis is the finding of Briggs et. al. (6) that calcium uptake increases in the presence of ouabain.

The literature cited suggests that vascular smooth muscle may regulate the level of intracellular ionic calcium by means of a transmembrane calcium transport system. Assuming that such a system exists the purpose of my thesis is to describe this system by answering the following questions: 1) What is the effect of metabolic blockade on calcium flux? 2) What effect does varying the ionic composition of the medium have on calcium flux? 3) Can the mechanical effects of vasoconstrictor and vasodilator drugs be explained from the effects of these agents on calcium flux?

The vascular smooth muscle chosen for this study was rabbit thoracic aorta and the following experiments were performed. To determine the energy dependency of calcium flux, radioactive calcium uptake and efflux as well as

total tissue calcium were determined under control conditions (37°C) and under conditions of low metabolic activity (2°C). To determine if extracellular ions are involved with either the uptake or efflux of calcium, uptake and efflux of calcium were followed in the presence and absence of extracellular sodium and efflux was followed in media of varying calcium concentration (0-6mM). Finally, to determine the site of action of vasoconstrictors and vasodilators, efflux, uptake and total tissue calcium were determined in the presence of the vasoconstrictor norepinephrine and the vasodilator isoproterenol.

METHODS

General

Albino rabbits of either sex weighing between one and two kilograms were killed by a blow to the back of the head. The thoracic cage was opened on the ventral side and the thoracic aorta was removed. The aorta was immediately placed in a physiological salt solution of the following composition: NaCl 137mM, $KHCO_3$ 5.9mM, $MgCl_2 \cdot 7H_2O$ 1.2mM, $CaCl_2$ 1.6mM, dextrose 11mM, sorbitol 1mM, versenate 0.026mM. The vessel was gently patted to remove blood from its lumen and the salt solution was replaced with fresh solution. This isolation procedure took approximately two minutes.

The aorta was trimmed of fat, slit open lengthwise and cut into transverse strips. A typical transverse strip measured 2mm wide by 7mm long by 250μ thick. Each transverse strip was blotted by placing it between two double layers of acid washed filter paper upon which was placed a one hundred gram weight for five seconds. The strip was removed from the filter paper and weighed to the nearest hundredth of a milligram on a Cahn Electrobalance. Each strip weighed between ten and twenty milligrams. From the time the transverse strip was removed from the filter paper and the weight determined, not more than thirty seconds elapsed. During this time there was no more than a two percent loss in weight due to water evaporation. After weighing, the transverse

strip was returned to the salt solution and mounted on a wire frame as described in the following sections.

Determination of radioactive calcium content

The following procedure is a modification of that described by Briggs and Melvin (5). Each transverse strip was hooked at one end by a silver wire and on the opposite end by a silver wire attached to a glass bead. The strips were suspended in a beaker of physiological salt solution with the glass bead hanging free and exerting a force on the strip of six hundred milligrams. The salt solution was maintained at a temperature of thirty seven degrees centigrade and was continuously bubbled with 99% oxygen and 1% carbon dioxide holding the pH at 7.4. The strips were incubated in this solution for two hours.

After this two hour incubation period the strips were transferred to a salt solution containing a trace amount of radioactive calcium (1uc/ml). The strips remained in the labeled salt solution for thirty minutes and at the end of this time they were transferred to test tubes containing ten milliliters of unlabeled salt solution. They remained in these tubes for five minutes and then were removed, blotted as described before, weighed and placed in graduated conical centrifuge tubes containing 0.25ml of glacialacetic-trichloracetic acid mixture (250grams trichloroacetic acid/ 250ml glacial acetic acid). This acid solution will henceforth be designated by the letters TCA. After all muscles

had been removed from the labeled salt solution, a 0.1ml aliquot of the solution was placed in 0.25ml of TCA.

Each muscle was digested according to the procedure described by Sparrow and Johnstone (36). The muscle-TCA mixture was heated for five minutes at 140°C and then approximately one milliliter of deionized water was added. The strips were reheated for an additional five minutes at 140°C. The tubes were allowed to cool to room temperature, were diluted to two milliliters with deionized water, briefly mixed on a vortex mixer and then placed in a clinical centrifuge. The strips were centrifuged at 1200g for thirty minutes and the resulting supernatants were decanted into individual nalgene containers.

The aliquots of labeled salt solution that had been placed in TCA were diluted to five milliliters with deionized water and mixed in a vortex mixer. The samples were not heated or centrifuged. This solution was also decanted into nalgene containers.

A one milliliter aliquot of the supernatant from the muscle digest or a one milliliter aliquot of the diluted labeled salt solution was placed in a glass counting vial and eleven milliliters of scintillation fluid were added (9ml 2-propanol, 2ml toluene-PPO mixture ((1g 2,5-Diphenyloxazole/200ml toluene)) ((2))). All samples were then placed in a Nuclear Chicago liquid scintillation counter and counted for ten minutes or twenty thousand counts.

To determine the background activity, several counting vials were prepared in the following way. Into a conical

centrifuge tube was placed 0.25ml of TCA and this was diluted to five milliliters with deionized water. A one milliliter aliquot of this solution was placed in a counting vial and eleven milliliters of scintillation fluid was added. This solution was counted for at least ten minutes and it was found that the amount of background activity amounted to twenty seven counts per minute. The automatic background subtractor was set to remove this background of twenty seven counts per minute from all samples.

The following formula was used to calculate the radioactive calcium content:

$$\text{Radioactive calcium content } \frac{\text{mM}}{\text{Kg wet weight}} = \frac{\text{CPM in muscle}}{\text{wet weight (mg)}} \times \frac{\text{mM/L Ca in medium}}{\text{CPM/L in medium}}$$

Radioactive calcium efflux

Each strip of thoracic aorta was mounted on an L-shaped silver wire and was placed for three hours in a physiological salt solution containing trace amounts of radioactive calcium (25uc/ml). As before, the solution was kept at 37°C and was continuously bubbled with 99% oxygen and 1% carbon dioxide. At the end of the loading period, each strip was removed from the labeled medium, blotted gently while still on the wire frame with strips of filter paper, dipped once in each of two individual counting vials containing 2.5ml of salt solution at room temperature, blotted again and placed in the first washout vial. At five minute intervals for the next two hours each strip was transferred to a new vial. All vials contained 2.5ml of unlabeled

salt solution at 37°C and were bubbled with 99% oxygen and 1% carbon dioxide for at least five minutes prior to receving a strip, though not bubbled while the strip was in the vial.

After all strips had been removed from the labeled salt solution, a 0.1ml aliquot was taken and handled as described in the previous section, Determination of radioactive calcium content.

After each strip had been washed in this way for two hours, it was removed from the frame, blotted, weighed and digested as described in the previous section.

To each of the washout vials, 11ml of scintillation fluid was added. A 1ml aliquot of the supernatant from the muscle digest or a 1ml aliquot of the diluted labeled salt solution was placed in a counting vial containing 1.5ml of physiological salt solution to which was added 11ml of scintillation fluid. The 1.5ml of physiological salt solution was added so that there was equal quenching in all counting vials. Counting vials containing aliquots of the labeled salt solution and the first three washout vials were counted for two minutes or one hundred thousand counts. All other vials were counted for ten minutes or twenty thousand counts. In all cases the automatic background subtractor was set so that it removed twenty seven counts per minute from each sample.

By adding the number of counts remaining in the muscle at the end of washout to the number of counts in the last washout vial, then adding this sum to the number of counts in the next vial and so on for each remaining vial, the

number of counts in the muscle at any time was determined. To correct for weight differences, the counts per minute in the muscle at the end of each five minute washout period were divided by the wet weight of the muscle. This number, counts per minute in the muscle per milligram wet weight, was plotted against time on semilogarithmic graph paper.

Determination of total calcium

The procedure for the determination of total calcium was similar to the procedure described by Sparrow et. al. (36). To the remaining 1ml of muscle digest 0.01ml of 2M potassium chloride was added and 0.02ml of 2M potassium chloride was also added to the remaining 4ml of diluted labeled salt solution. Total calcium was determined from aliquots of these solutions by atomic absorption spectrophotometry. For each experiment a standard curve was constructed from which was obtained the calcium concentration of each unknown sample in millimoles per liter. The total calcium in the muscle strip was calculated in the following way:

$$\text{Calcium mM/Kg wet weight} = \frac{\text{Calcium mM/L} \times \text{Dilution factor}}{\text{wet weight}}$$

The calcium content of the incubation medium was calculated in the following way:

$$\text{Calcium mM/L} = \text{Calcium mM/L} \times \text{Dilution factor}$$

Determination of sorbitol-^{14}C space

Transverse strips were suspended as described in Determination of radioactive calcium content for two hours in a physiological salt solution at 37°C and a pH of 7.4. At the end of two hours the strips were transferred to a salt solution containing trace amounts of sorbitol-^{14}C (1uc/ml) and remained in this solution for thirty minutes. At the end of thirty minutes the strips were removed, blotted, weighed and digested as described in Determination of radioactive calcium content. After all strips had been removed from the labeled salt solution, a 0.1ml aliquot of this solution was placed in 0.25ml of TCA and diluted to 5ml with deionized water.

A one milliliter aliquot of the muscle digest or the diluted labeled salt solution was placed in a counting vial and eleven milliliters of scintillation fluid was added. All samples were counted for ten minutes or twenty thousand counts with the automatic background subtractor set to remove twenty seven counts per minute.

Glassware

All glassware used in these experiments was pyrex. To remove calcium adhering to the glass, thoroughly washed glassware was soaked for at least thirty minutes in a 1:6 solution of hydrochloric acid and deionized water and then was thoroughly rinsed in deionized water.

Drugs, isotopes and statistics

Radioactive calcium forty five and sorbitol-^{14}C were

obtained from New England Nuclear. The radioactive calcium was supplied as $CaCl_2$ in 0.5N HCl and the sorbitol-^{14}C in a 9:1 ethanol:water solution.

Norepinephrine, isoporterenol and phentolamine were supplied by the Winthrop Laboratory.

The Student-t test was used to determine the statistical significance of all results. A "p" value equal to or less than 0.05 was considered to be statistically significant.

RESULTS

Equilibration of total tissue calcium with extracellular radioactive calcium

The rate at which extracellular radioactive calcium equlibrates with total tissue calcium is depicted in figure one. Each strip of aorta was incubated in a nonradioactive salt solution for two hours prior to exposure to radioactive salt solution to allow time for the normal ion gradients to be reestablished after the trauma of dissection. As can be seen, radioactive calcium equilibrates with total tissue calcium within ninety minutes and both the radioactive calcium content and total tissue calcium content remain constant for as long as four hours. The average total tissue calcium was 3.58 ± 0.09mmoles/Kg wet weight ($n = 29$).

Factors that influence radioactive calcium content

Drugs

Figure two describes the effect of norepinephrine (10^{-6}g/cc) on radioactive calcium equilibration in aortic strips incubated up to four hours in a radioactive calcium salt solution. Only after four hours of exposure to norepinephrine does the equlibration of radioactive calcium differ significantly ($0.05 > p > 0.02$) from the control. Total tissue calcium is greater at the end of four hours in the

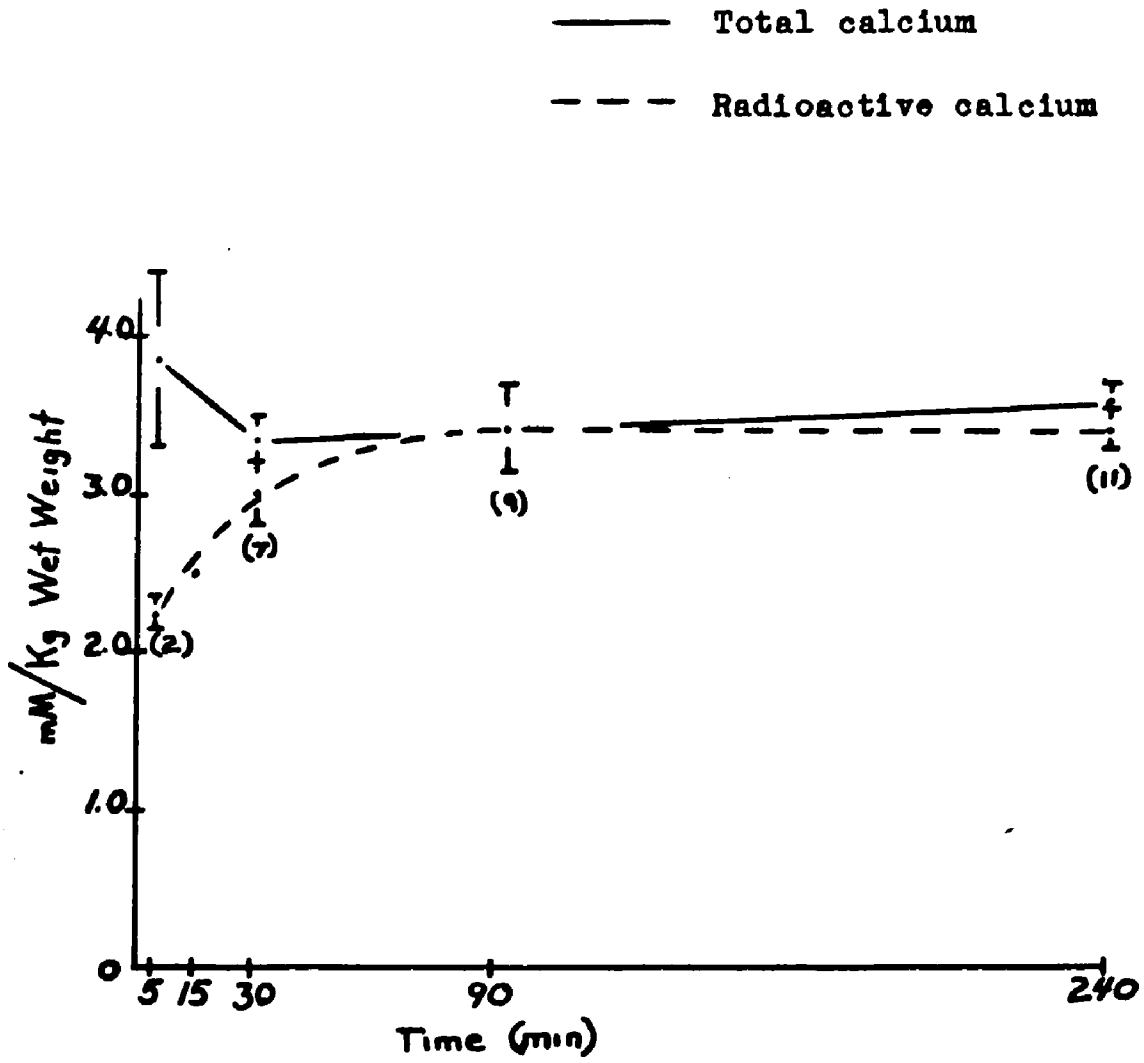

Fig. 1.--Equilibration of radioactive calcium with total calcium. Values in () equal number of observations. Brackets equal S.E.M.

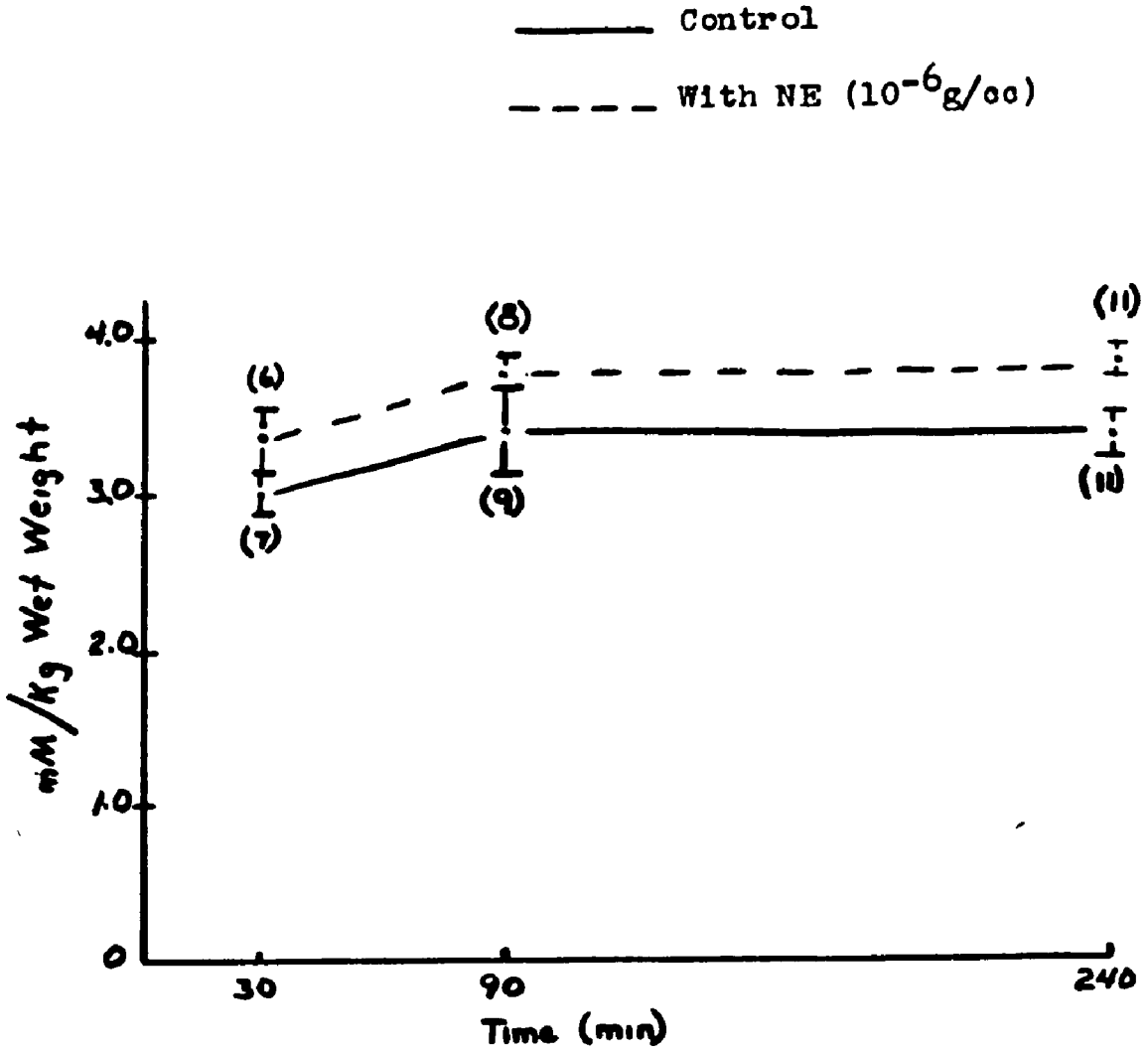

Fig. 2.-- Radioactive calcium equilibration in the presence and absence of norepinephrine. Values in () equal number of observations. Brackets S.E.M.

presence of norepinephrine (4.12 ± 0.28 vs. 3.56 ± 0.15 mmoles/Kg wet weight, n= 11) but not significantly (0.10>p>0.05).

In their 1961 paper, Briggs and Melvin (5) demonstrated that within thirty minutes epinephrine increased the uptake of radioactive calcium in rabbit thoracic aorta. In an attempt to demonstrate a similar effect by norepinephrine, a modification of their technique was used. Strips were incubated for two hours in a physiological salt solution, thirty minutes in a salt solution containing trace amounts of radioactive calcium and finally five minutes in a non-radioactive salt solution. When drugs were used they were present during the thirty minute incubation in radioactive calcium salt solution and the five minute incubation in non-labeled salt solution.

The term "radioactive calcium content" used in figures three and five means the calcium concentration calculated from the radioactive calcium activity of the muscle strip at the end of the above procedure. It does not represent a measure of calcium influx alone but rather an equlibration concentration, for within thirty minutes radioactive calcium has equilibrated with 84% of total tissue calcium (figure 1).

The control radioactive calcium content is indicated by the first bar in figure three and equals 0.87 ± 0.04mmoles /kg wet wt.. From figure one, a content of approximately 3mmoles/Kg wet wt. would have been expected at the end of a thirty minute incubation in radioactive calcium. The difference between figure one and figure three means that the

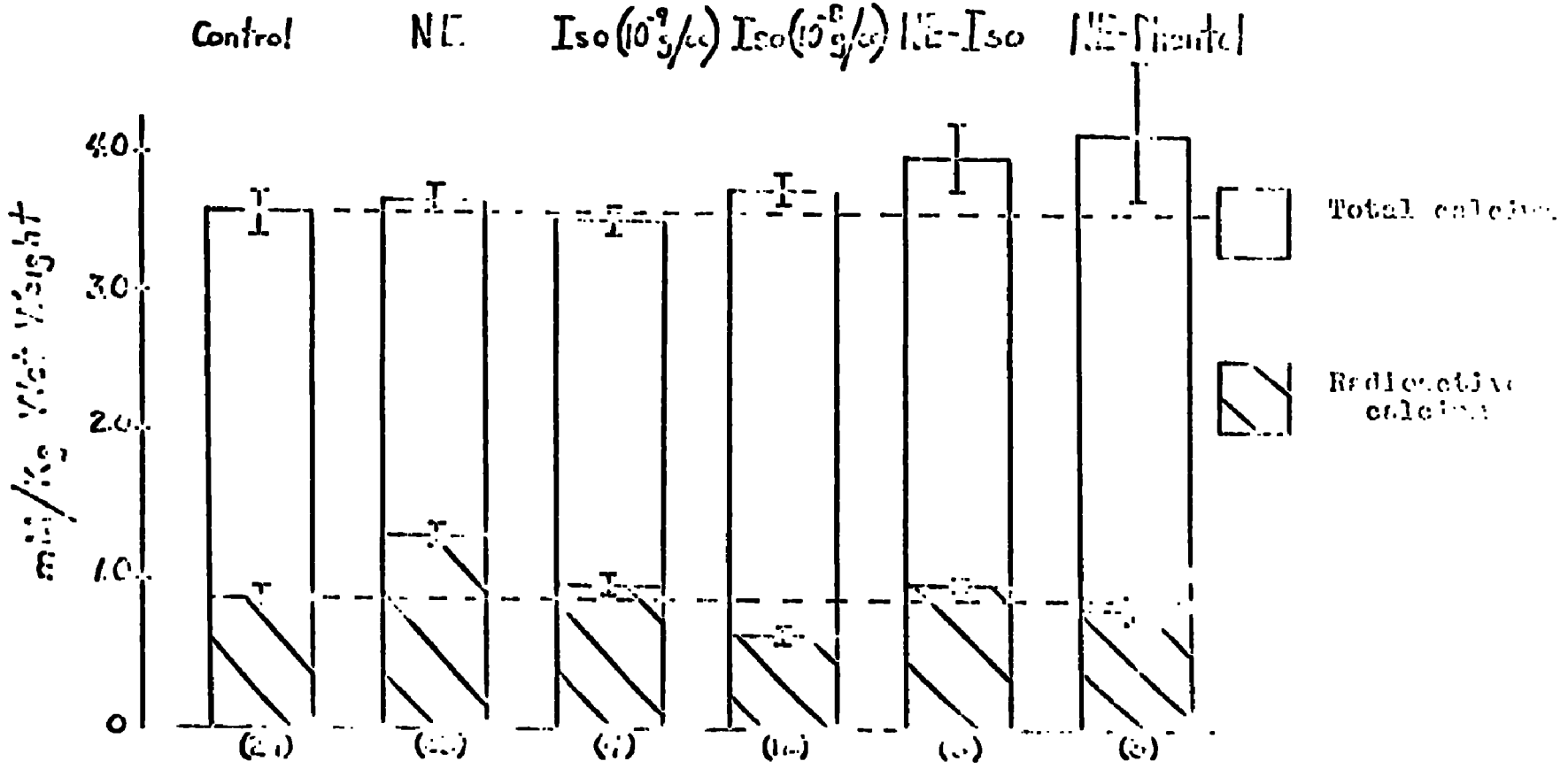

Fig. 3.- Effect of [illegible] on radioactive calcium or [illegible] total calcium. Values in () equal number of observations. Bars = S.E.M.

five minute wash in non-radioactive salt solution removed over 70% of the radioactive calcium.

If one assumes that sorbitol distributes only in the extracellular space, figure four indicates that the extracellular space of rabbit thoracic aorta is 538 ± 28ml/Kg wet weight. Assuming an extracellular calcium concentration of 1.6mM/L, 0.86mmoles/Kg wet wt. of the total tissue calcium can be thought to exist in the extracellular space. Figure four also indicates that within five minutes 85% of the extracellular space is washed out. Even if all the extracellular space was washed out in five minutes, insufficient calcium woule be lost (0.86mmoles/Kg wet wt.) to account for the 70% decrease in radioactive calcium content shown in figure three. The remainder of the calcium (46%) must be coming from compartments in which sorbitol does not equilibrate. These compartments could represent intracellular or extracellular calcium pools.

The remainder of figure three describes the effects of various drugs on the radioactive calcium and total tissue calcium contents. Norepinephrine (10^{-6}g/cc) causes a significant ($p<0.01$) increase in the radioactive calcium content, while isoproterenol (10^{-8}g/cc) causes a significant ($p<0.01$) decrease. Isoproterenol at a concentration of 10^{-9} g/cc has no effect on the radioactive calcium content. In the presence of isoproterenol (10^{-8}g/cc) or phentolamine (10^{-6}g/cc) norepinephrine is unable to cause an increase in radioactive calcium content. Phentolamine alone had no effect on radioactive calcium content. None of the drugs

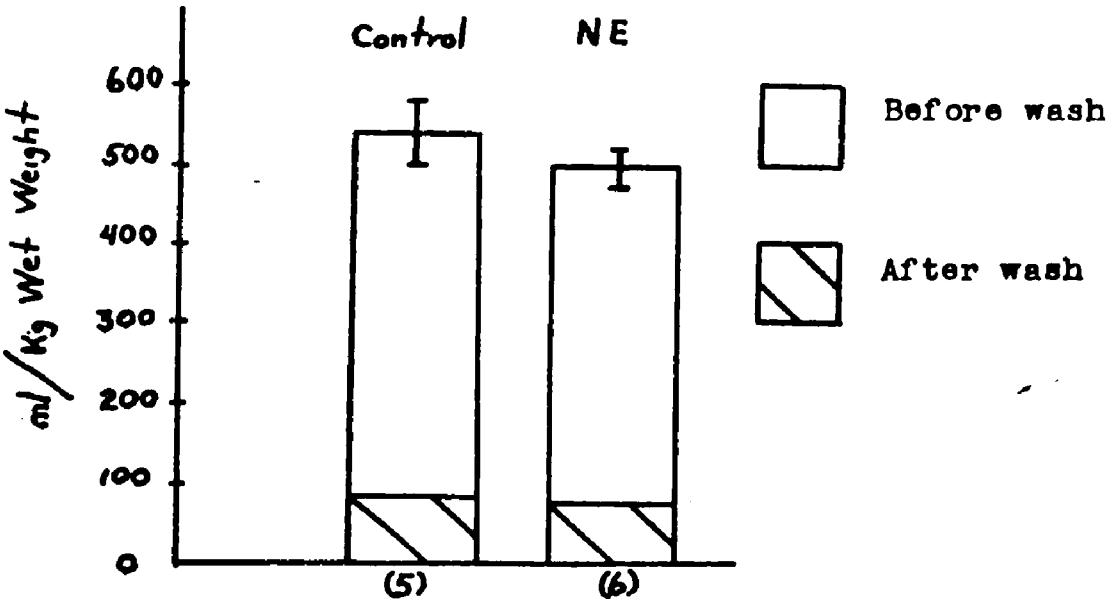

Fig. 4.-- Thirty minute sorbitol-^{14}C space before and after a five minute wash in non-radioactive solution. Values in () equal number of observations. Brackets S.E.M.

produced a significant change in total tissue calcium.

It has been reported by others that norepinephrine induced relaxation in intestinal smooth muscle is associated with an increase in the extracellular space (7). If norepinephrine causes a decrease in aortic extracellular space as a result of contraction, the rate of release of radioactive calcium during the last five minute wash in non-radioactive salt solution may be reduced. Such trapping of radioactive calcium may explain the increased calcium content seen in the presence of norepinephrine. To test this possibility, the washout of sorbitol-^{14}C was followed in the presence and absence of norepinephrine.

Figure four describes the thirty minute sorbitol space before and after a five minute wash in non-labeled salt solution. The sorbitol space after thirty minute incubation with norepinephrine is slightly smaller but the difference from control is not significant ($p=0.20$). The sorbitol space is the same after a five minute wash in non-labeled salt solution with or without norepinephrine. The observation that sorbitol-^{14}C is lost as rapidly in the presence and absence of norepinephrine suggests that norepinephrine does not cause an increase in the radioactive calcium content by decreasing the washout of radioactive calcium from the extracellular space.

Sodium-free medium

The effects of sodium-free medium on the radioactive calcium content and total tissue calcium as well as its

effect on the ability of norepinephrine (10^{-6} g/cc) to increase the radioactive calcium content is described in figure five. In these experiments the muscle strips were incubated in sodium-free medium for the total incubation time (two hours and thirty five minutes). As can be seen in figure five incubation in a sodium-free medium (no significant difference between LiCl or choline chloride substitution) causes a significant increase ($p<0.01$) in the radioactive calcium content but does not cause a significant change in total tissue calcium content. Norepinephrine is unable to cause a further increase in radioactive calcium content in a sodium-free medium.

Low temperature

Figure five also illustrates the effects of low temperature ($2^{\circ}C$) on radioactive calcium content and total tissue calcium content as well as its effect on the ability of sodium-free medium and norepinephrine to cause an increase in radioactive calcium content. As in the sodium-free experiments, the strips were exposed to $2^{\circ}C$ temperature for the total incubation time of two hours and thirty five minutes. Incubation at $2^{\circ}C$ produced a significant increase in radioactive calcium content ($p<0.01$) as well as total tissue calcium content ($0.05>p>0.02$). Neither sodium-free medium nor norepinephrine were able to cause a further increase in radioactive calcium content at $2^{\circ}C$.

As shown in figure one, within thirty minutes 84% of total tissue calcium has exchanged with radioactive calcium.

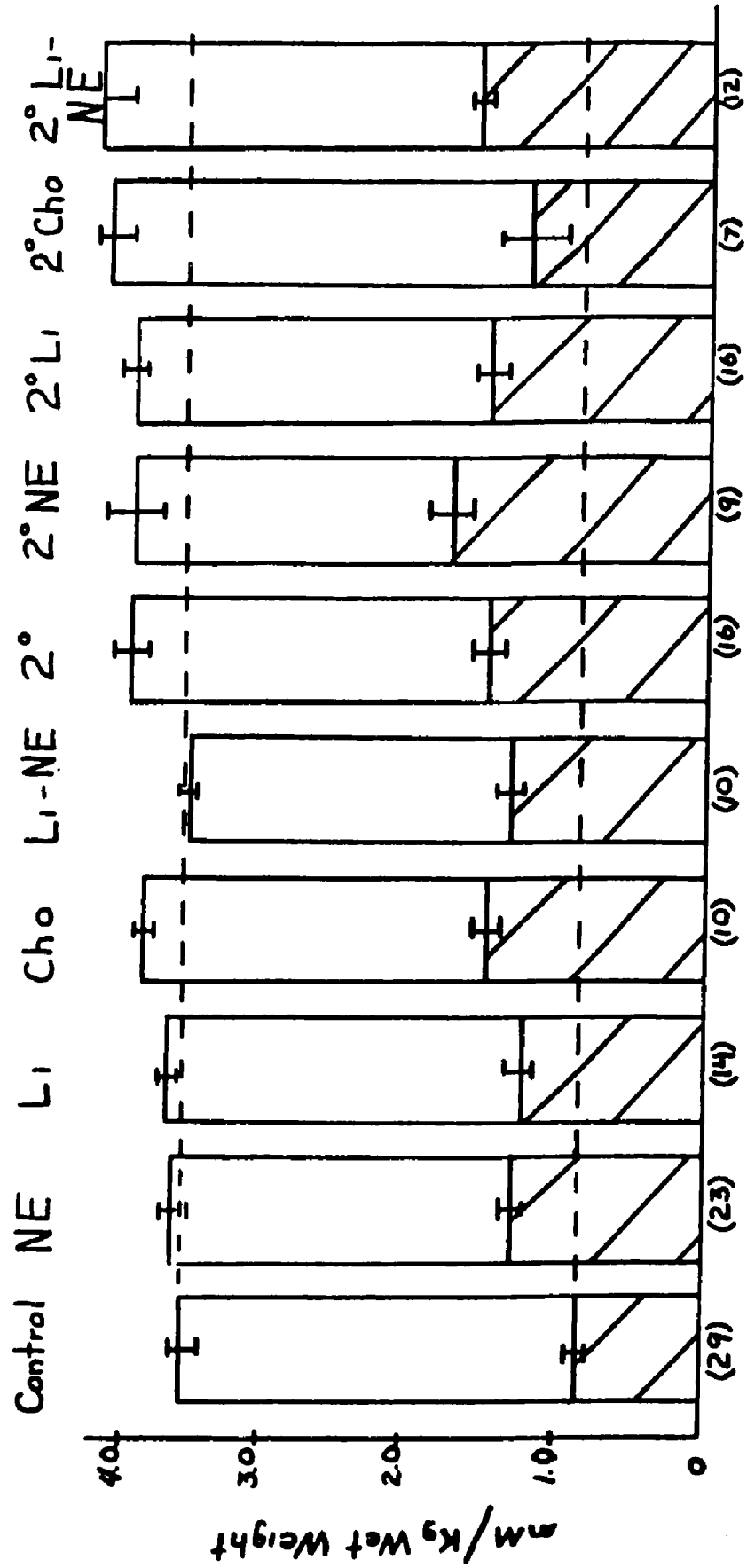

Fig. 5.--Effect of sodium-free medium and 2°C on radioactive calcium content and total calcium. Values in ( ) equal number of observations. Legend same as figure 3. Brackets S.E.M.

The changes in radioactive calcium content produced by drugs, sodium-free medium, or low temperature do not represent changes in calcium influx but represent changes in the equilibrium distribution of radioactive calcium. Changes in the equilibrium distribution may be brought about by changes in influx and/or efflux. To better be able to describe the effects of drugs, sodium-free medium and low temperature on calcium flux, calcium efflux was studied under various conditions.

Factors that influence radioactive calcium efflux

Normal radioactive calcium efflux

A typical curve of the loss of radioactive calcium from aortic strips into a medium of normal calcium concentration (1.6mM) is shown in figure six. There is an initial rapid loss of calcium which slows after approximately forty minutes, after which there is no further change in the proportion lost.per unit time. By the statistical technique of least squares and accepting a confidence interval no smaller than 0.99, bhe best fit line for the points after forty minutes was obtained. In four such experiments the average slope of the best fit line was $0.016 min.^{-1} \pm 0.001$ (S.E.M.). This slope represents the rate constant or fraction of calcium lost per minute. Modifying the above procedure so that during the first forty five minutes the tissues are washed in a calcium-free medium and in 1.6mM calcium for the last 75 minutes, transient changes in the rate constants are produced.

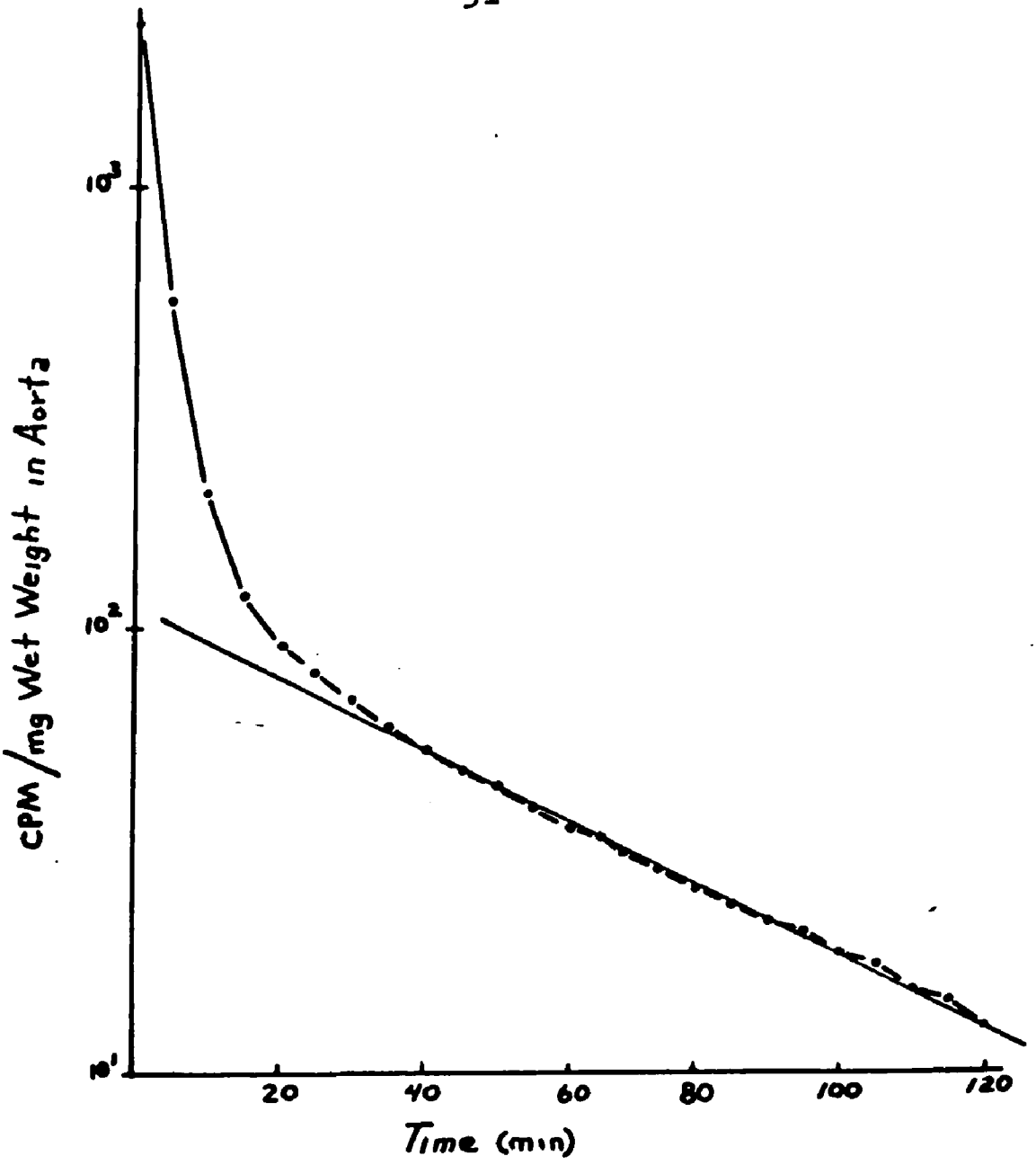

Fig. 6.--Washout of radioactive calcium into 1.6mM calcium salt solution.

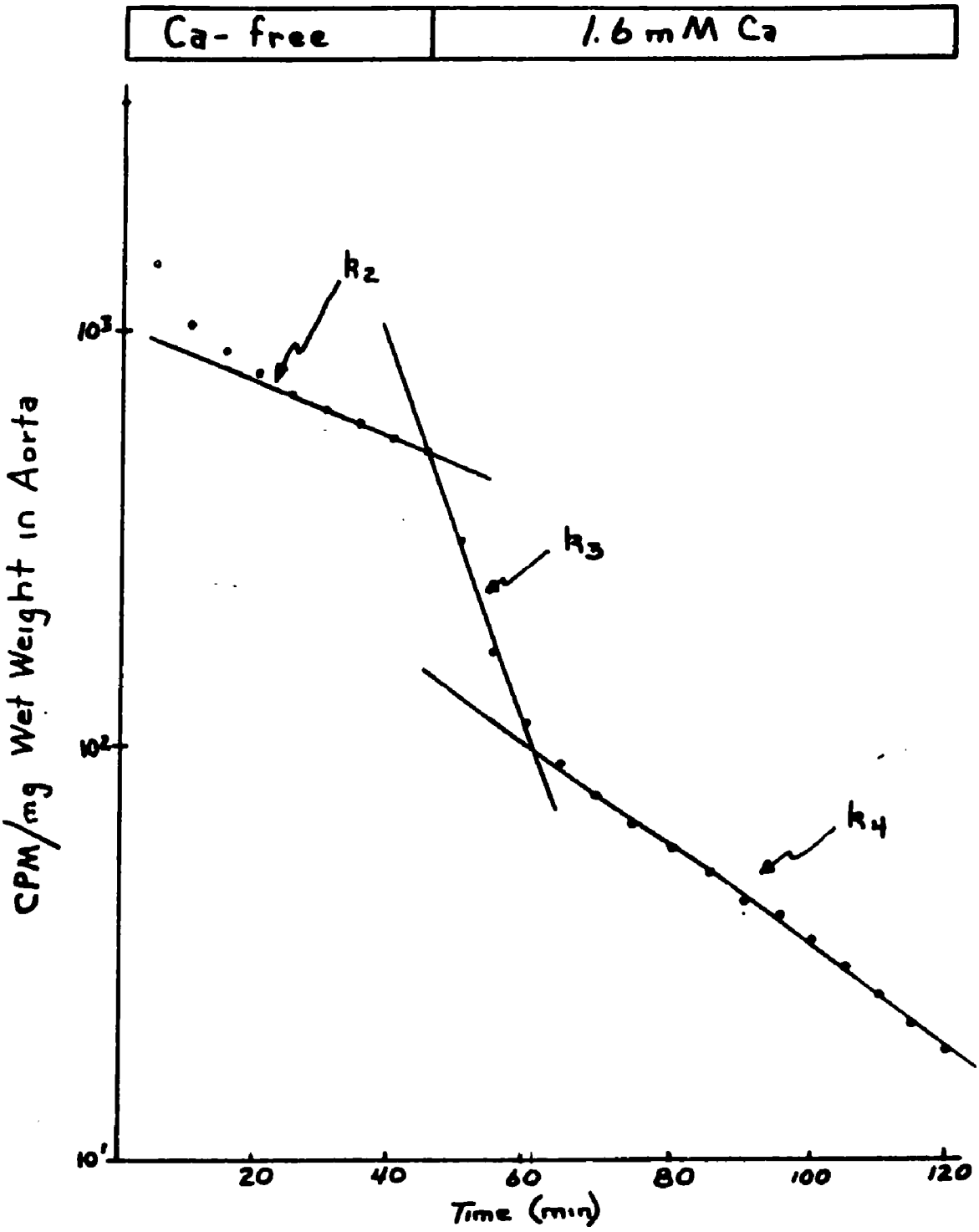

Fig. 7.--Washout of radioactive calcium into calcium-free and normal calcium medium.

Figure seven represents a typical curve from such an experiment. There is an initial rapid loss of radioactive calcium which reaches a constant proportion after twenty to twenty five minutes. I have arbitrarily designated the constant proportion lost in calcium-free medium as rate constant k_2. In the presence of normal extracellular calcium (from 45 minutes on) there is a rapid loss of radioactive calcium designated by rate constant k_3 followed by a slower loss for the remainder of the two hours designated by rate constant k_4. Twenty one such experiments indicate that rate constant $k_2 = 0.017 \pm 0.001 min.^{-1}$, rate constant $k_3 = 0.101 \pm 0.004 min.^{-1}$ and rate constant $k_4 = 0.028 \pm 0.001 min.^{-1}$. Measurement of total tissue calcium at the end of such experiments indicates that it equals the normal value of 3.58mmoles/Kg wet wt.. It is therefore surprising that rate constant k_4 does not equal the rate constant determined from a two hour washout in normal calcium (figure 6).

The washout technique used to produce figure seven provides a means to study the effect of various conditions on the loss of calcium into calcium-free medium and on the exchange of calcium associated with the muscle (extracellular and intracellular) with calcium in the bathing medium.

Extracellular calcium concentration

Experiments were performed to determine the effect of increasing extracellular calcium on rate constants k_3 and k_4. Figure eight summarizes these results. It can be seen that rate constant k_3 increases as extracellular calcium

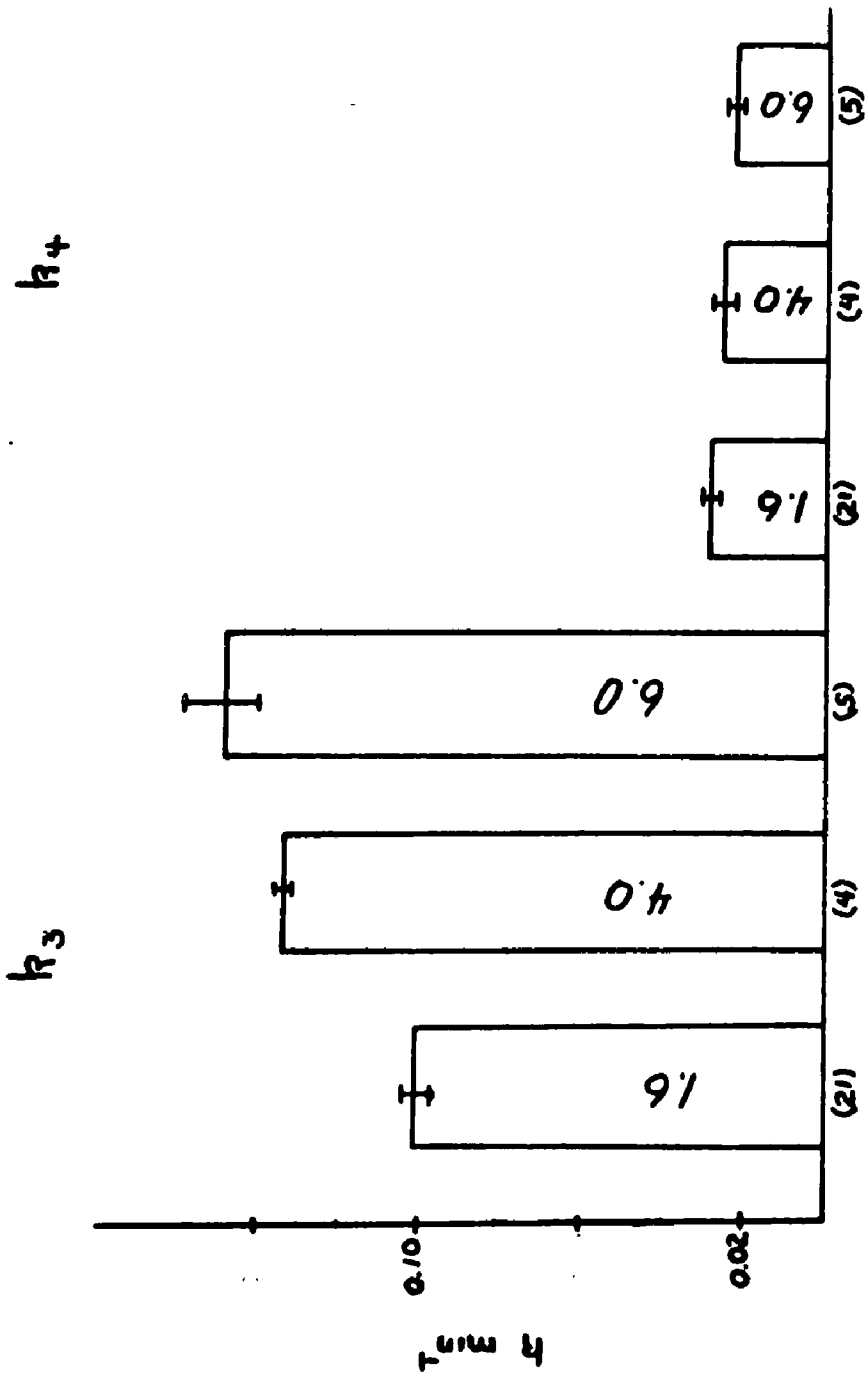

Fig. 8.--Effect of extracellular calcium on efflux rate constants. Values in ( ) equal number of observations. Brackets S.E.M.

increases. However, rate constant k_4 continues to decrease as calcium concentration increases (6mM significantly less than 4mM, $p = 0.05$). This suggests that the loss of calcium is influenced by extracellular calcium and that it may be coming from different compartments since rate constants k_3 and k_4 are affected differently by extracellular calcium.

If strips are washed in normal extracellular calcium (1.6mM) for forty five minutes and then exposed to 4mM calcium, there is no significant change in the slope of the washout curve. This suggests that during the first forty five minutes the calcium pool associated with rate constant k_3 has been depleted.

It appears that the efflux of calcium from strips of thoracic aorta can be thought of as coming from as many as three compartments. Loss of calcium from two of these compartments (k_3 and k_4) is influenced to a different extent by extracellular calcium. The rapid loss of calcium into a normal calcium medium (k_3) can be increased further by increasing the extracellular calcium, while the slow loss (k_4) is depressed by increasing extracellular calcium.

Drugs

Using the procedure as for figure seven, the effect of norepinephrine, isoproterenol, sodium-free medium and low temperature on rate constants k_2, k_3 and k_4 was determined. Figure nine illustrates the effects of norepinephrine and isoproterenol on these rate constants.

Norepinephrine (10^{-6} g/cc) causes a significant

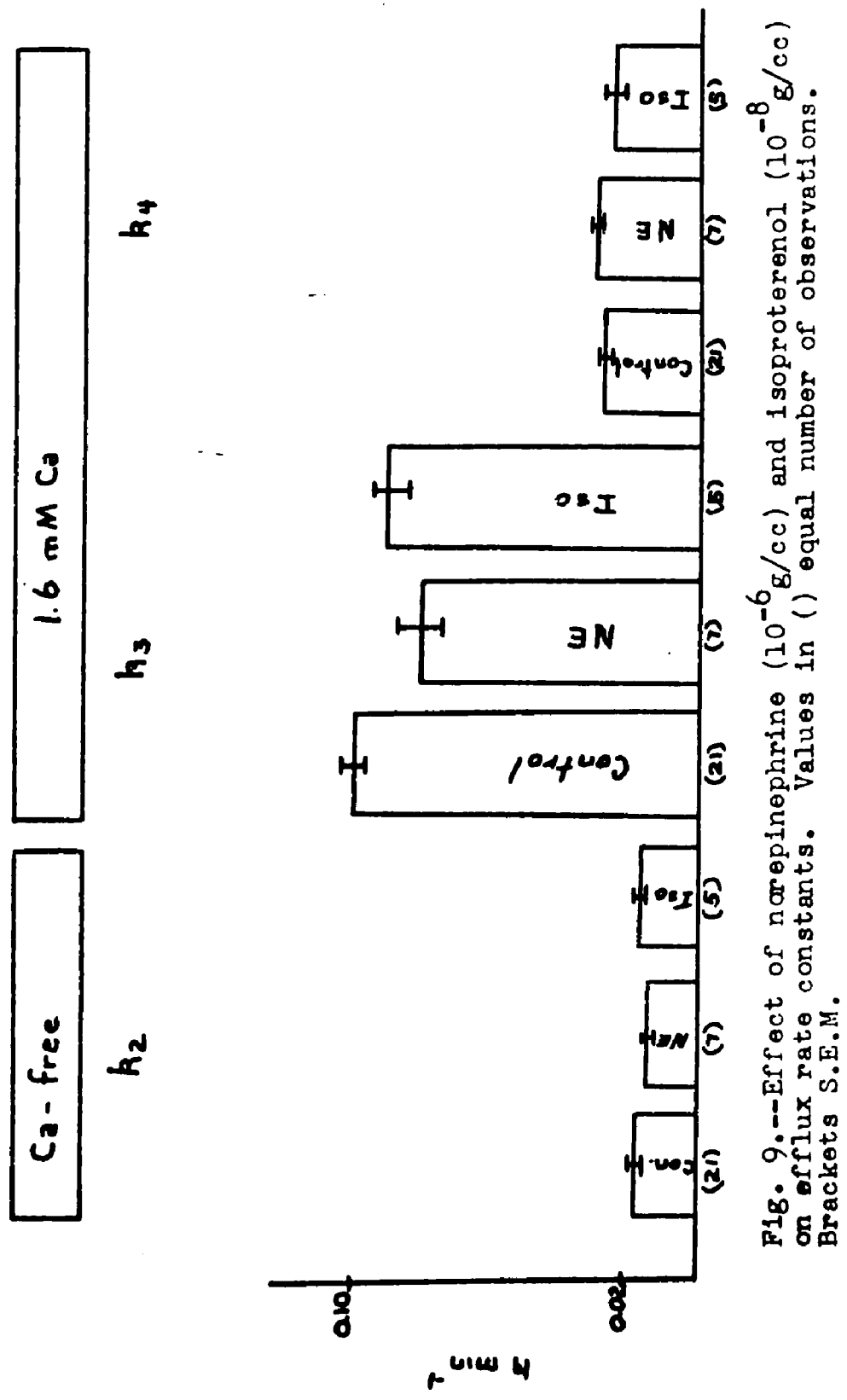

Fig. 9.--Effect of norepinephrine (10^{-6}g/cc) and isoproterenol (10^{-8}g/cc) on efflux rate constants. Values in () equal number of observations. Brackets S.E.M.

($p = 0.02$) decrease in the rate constant for washout of calcium into calcium-free medium (k_2 as well as a significant ($0.02 > p > 0.01$) decrease in the rate constant for the initial rapid washout of calcium into 1.6mM calcium medium (k_3). However, norepinephrine does not cause a significant change in rate constant k_4. Isoproterenol (10^{-8}g/cc) had no effect on any of the rate constants.

Sodium-free medium

The effect of sodium-free medium on rate constants k_2, k_3 and k_4 was determined on two different tissue preparations. In one preparation the strips were loaded with radioactive calcium in a normal sodium medium and the radioactive calcium washout followed in a sodium-free medium. Under these conditions no significant ($p > 0.05$) change in the rate constants was observed. In the second preparation the strips were loaded with radioactive calcium while in a sodium-free medium and the washout of radioactive calcium followed in a sodium-free medium. The results are illustrated in figure ten. Rate constants k_3 and k_4 were significantly decreased ($0.05 > p > 0.02$).

Low temperature

Figure eleven illustrates the effect of low temperature ($2^{\circ}C$) on rate constants k_2, k_3, and k_4. In these experiments the muscle strips were incubated at $2^{\circ}C$ during both the loading and efflux periods. Unlike either norepinephrine or sodium-free medium, $2^{\circ}C$ incubation causes a significant ($p < 0.01$) decrease in all three rate constants.

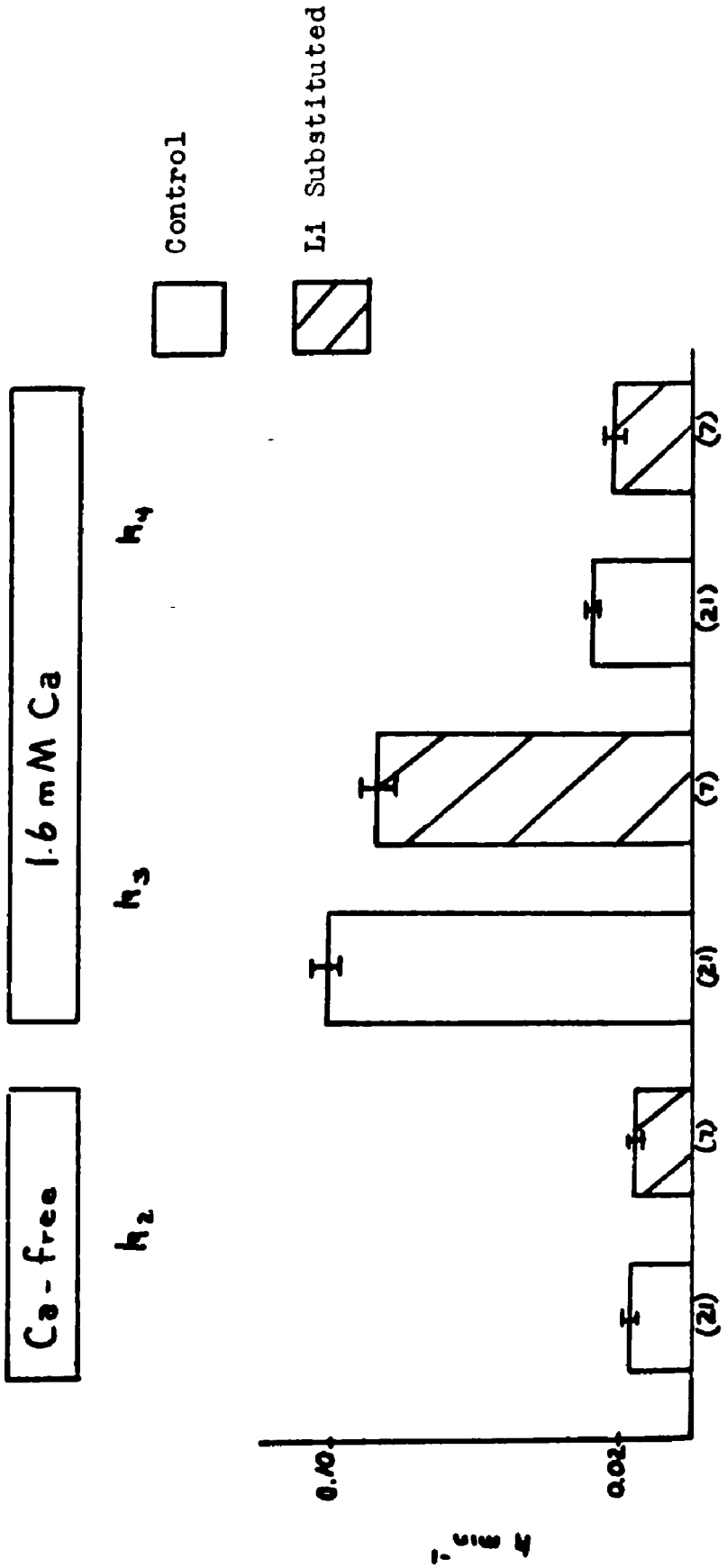

Fig. 10.--Effect of extracellular sodium on efflux rate constants. Values in ( ) equal number of observations. Brackets S.E.M.

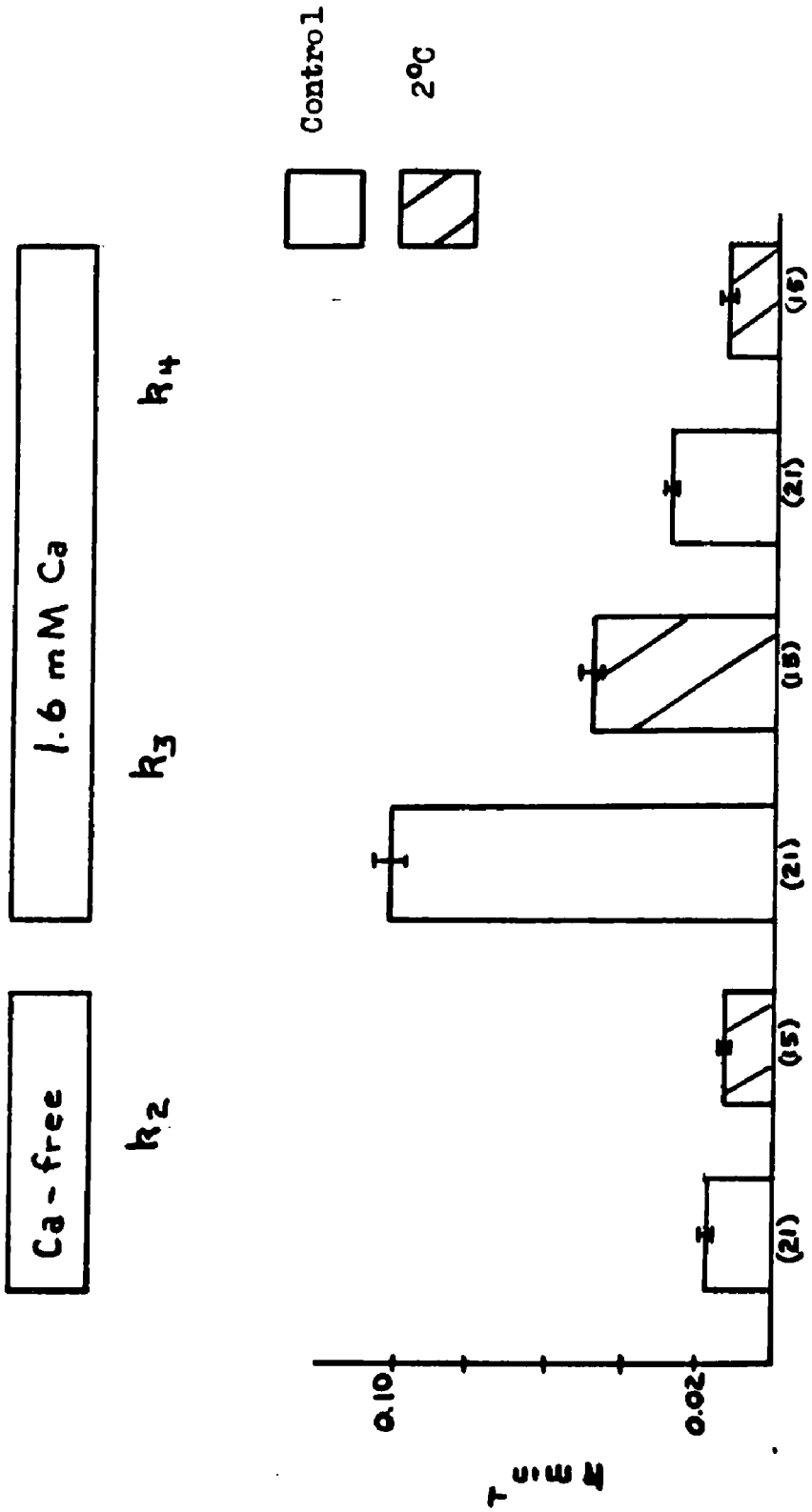

Fig. 11.--Effect of temperature on efflux rate constants. Values in ( ) equal number of observations. Brackets S.E.M.

DISCUSSION

Control equilibration and efflux of radioactive calcium

Figure one indicates that extracellular radioactive calcium equilibrates with total tissue calcium within ninety minutes. This means that all pools of calcium exchange with extracellular radioactive calcium and that there is not an unexchangeable pool as found by investigators in other types of smooth muscle (39).

Figure 12 is a typical cross section of the thoracic aorta used in these studies. The stippled area represents the adventitial layer and the non-stippled area the medial layer. Using planimetry it was determined that the medial layer represented 70% of the cross section while the adventitial layer represented 30%. It has been determined (25) that the medial layer of dog carotic artery is 56% smooth muscle cells with the remainder consisting of collagen and elastin. If one assumes that the medial layer of rabbit thoracic aorta contains at least the same percentage of smooth muscle cells, then 39% of the aorta is smooth muscle cells.

Since the smooth muscle cells are located in the medial layer, the calcium associated with this layer may be most important in control of contractility. Is it possible to associate one of the rate constants (figure 7) with washout

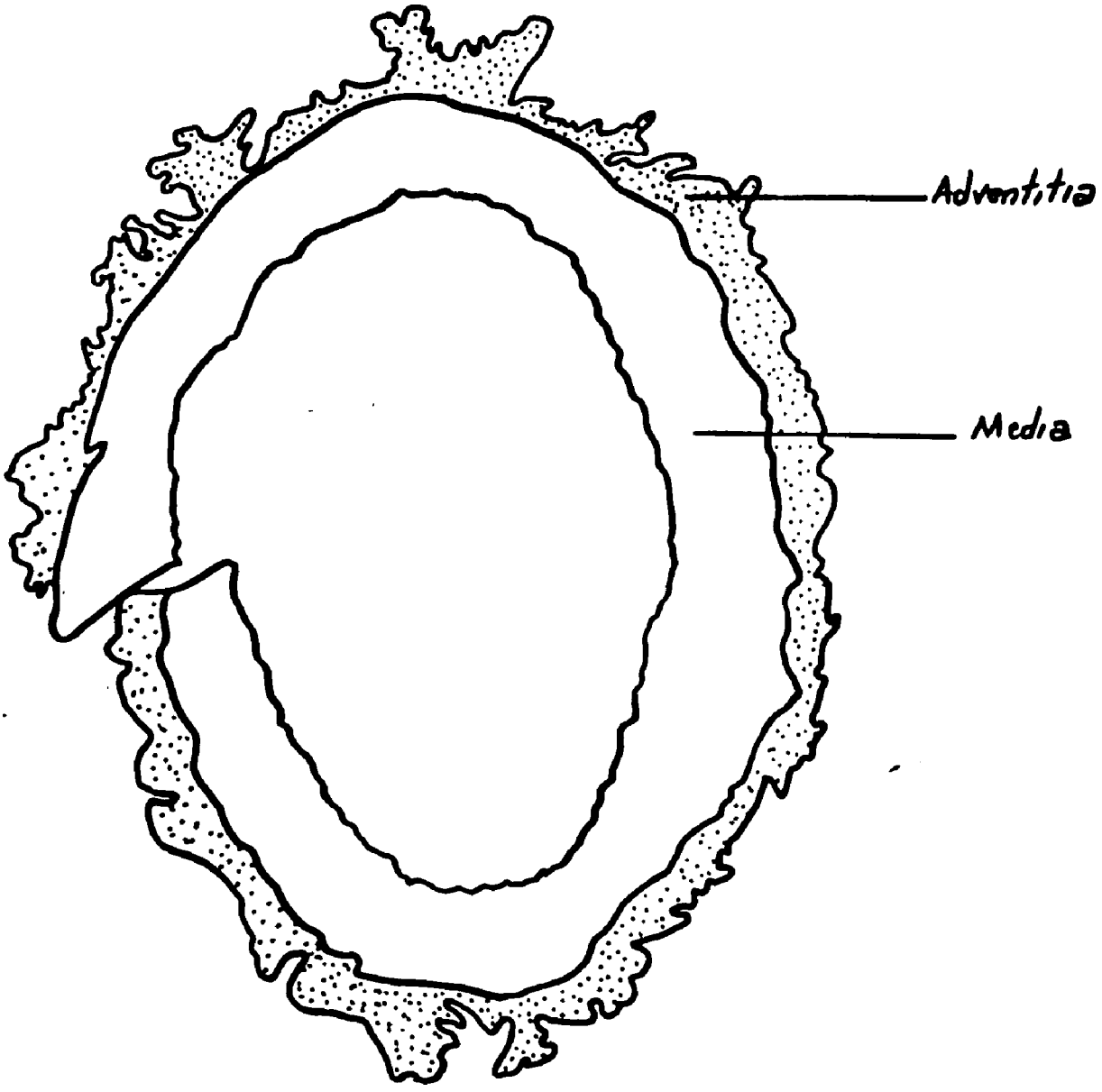

Fig. 12.-- Cross section of rabbit thoracic aorta

from adventitial and medial layers?

Hudgins and Weiss (20) studied the washout of radioactive calcium from isolated medial and adventitial layers of rabbit thoracic aorta. They observed that the washout of calcium from the adventitial layer into calcium-free salt solution was slightly faster than from the medial layer. The washout of whole aorta under the same conditions was identical to the washout from the medial layer. Upon addition of calcium to the washout medium there was a similar increase in calcium loss from both medial and adventitial. layers. The similarities of washout rates between adventitial and medial layers in either a calcium or calcium-free salt solution make it impossible to seperate these two pools of calcium in terms of rate when the washout from whole aorta is studied.

Even though the rates of washout from medial and adventitial layers are similar, one could study washout from the medial layer by washing the whole aorta sufficiently so that the calcium associated with the adventitial layer was insignificant compared to that remaining in the medial layer. This requires that the amount of calcium associated with the medial layer be much larger than the calcium associated with the adventitial layer.

Hudgins and Weiss (20) also compared the amount of radioactive calcium taken up by one gram of media with that taken up by one gram of adventitia and determined that 1.77 times more calcium is taken up by the media. Assuming that 70% of the aorta is media and the total tissue calcium

concentration is 3.58mmoles/Kg wet weight, calculations indicate that the media contains 3.19mmoles of calcium/Kg wet weight of aorta while the adventitia contains 0.39mmoles/Kg. Because of the large calcium content of the medial layer, it is possible that rate constant k_4 may represent the loss of calcium from the medial layer and therefore from the smooth muscle cells.

The pools of calcium described by rate constants k_2, k_3 and k_4 may not be bound superficially. EGTA (ethylene glycol bis(b-aminoethyl ether)-N,N'-tetraacetic acid) is thought to be unable to cross the cell membrane (20, 39) and therefore, only forms a complex with superficial calcium. If the pools of calcium which washout at rate constant k_2, k_3 or k_4 are superficial they should be removed by exposure to EGTA and the subsequent addition of extracellular calcium should not cause a change in the rate constant of radioactive calcium efflux. Exposure of strips for 45 minutes to 0.1mM EGTA does not change the percent of calcium lost into calcium-free medium or remove the calcium desplaced by extracellular calcium. Figure 13 represents one of three such experiments. This suggests that the calcium lost at rate constant k_2, k_3 or k_4 is intracellular calcium.

Further evidence in favor of the suggestion that k_2, k_3 and k_4 describe the washout of intracellular calcium is derived from a comparison of the amount of extracellular radioactive calcium present at the begining of washout with the amount of calcium lost during the initial washout period. Within the first five minutes of washout an amount of

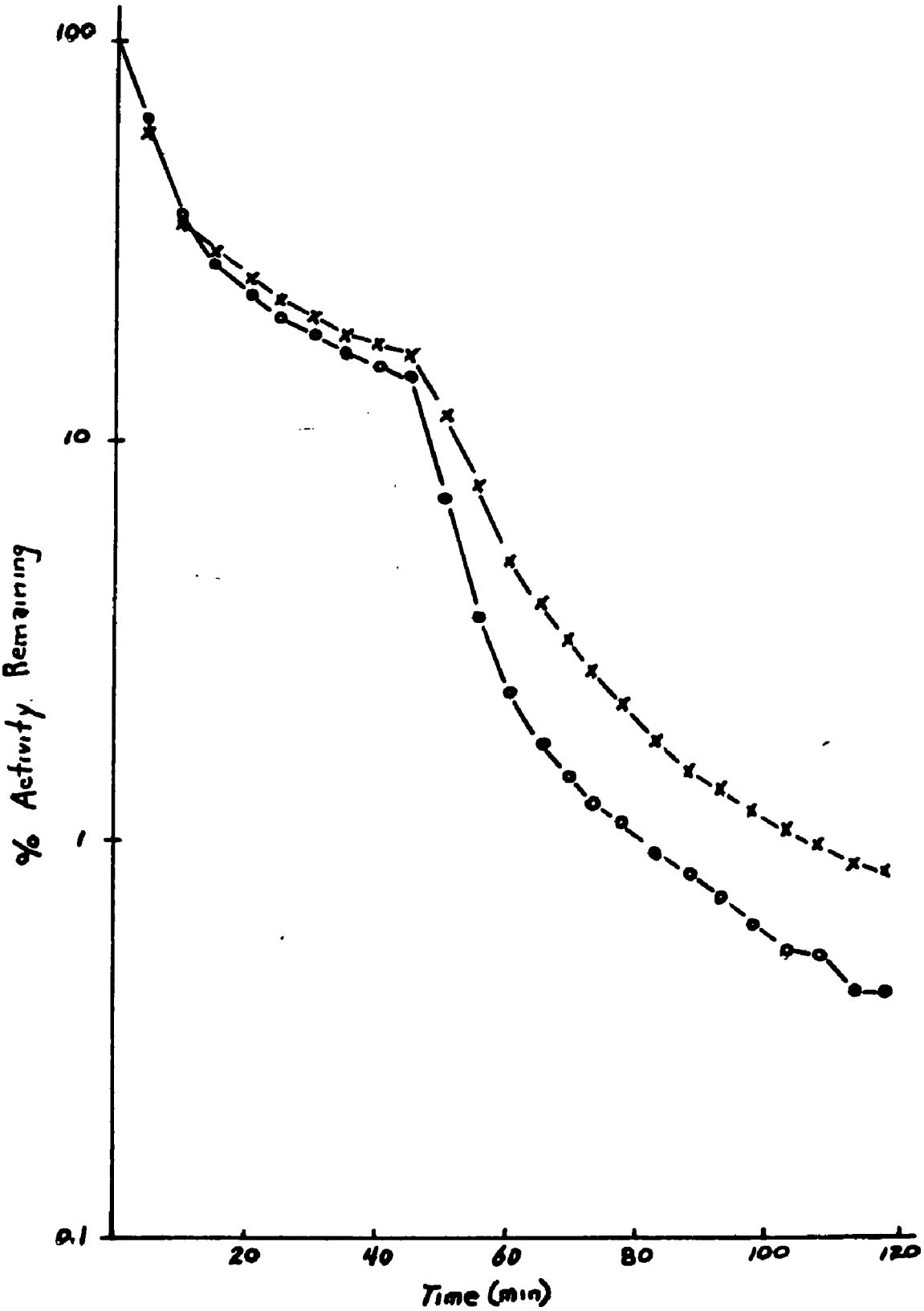

Fig. 13.--Percent of activity remaining in muscle during washout into EGTA-1.6mM Ca (X) or Ca-free-1.6mM Ca (o).

radioactive calcium is lost which is equal to the amount of radioactive calcium present in the extracellular space. This suggests that all calcium lost after the first five minutes is derived from non-extracellular space.

The increase in k_3 with increasing extracellular calcium (figure 8) is characteristic of release from a pool of bound calcium or the exchange of calcium across the cell membrane via a mediated transport system. As extracellular calcium concentration increases, the proportion of radioactive calcium lost would increase if either system existed. The present data does not differentiate between the two systems. However, the lack of effect of EGTA discussed above suggests that if the pool of calcium exchanging with extracellular calcium at rate constant k_3 is bound, it may be bound intracellularly.

The effect of calcium concentration (figure 8) on k_4 can be interpreted in at least two ways. Measurement of the total tissue calcium by atomic absorption spectrophotometry at the end of an efflux experiment indicates that the non-extracellular calcium (extracellular calcium subtracted) increases with increasing extracellular calcium concentration. The fact that k_4 decreases under these conditions may be interpreted as indicating the existence of a mediated transport system. If there is a limited number of avenues by which radioactive calcium may leave the cell, that is a limited number of carrier molecules, an increase in intracellular calcium would change the competition between radioactive and non-radioactive calcium molecules in favor

of non-radioactive calcium. Such a change in competition would reduce the number of radioactive calcium molecules leaving and therefore, rate constant k_4.

An alternative explanation may be related to the membrane permeability. If the membrane permeability of the cells is the same after the tissues are washed in normal extracellular calcium (1.6mM) for two hours (figure 6) as it is after washing for 75 minutes in normal calcium (figure 7), I would expect that the rate constant from figure 6 would equal k_4 determined in the presence of 1.6mM calcium (figure 7). The observation that they are not equal suggests that the membrane permeability is not the same in both types of experiments. It has been generally accepted that decreased extracellular calcium increases membrane permeability. It is possible that exposing the strips to calcium-free salt solution (figure 7) changes the membrane permeability and that this permeability has not returned to normal when rate constant k_4 is determined. As extracellular calcium concentration is increased, the permeability may be returned to normal more rapidly which may explain why with increasing extracellular calcium rate constant k_4 approaches the rate constant determined in figure 6.

In summary, the only conclusions that can be drawn are that rate constant k_2 describes the washout of calcium in the absence of extracellular calcium and rate constants k_3 and k_4 describe the exchange of non-radioactive calcium with radioactive calcium. The observation that k_2 is smaller than k_3 or k_4 indicates that radioactive calcium exchanges

less readily with other ions in the calcium-free solution than with calcium. I interpret the existence of two rate constants, k_3 and k_4 as indicating that a pool of calcium, which washes out at rate constant k_3, has been depleted and the washout from another pool, at rate constant k_4 remains.

Effect of sodium

In the presence of sodium-free medium there is an increase in the radioactive calcium content (figure5) and a decrease in the efflux rate constants k_3 and k_4 (figure 10). Since there is no change in k_2 there is no sodium outside-calcium inside exchange system in rabbit thoracic aorta. If there was a sodium inside-calcium outside exchange system, one would expect that radioactive calcium content would decrease in the absence of sodium. The increased content observed suggests that such a system does not exist.

Combining the observation that sodium-free salt solution increases radioactive calcium content with the observation that it decreases its exchange with extracellular calcium suggests that the lack of sodium impairs calcium efflux. Since sodium-free medium only affects calcium efflux in the presence of extracellular calcium, I suggest that sodium acts to couple a calcium outside-calcium inside exchange system. In the absence of sodium, calcium is able to enter the tissue, but is less able to leave. This results in an increase in cellular calcium. The sodium needed to couple calcium-calcium exchange is lost slowly for, sodium-free medium only decreased k_3 and k_4 after the muscle strip

had been exposed to sodium-free medium during both loading and washout periods (a total of 3hours and 45 minutes). The observation that prolonged exposure to sodium-free medium is necessary to produce a change in rate constants suggests that the pool of sodium involved is probably not extracellular sodium but is intracellular sodium or sodium bound to the cell membrane.

In a recent paper (3) it has been demonstrated that removal of extracellular sodium produces an increase in tension of rabbit thoracic aortic strips. This suggests that the increased radioactive calcium content observed in sodium-free medium (figure 5) is available for activation of the contractile proteins.

Effect of norepinephrine and isoproterenol

In the presence of norepinephrine (10^{-6}g/cc) there is a significant increase in the radioactive calcium content (figure 3), in total tissue calcium content after four hours exposure and a significant decrease in rate constants k_2 and k_3 (figure 9). The observation that radioactive calcium is 84% equilibrated with total tissue calcium within thirty minutes (figure 1) means that the radioactive calcium content as measured for figure three does not reflect changes in calcium influx only, but also reflects changes in the calcium equilibrium distribution. The increased radioactive calcium content seen in the presence of norepinephrine could therefore be due to an increase in uptake or a decrease in efflux. If one assumes that the small decrease in rate

constant k_2 produced by norepinephrine is truly significant, the observation that total tissue calcium increases in the presence of norepinephrine suggests that norepinephrine acts to decrease efflux rather than to increase influx.

The decline of the mechanical response to norepinephrine in a calcium-free salt solution is slow (figure 14) suggesting that the calcium utilized by norepinephrine is firmly bound. The observation that 50% of the original tension remains at a time (45 minutes) when only 15% of the radioactive calcium remains suggests that the pool of calcium utilized by norepinephrine may be small relative to the total tissue calcium.

The observation that norepinephrine is unable to cause a further increase in radioactive calcium content in sodium-free medium suggests that sodium-free medium and norepinephrine may be working on the same calcium-calcium exchange system. The lack of sodium uncouples the exchange system so that efflux is reduced. Since efflux is already reduced, norepinephrine is unable to reduce it further and there is no further increase in calcium content. However, it is possible that both sodium and norepinephrine are working at different sites with each site producing similar changes in calcium flux.

Isoproterenol (10^{-8}g/cc) caused a significant decrease in the radioactive calcium content (figure 3) but no change in total tissue calcium (figure 3) or efflux rate constants (figure 9). The observation that radioactive calcium content decreases without a change in efflux suggests that

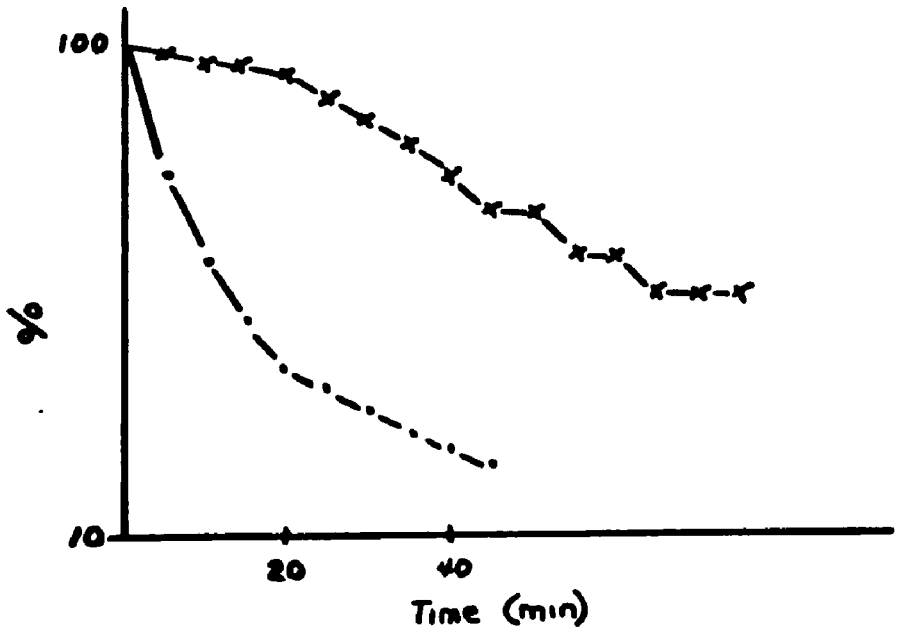

Fig. 14.--Comparison of the percent of control tension remaining (X) with the percent of radioactive calcium remaining (o) as the length of exposure to calcium-free medium increases.

isoproterenol causes a decrease in calcium influx. If this is true, one would expect that the washout of calcium in the presence of extracellular calcium (k_3 and k_4) would also be reduced if extracellular calcium could not exchange with the radioactive calcium. There was a decrease in both k_3 and k_4 in the presence of isoproterenol but the decrease was not significant.

Effect of low temperature

When the temperature is lowered to 2°C there is an increase in total tissue calcium (figure 5), an increase in radioactive calcium content (figure 5) and a decrease in all three rate constants (figure 11). These changes suggest that low temperature reduces efflux instead of stimulating influx. Low temperature appears to depress k_3 and k_4 more than k_2. This may mean that the calcium-calcium exchange system is more temperature sensitive than the system involved with the exchange of other ions in solution with calcium. The decrease in rate constant produced by low temperature is insufficient to indicate an energy requiring efflux process. I suggest that calcium efflux is driven in part by the energy supplied by the movement of extracellular calcium into the cell down its electrochemical gradient. Like sodium-free medium, 2°C uncouples this calcium-calcium exchange system and in addition reduces the exchange of calcium with other ions in solution (k_2).

It has been observed (24) that sheep carotid arteries contract when the temperature of the medium in which they

are bathed is lowered from 35°C to 5°C. After 10-15 minutes the contraction dies away and at this time norepinephrine is unable to cause its usual mechanical response of contraction. These observations suggest that like sodium-free medium the increased radioactive calcium content (figure 5) produced by low temperature is available to activate the contractile proteins. However, with prolonged exposure to low temperature metabolic inhibition may be sufficient to block further maintenance of tension and the initiation of tension by norepinephrine.

Conclusion

In conclusion (figure 15) I feel that the data indicates that there is neither a sodium-inside calcium-outside nor a sodium-outside calcium-inside exchange system in rabbit thoracic aorta but a calcium-calcium exchange system. The function of this exchange system is to match calcium efflux with the influx of calcium produced by the movement of calcium down its electrochemical gradient. Sodium acts to couple the exchange and in the absence of sodium calcium influx proceeds, but efflux is reduced. This exchange system does not require a direct supply of metabolic energy, but may be driven in part by the movement of extracellular calcium down its electrochemical gradient. Norepinephrine causes an increase in intracellular calcium and therefore tension by uncoupling the exchange system so that efflux is reduced. Isoproterenol causes a reduction in intracellular calcium and therefore relaxation by decreasing calcium influx.

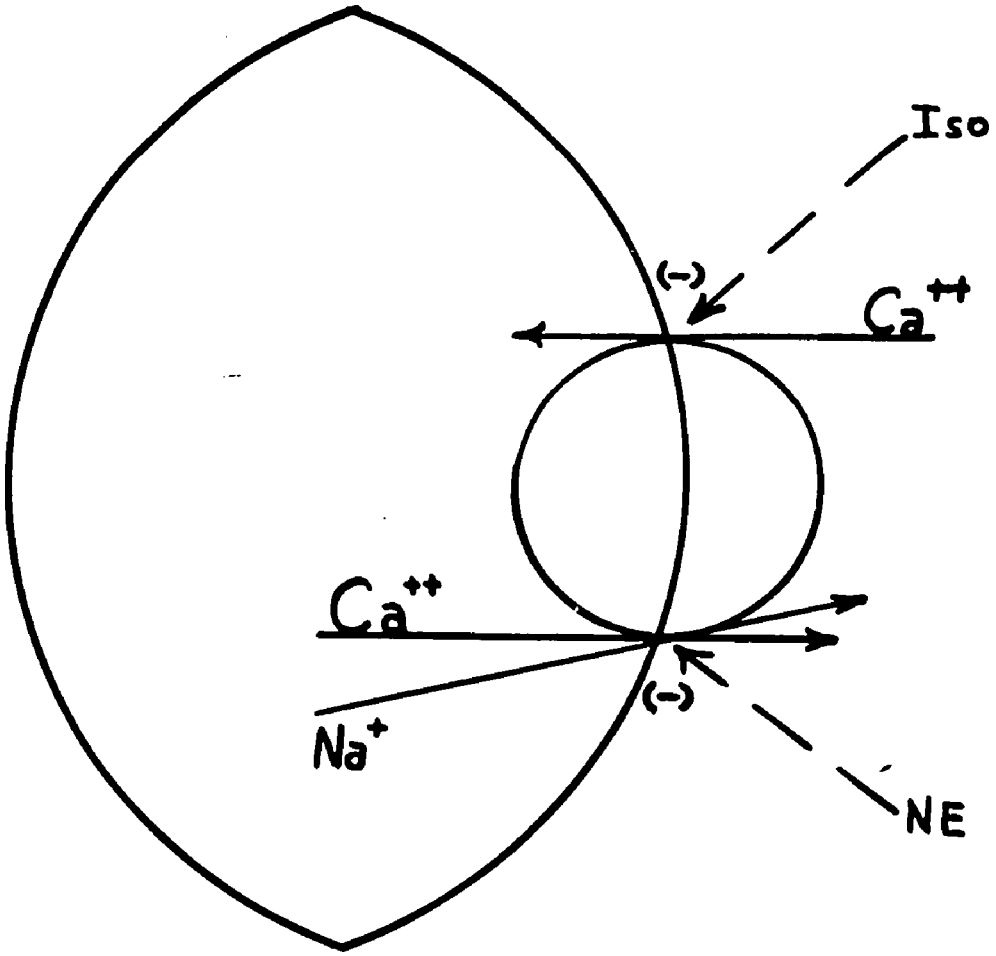

Fig. 15.-- Proposed schema for the control of intracellular ionic calcium concentration.

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