A Study of Releasable Ca Fractions in Smooth Muscle Cells of the Rabbit Aorta

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ABSTRACT The distribution of Ca in the cellular compartment of smooth muscle cells of the rabbit aorta has been studied by analyzing the effect of norepinephrine, caffeine, and DNP on ^{45}Ca exchange and on the pattern of tension development. These three substances increase the release of ^{45}Ca from the tissue, but DNP acts more slowly than norepinephrine or caffeine. Also, the effect of norepinephrine and caffeine on tension development occurs almost immediately, while that of DNP appears only after a delay of 5 min. Study of the effect of these substances on the Ca efflux has shown that norepinephrine and caffeine act probably on the same Ca compartment, while DNP seems to act on a different compartment with a slower exchange rate. The difference between these two pools could be further demonstrated by studying Ca release after loading the tissues with tracer in either K-rich solution or in a solution with reduced $[Ca]_0$. The K depolarization results in an excessive loading of the cells with ^{45}Ca. Exposing these cells during the efflux procedure to a solution containing DNP causes a much larger release of ^{45}Ca than that observed after a loading procedure in normal solution. In contrast, the release of ^{45}Ca elicited in such tissues by norepinephrine or caffeine disappears. This disappearance is due to the prolonged increase of the Ca exchangeability induced by K depolarization. During initial exposure to PSS the increased exchangeability causes an accelerated loss of tracer from the tissue compartment on which norepinephrine and caffeine act, while the DNP sensitive compartment is not affected. It is suggested that noradrenaline and caffeine act on the same calcium pool close to the membrane and that DNP acts mainly on the mitochondria.

INTRODUCTION

A number of intracellular binding sites for Ca have already been identified in smooth muscle. These include the sarcoplasmic reticulum (Somlyo and Somlyo, 1971; Devine et al., 1973), mitochondria (Somlyo and Somlyo, 1971; Batra, 1975; Vallières, et al., 1975) and the plasma membrane (Devynck et al., 1973; Popescu et al., 1974; Debbas et al., 1975). However, up till now it has not been possible to clarify which role each of these structures plays in determining the cytoplasmic calcium concentration.

Recent studies of the Ca binding properties of smooth muscle microsomes (Carsten, 1969; Batra and Daniel, 1971; Fitzpatrick et al., 1972; Baudouin et al., 1972; Hess and Ford, 1974), purified membrane fractions (Hurwitz et al., 1973; Devynck et al., 1973), and isolated mitochondria (Batra, 1975; Vallières et al., 1975) have not yielded conclusive answers. This is largely due to uncertainty about the origin and purity of the microsomes and to the limited coupling

efficiency of the mitochondria (Vallières et al., 1975). Moreover, from such experiments it is not possible to infer with certainty the contributions of these organelles to Ca homeostasis in the intact cells.

Studies of tension development in arterial smooth muscle have led to the hypothesis that the phasic response to norepinephrine, histamine, and angiotensin depends on the release of an intracellular Ca fraction (Hinke et al., 1964; van Breemen, 1969; Steinsland et al., 1973; Deth and van Breemen, 1974). An influx of Ca accompanying such stimulation apparently serves to replenish the released Ca fraction and induces a tonic contraction. This entry of Ca has been demonstrated by studying the uptake of ^{45}Ca into the cellular compartment (Deth and van Breemen, 1974). However, the release of Ca from its intracellular stores is not amenable to uptake studies. A possible approach to this problem is the analysis of ^{45}Ca effluxes, but such data do not yield a pattern that would allow the identification of different cellular fractions. This is not surprising in view of the number of potential ^{45}Ca sources, both intra- and extracellular. Retention of ^{45}Ca in the extracellular compartment can be minimized by a medium containing Ca-EGTA (van Breemen and Casteels, 1974) or ionized Ca. With either medium, highly reproducible efflux data can be obtained.

We have compared the effect on ^{45}Ca efflux of three substances which are known to release intracellular calcium: 2,4 dinitrophenol; norepinephrine; and caffeine. In addition, we have studied the relation between release from and uptake into the cellular compartment. From these data we have tried to characterize the respective compartments on which these substances act and to correlate the movements of ^{45}Ca with the observed contractile behavior.

MATERIALS AND METHODS

Tissue Preparation

Rabbits weighing 2–3 kg were sacrificed by a blow to the neck and bled. The thoracic aorta was rapidly excised, transferred to a dissection bath containing normal physiological salt solution (see below) bubbled with oxygen, and kept at 37°C. The aortas were cleansed of adherent fat and connective tissue and divided into 16–20 cross-sectional rings. Contraction experiments were carried out with such rings. For efflux and influx studies the rings were cut open into strips each weighing about 15 mg. Before any experiments were begun, tissues were allowed to equilibrate for 1 h.

Solutions

The normal physiological solutions (PSS) we used for most experiments contained (mM): NaCl, 160; KCl, 4.5; $CaCl_2$, 1.5; $MgCl_2$, 1.0; D-glucose, 10; HEPES, 5. The pH of all solutions was kept at 7.2. In some experiments a K^+-rich solution (144.5 mM K) was used, obtained by substituting 140 mM KCl for an equivalent amount of NaCl. The drugs added to these solutions were norepinephrine (NE) at concentrations varying between 10^{-8} and 10^{-4} M, caffeine at 10^{-2} M, and 2,4 dinitrophenol (DNP) at a concentration of 10^{-4} M.

^{45}Ca Efflux Measurements

For routine efflux experiments the tissues were loaded for 3 h in 10 ml normal physiological solution containing 1.5 mM labeled Ca (5×10^6 cpm/ml).

We also used two additional loading procedures. The first of these consisted of a 20-

min exposure to a large volume (150 ml) of physiological saline which had a reduced Ca concentration (0.2 mM) but which was also labeled with 5×10^6 cpm/ml ^{45}Ca. This loading procedure was used to decrease the nonspecifically bound label, and also served to reduce the amount of ^{45}Ca penetrating into the different intracellular compartments. The second of the procedures consisted of a sequential exposure for 2 h to radioactive PSS containing 1.5 mM Ca, for 45 min to a K-rich solution with the same concentration and specific activity of Ca, and finally for 15 min to the initial labeled PSS. This procedure was designed to increase the amount of ^{45}Ca in the cellular compartments by the K depolarization. The purpose of the final exposure to labeled PSS was to eliminate an interference of increased external K with further experimental procedures.

Before the efflux was started, the tissues were rinsed for 5 s in a large volume of PSS to remove the adherent loading solution. Thereafter they were transferred at 5- or 10-min intervals through a series of previously washed plastic scintillation vials containing the efflux medium. The tissues were allowed to move freely in 5 ml of PSS containing 1.5 mM Ca and bubbled by a Teflon catheter with O_2. It was found that a transfer of the tissue by means of a small forceps did not cause any efflux artefacts. The transfer of extraneous label by the Teflon catheters was minimized by wiping them during the transfer. In experiments comparing the effects of norepinephrine, DNP, and caffeine, strips of the same aorta were used.

The solutions used for efflux contained Ca to maintain normal physiological conditions and to avoid possible deleterious effects on membrane function of a sustained exposure to Ca-free solution. In such solutions there occurs an exchange of ^{40}Ca with ^{45}Ca, at extracellular and intracellular binding sites, rather than a net loss of tissue calcium.

At the end of the efflux experiment tissues were blotted, weighed, and placed in scintillation vials containing 1 ml of 30% H_2O_2. They were then digested by warming in an oven overnight at 95°C. This digest was then dissolved in 5 ml of isotonic NaCl containing 5 mM EDTA. The amount of ^{45}Ca was determined by liquid scintillation counting using a scintillation mixture described by Patterson and Greene (1965). The quenching by DNP was minimized by the addition of 100 μl of 1 N HCl to each sample. The small residual quenching (<3%) was determined by using an internal standard.

Cellular ^{45}Ca Uptake

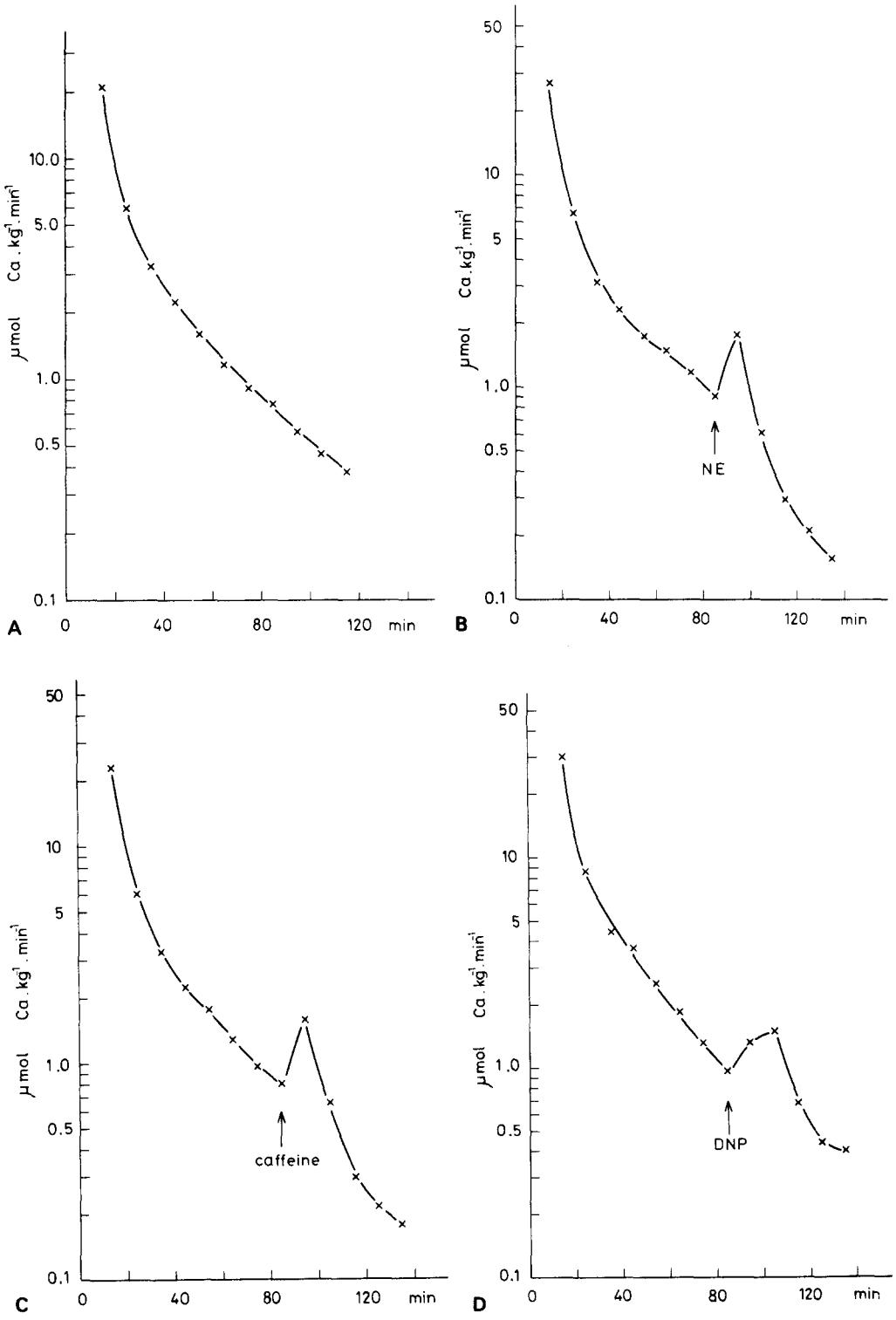
^{45}Ca uptake by the cellular compartment was measured by the lanthanum (LA) method as described by van Breemen et al., (1972). After exposure of the aorta strips to the radioactive solutions, they were rinsed in solutions containing 10 mM $LaCl_3$ for 1 h, weighed, digested, and counted as described above.

Contraction Experiments

Aorta rings were mounted in an organ bath (8 ml) between a fixed glass rod and an isometric force transducer. An initial passive tension of 2 *g* was applied to the rings and during the equilibration period of 1 h the bathing solution was changed at 20-min intervals. The solutions used were identical to those used in the flux experiments and addition of drugs was made directly in the organ bath, stirring being provided by the bubbling action of O_2.

RESULTS

Aortic strips which have been loaded for 3 h in PSS containing 1.5 mM Ca exhibit a typical efflux pattern, as shown in Fig. 1*a*. The addition of 10^{-5} M norepinephrine, 10^{-2} M caffeine, or 10^{-4} M 2,4-dinitrophenol is shown, respectively, in Fig. 1*b*, *c*, and *d*. Addition of norepinephrine and caffeine increases the


μmol Ca.kg⁻¹.min⁻¹
10.0
5.0
1.0
0.5
0.1
0
40
80
120
min
A
50
10
5
NE
B
caffeine
C
DNP
D

rate of efflux to its maximum within 10 min. Earlier experiments using efflux intervals of 2 min showed the peak to be between 2 and 4 min. In contrast, the response to DNP appears more slowly, reaching a maximum only after 20 min. A similar difference in the time course of action is found for the contractile effect of these substances. Norepinephrine and caffeine affect tension development almost immediately; norepinephrine causes a rapid and large increase of tension development, while caffeine induces a small transient response. DNP increases the tension development after a delay of 5 min (Fig. 2 *left*).

Relation between the Amount of ^{45}Ca Released and the Contractile Response Induced by Norepinephrine

Strips of the same aorta were stimulated after 60 min of efflux with different concentrations of norepinephrine. The amount of ^{45}Ca released by NE (μmol/kg wet wt) was estimated from the area above the curve obtained by extrapolating the rate measured before stimulation and plotted on linear coordinates. Other tissues were tested for the contractile response to the same norepinephrine concentrations. Both sets of data have been expressed as a percentage of the maximum response and they are represented in Fig. 3. Some parallel between stimulation of ^{45}Ca efflux and contraction is observed. In the aorta used for these experiments both contraction and ^{45}Ca release reached their maximum only at 10^{-4} M norepinephrine and no response could be observed at 10^{-8} M.

^{45}Ca Exchange in the Compartments Affected by NE, Caffeine, and DNP

In order to define the compartments affected by these three substances we determined the amount of ^{45}Ca released after 60, 80, 100, or 120 min of efflux by either norepinephrine or caffeine or by DNP. Since during the efflux procedure ^{45}Ca present in the cellular compartment exchanges with ^{40}Ca, the amount of ^{45}Ca released by one of the substances should decrease as a function of its rate of exchange with the external medium. An estimate of this rate of exchange might give some indication as to the identity of these compartments. By plotting the logarithm of these amounts of ^{45}Ca as a function of time at which the stimulus was applied we can estimate an exchange rate. This value should be similar for different pharmacological substances acting on the same compartment. In order to facilitate the comparison of these data, the effect of norepinephrine was determined for strips of each aorta and then compared to the effect of DNP or of caffeine in analogous aorta strips. The averages of these calculated exchange rates after a 3-h loading period in PSS containing 1.5 mmol Ca are summarized in Table I*a*. From these data it can be concluded that the ^{45}Ca of the compartments on which NE and caffeine act exchanges at a similar rate, while the rate of the DNP-sensitive compartment is significantly lower.

FIGURE 1 (opposite). Effects of norepinephrine, caffeine, and DNP on the rate of ^{45}Ca efflux ($\mu mol \cdot kg^{-1} \cdot min^{-1}$) from rabbit aorta. The tissues were loaded in PSS containing ^{45}Ca for 3 h, then washed in a medium containing 1.5 mM Ca in order to measure the efflux. Each curve is the average of four tissues dissected from a single aorta. The control efflux is represented in A, the effect of 10^{-5} M NE added after 80 min of efflux in B, the effect of 10^{-2} M caffeine in C, and the effect of 10^{-4} DNP in D.

The similarity of the rates of exchange for the Ca released by norepinephrine and by caffeine might be due either to the fact that the same compartment is affected by both substances or to a fortuitous circumstance. The reliance of caffeine and NE on a single Ca pool is also suggested by the observation that in

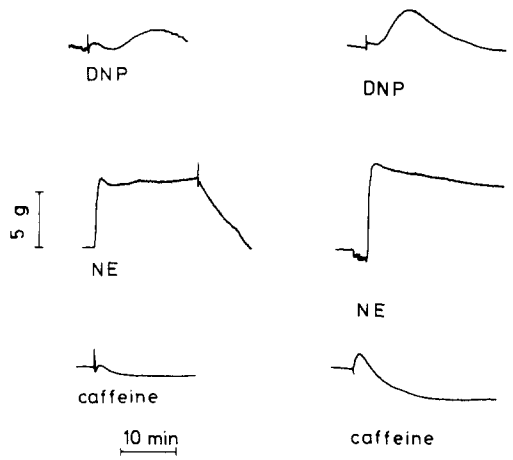


FIGURE 2. The effects of norepinephrine, caffeine, and DNP on the tension development of aorta rings. *Left,* Control responses in PSS containing 1.5 mM Ca to DNP (10^{-4} M), NE (10^{-5} M), and caffeine (10^{-2} M). *Right,* Responses of the same tissues to these drugs after 45 min of exposure to a 140 mM K solution, followed by 15 min of exposure to PSS.

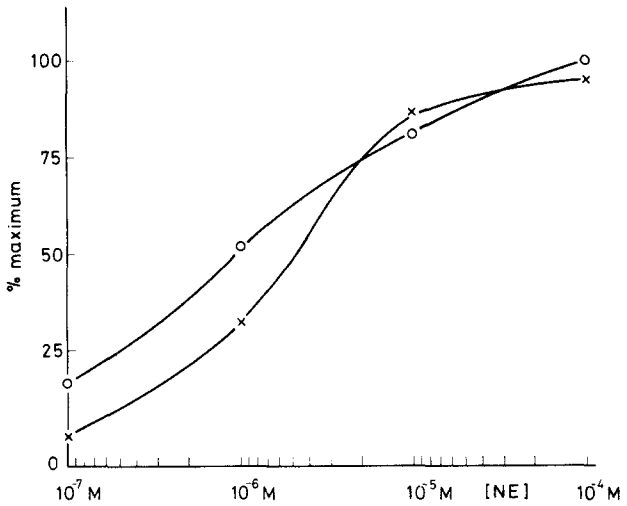


FIGURE 3. Dose-response relationships for the contraction and ^{45}Ca release caused by NE. Tissues from the same aorta were stimulated with increasing concentrations of NE. The tension response is indicated by the crosses (×) and the release of ^{45}Ca after 60 min of exposure to PSS by the open circles (○). The increase in tension (average of four) and the release of ^{45}Ca (average of three) are represented on the ordinate as a percentage of the maximum. The norepinephrine concentration is given on the abscissa on a logarithmic scale.

Ca-free conditions, transient caffeine treatment eliminates subsequent NE responsiveness and that transient caffeine exposure was found to eliminate NE stimulation of ^{45}Ca efflux. To test this hypothesis further we have studied calcium release after loading procedures which are likely to yield a different pattern of ^{45}Ca distribution. The first procedure, a 20-min exposure to a PSS containing 0.2 mM Ca, will produce an incomplete exchange of Ca because the transmembrane Ca gradient and the loading period have been reduced. Such an incomplete exchange might affect the various compartments differently. These data are represented in Table I *b*, and they again indicate a similarity for the compartments affected by norepinephrine and caffeine while the DNP-sensitive compartment has a lower exchange rate. A second altered loading procedure

TABLE I

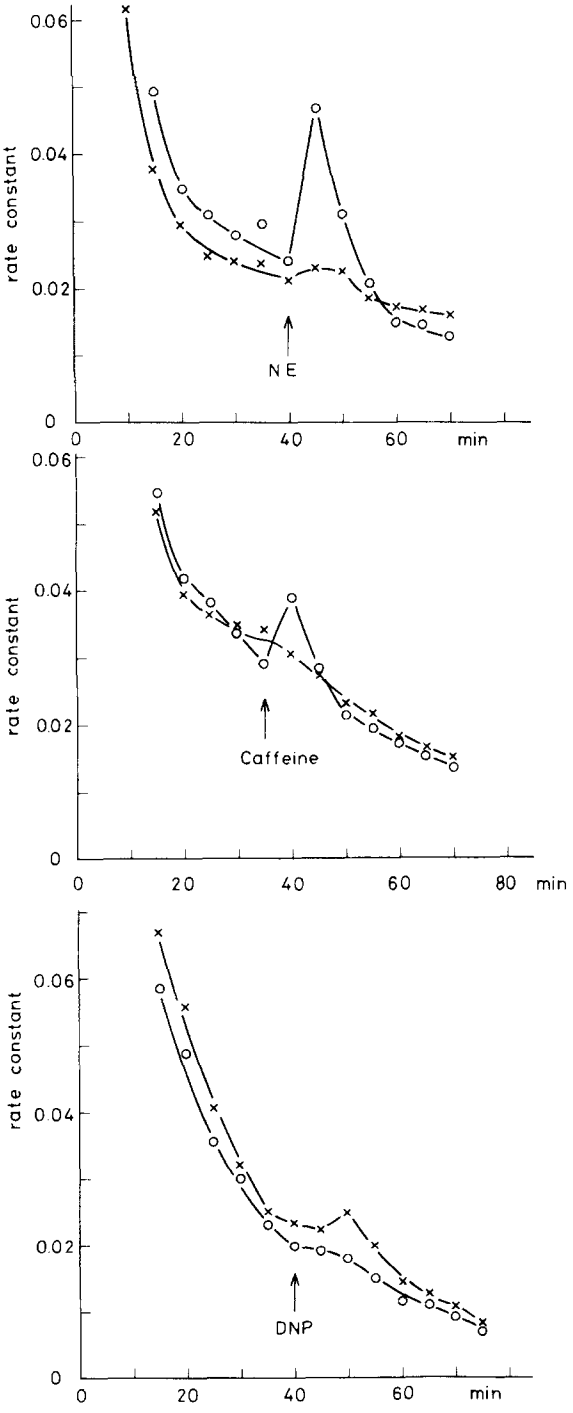
ESTIMATES OF THE POOL SIZES AND OF THE RATE COEFFICIENTS OF THE ^{45}Ca FRACTIONS RELEASED BY NE, DNP, AND CAFFEINE.

	μmol Ca	*k*	*n*
A			
NE	47.0±9.0	0.0281±0.0016	8
DNP	29.1±5.3	0.0221±0.0018	4
Caffeine	39.0±6.1	0.0279±0.0021	4
B			
NE	17.8±6.7	0.0322±0.0021	8
DNP	4.3±1.8	0.0152±0.0030	4
Caffeine	15.4±7.8	0.0295±0.0025	4

The amount of ^{45}Ca released by each agent was measured after different times of efflux. These values could be fitted by the first order relation: $y=a.e^{-kt}$ where y represents the amount of ^{45}Ca released and t the time of the efflux at which the agent was added, a represents the pool size at $t=0$ and k the rate of decrease of ^{45}Ca in this pool. These values were obtained by applying NE and either DNP or caffeine on aortas which were loaded for 3 h in PSS with 1.5 mM Ca containing ^{45}Ca (A) and on aortas which were loaded for 20 min in a PSS with 0.2 mM Ca containing ^{45}Ca (B). Values are expressed as mean ± SEM. n=number of observations.

was to transfer tissues to a 140 mM K solution after 2 h in normal medium. After 45 min of depolarization the tissues were returned to normal radioactive PSS for 15 min to avoid a sudden entry of ^{40}Ca during the initial stage of the efflux before full repolarization had occurred. Since high K depolarization causes a large increase in cellular ^{45}Ca uptake (van Breemen et al., 1972), it was expected that the cellular fractions released by these agents would be increased in size after exposure to a K-rich solution. This hypothesis was supported by the finding that the effect of norepinephrine, caffeine, and DNP on the contractile response is enhanced after such K depolarization (Fig. 2 *right*).

Rather surprisingly, after depolarization during loading, the normal stimulation of ^{45}Ca efflux produced by NE and caffeine was almost absent, while it was increased for DNP by a factor of 3 (Fig. 4). The reason for the reduction of the ^{45}Ca release by NE and caffeine was not immediately apparent, since the contractile response to both agents was actually larger than under control


0.06
0.04
0.02
0
rate constant
NE
0
20
40
60
min
0.06
0.04
0.02
0
rate constant
Caffeine
0
20
40
60
80
min
0.06
0.04
0.02
0
rate constant
DNP
0
20
40
60
min

conditions. The increased release of Ca by DNP in these tissues could be related to the fact that K depolarization augments the Ca content of the mitochondria as indicated be electron microscope observations (Somlyo et al., 1974). An attractive hypothesis to explain this set of seemingly contradictory results is that during the first stage of the efflux ^{40}Ca has replaced the ^{45}Ca in the compartment affected by NE and caffeine.

To test this possibility the cellular uptake of ^{45}Ca was measured for normal tissues and tissues which, after 45 min of exposure to high-K solution, have been repolarized for 15 min in PSS. The uptake of ^{45}Ca during these uptake experiments is analogous to the uptake of ^{40}Ca during the preceding efflux experiments. Fig. 5 shows that in the previously depolarized tissue the amount of ^{45}Ca taken up during the initial 15 min is three times larger than the amount taken up during the same period by control tissues. After 120 min the ^{45}Ca content of the tissues which had been depolarized gradually declined to the level of the control tissue. This effect of a preceding K depolarization depends on the presence of Ca in the depolarizing solution. Depolarization in a Ca-free medium is not followed by such a prolonged modification of the Ca-exchange.

These data clearly indicate that even 15 min after the end of the K depolarization much greater quantities of Ca are entering into the cells than under control conditions. Moreover, this finding again indicates that the compartment on which NE and caffeine act and that on which DNP acts are affected quite differently by a preceding K depolarization.

Changes in Cellular ^{45}Ca Content Induced by NE, DNP, and Caffeine

In order to achieve a clear insight into the overall effect of NE, DNP, and caffeine on Ca distribution, changes in the cellular ^{45}Ca content were measured with the La method under the experimental conditions that had been used in the efflux studies. Tissues were equilibrated in PSS containing ^{45}Ca for 2 h, a period during which a plateau of uptake was reached. At this stage the effect of each agent on the cellular ^{45}Ca content was determined. Fig. 6 shows that 10^{-5} M NE increases the cellular ^{45}Ca content by about 0.7 $\mu mol \cdot kg^{-1} \cdot min^{-1}$. This uptake probably represents a portion of the Ca responsible for the tonic phase of contraction. The large uptake of ^{45}Ca that follows high K depolarization (6 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) is shown for comparison. It was found that DNP caused no net change in the cellular ^{45}Ca content. Because we have observed that the outward movement of ^{45}Ca is stimulated by DNP, we must assume that there is a balancing amount of inward movement. Caffeine (10^{-2} M) causes a rapid net extrusion of cellular ^{45}Ca. This effect is evident 1 min after drug addition, but, as

FIGURE 4 (opposite). Crosses represent the effect of 10^{-5} M NE, 10^{-2} M caffeine, and 10^{-4} M DNP on ^{45}Ca efflux from tissues which have been exposed sequentially for 2 h to PSS, for 45 min to K-rich solution, and for 15 min to PSS, all solutions containing 1.5 mM ^{45}Ca of the same specific activity. The control conditions, consisting of a 3-h exposure to PSS containing 1.5 mM ^{45}Ca, are represented by open circles (O). The rate coefficient (min^{-1}) is plotted on the ordinate as a function of time on the abscissa (min).

shown in Fig. 7, it is transient. This net extrusion is due to the stimulation of the ^{45}Ca efflux by caffeine, without an accompanying uptake of Ca. It is very unlikely that the action of caffeine is caused by its action on cyclic nucleotide levels, since the more potent phosphodiesterase inhibitors, papaverine and theophylline, do not cause an increase in ^{45}Ca efflux or a net extrusion of cellular Ca in the aorta.

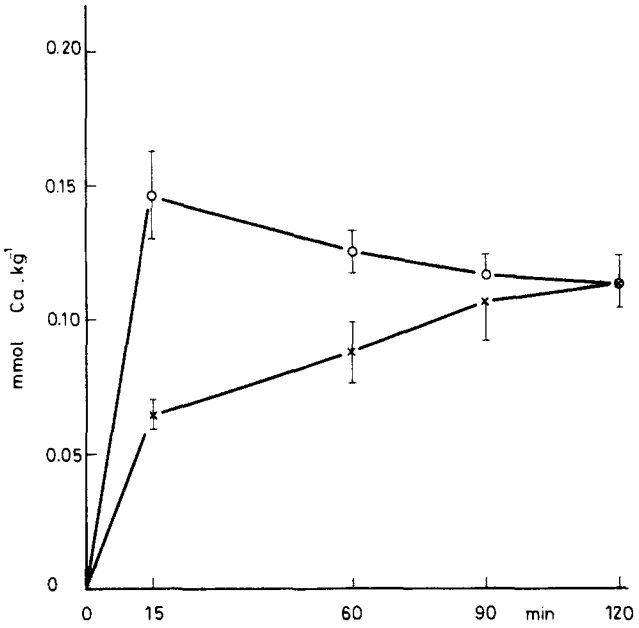


FIGURE 5. The increase of the amount of ^{45}Ca (mmol·kg^{-1}) entering into the cellular compartment of the aorta as a function of the time of exposure (min) to the radioactive solutions. The control tissues (×) were exposed for 3 h to PSS, before the loading procedure was started in PSS containing 1.5 mM ^{45}Ca. The other tissues were pretreated by an exposure of 2 h to PSS, of 45 min to K-rich solution, and of 15 min to PSS before they were loaded in the same radioactive PSS (○). Groups of tissues were removed at the times indicated on the abscissa and their cellular ^{45}Ca content was determined with the La method.

DISCUSSION

The free calcium concentration in the cytoplasm of smooth muscle is controlled by changes in the rate of Ca supply and the rate of Ca removal. Increased supply can be achieved by increasing the Ca permeability of the plasma membrane, allowing an influx, or by releasing intracellular Ca from its binding sites. Conversely, a decrease in cytoplasmic Ca can be brought about by reversing each of these processes, resulting in Ca extrusion and increasing intracellular binding.

The role of Ca influx in the activation of contractile proteins has been investigated by measuring the changes in cellular ^{45}Ca content by the La method

(van Breemen et al., 1972). However, physiological agonists such as norepinephrine, angiotensin, and histamine initiate contraction by releasing intracellular Ca (Deth and van Breemen, 1974).

In the present studies we have carried out ^{45}Ca effluxes in solutions containing 1.5 mM ^{40}Ca. These experiments are therefore characterized by a steady state and a gradual replacement of ^{45}Ca by ^{40}Ca or by a decrease in specific activity in

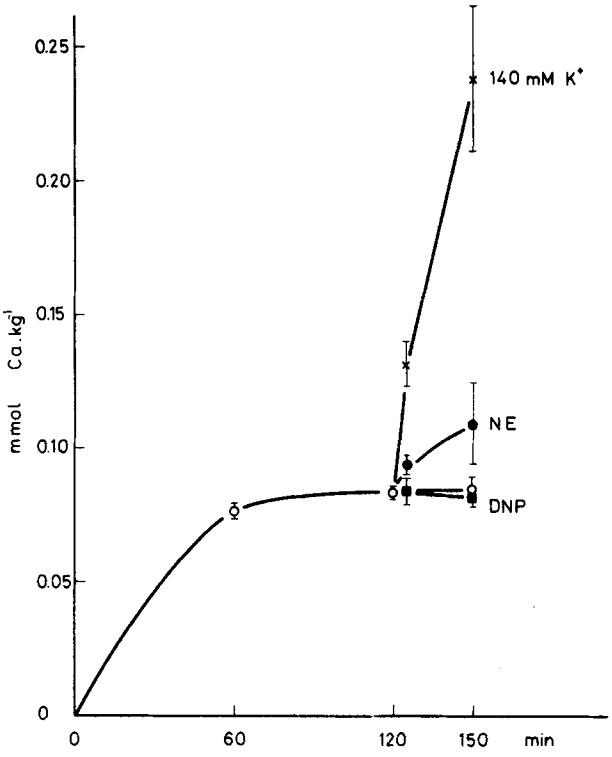


FIGURE 6. Effects of NE, DNP, and high K on the cellular ^{45}Ca content. After 120 min of uptake in PSS labeled with ^{45}Ca, tissues were transferred to a medium of the same specific activity but containing either 10^{-5} M NE (●), 10^{-4} (■), 140 mM K^+ (×), or the normal medium (○). The cellular ^{45}Ca content was determined by the La method.

the various tissue compartments. A net loss of cellular Ca, as induced in a Ca-free solution, does not occur. The amount of ^{45}Ca which is released from the intracellular pools upon exposure to drugs will therefore decrease as a function of the time at which the drug is admitted during the efflux procedure. We can therefore assume that under such steady-state conditions the decrease in the amount of ^{45}Ca released by some substance can given an estimate of the exchange rate for the Ca pool on which the drug is acting (van Breemen and Casteels, 1974). Because we have observed that after a loading procedure in PSS these estimates of the half-times for the ^{45}Ca exchange had a maximum value of

31 min, we can consider that a 3-h exposure to a ^{45}Ca medium is sufficient to obtain a specific activity in the intracellular pools which is similar to that of the loading medium. Short loading times can result in an uneven distribution of ^{45}Ca, consisting of a relatively more extensive labeling of the rapidly exchanging fractions.

Our experiments reveal that after equilibrium distribution of ^{45}Ca, the Ca

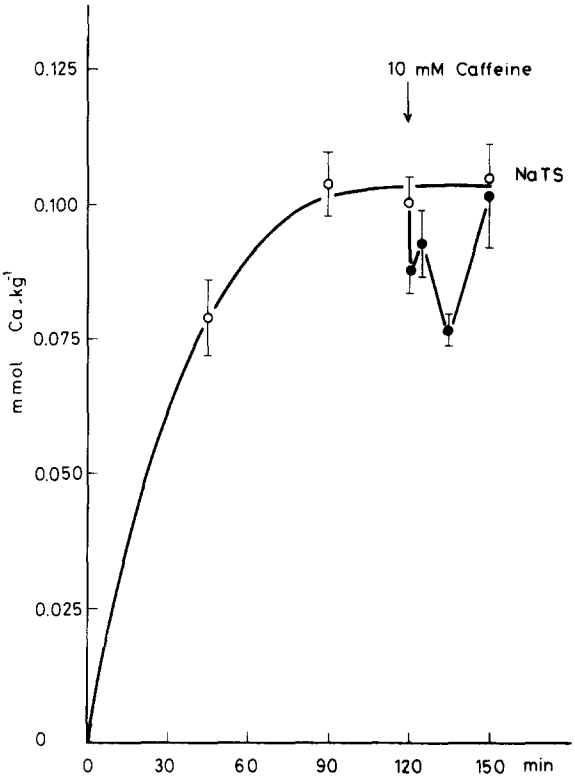


FIGURE 7. Effect of caffeine on the cellular ^{45}Ca content. After 120 min of uptake of ^{45}Ca in PSS, the tissues were transferred either to a similar solution of equal specific activity for ^{45}Ca and containing 10 mM caffeine (●) or the same medium without caffeine (○). The cellular ^{45}Ca content was determined with the La method.

compartments affected by norepinephrine and caffeine exhibited similar exchange rates, while the Ca pool sensitive to DNP exchanged more slowly. The disparity between the estimates of the rate constants obtained by application of norepinephrine and of DNP becomes more pronounced after a short period of labeling carried out in a solution containing 0.2 mM Ca. Because we can assume that such a brief exposure to a radioactive solution will preferentially label peripheral binding sites, these findings suggest that the Ca sites sensitive to norepinephrine and caffeine are more superficially located than those affected by DNP.

Caffeine has been shown to have a dual effect on Ca binding to skeletal muscle

sarcoplasmic reticulum. At a caffeine concentration of 10 mM, Ca release is enhanced and Ca uptake is impaired (Weber and Herz, 1968), resulting in the well-known irreversible caffeine contracture (Axelsson and Thesleff, 1958). Its effects are associated with an increase in both ^{45}Ca influx and ^{45}Ca efflux (Bianchi, 1961). In vascular smooth muscle 10 mM caffeine causes a "nonspecific" inhibition of a wide range of physiological and nonphysiological stimuli (Sakai and Iizuka, 1974; Nasu et al., 1975; van Breemen, 1976) after an initial transient increase in tension (Somlyo and Somlyo, 1968). Similar combinations of inhibitory and excitatory effects of caffeine have been reported in other smooth muscles (Ito and Kuriyama, 1971).

The fact that NE and caffeine use the same Ca source is also suggested by the experments in which tissues were transiently depolarized during ^{45}Ca loading. After such treatment, a stimulation of ^{45}Ca efflux by either agent was almost absent, while the DNP response was increased threefold. Caffeine (10 mM) also causes a transient net extrusion of about 20 μmol of calcium per kilogram, which largely accounts for the stimulation of ^{45}Ca efflux by this substance. One explanation for the stimulation of the efflux and the simultaneous appearance of only a small contraction would be that caffeine releases calcium from the sarcoplasmic reticulum, and the calcium is then rapidly extruded from the cell before it can fully activate the myofilaments. This possibility receives support from the observation of Huddart and Syson (1975) that caffeine does not stimulate ^{45}Ca efflux from rat ileal longitudinal muscle, which has little or no sarcoplasmic reticulum. Alternatively, caffeine could also be acting at the level of the plasma membrane, causing the extrusion of a membrane-bound Ca^{++} fraction. This latter mechanism would be consistent with the conclusions of Ito and Kuriyama (1971) and Sunano and Miyazaki (1973), that caffeine exerts its effects at the cell membrane. Since both intracellular binding and extrusion of Ca^{++} could contribute to the relaxation of vascular smooth muscle (Deth and van Breemen, 1974), caffeine may affect both systems.

DNP, which is known to be an uncoupler of mitochondrial energy conservation also causes a release of Ca from isolated mitochondria (Vasington and Murphy, 1962). The same mechanism may be responsible for the release of cellular Ca by DNP in taenia coli (van Breemen et al., 1975). This action of DNP is associated with a moderate decrease in ATP levels (Born and Bülbring, 1955; van Breemen et al., 1975). The finding that in normal Krebs solution much more Ca is released by DNP after exposure to a K-rich solution suggests that this substance acts on the mitochondria which have been loaded with ^{45}Ca during the preceding depolarization. This is in agreement with electron microscope studies which have shown Ca to be deposited in mitochondria after periods of depolarization (Somlyo et al., 1974). This massive gain in cellular Ca induced by high K depolarization is therefore probably due to mitochondrial Ca accumulation. The delay between the addition of DNP to the solution and the appearance of maximum Ca release and tension development can be explained either by the slowness of DNP in reaching the mitochondria and/or the inefficiency of the Ca release by the mitochondria in activating the contractile proteins and the Ca extrusion pump.

This increased uptake of ^{45}Ca which was shown to persist for some time in

normal Krebs solution after a period of depolarization by high $[K]_0$ is not caused by the K depolarization itself, but probably by the Ca gain that occurs during this depolarization. This conclusion is supported by the finding that depolarization in Ca-free solution does not modify a subsequent Ca uptake. The finding that norepinephrine induces a larger tension development after an earlier K depolarization suggests that the Ca pool which is affected by norepinephrine becomes larger during this treatment. However, the concomitant release of ^{45}Ca by norepinephrine is very much reduced. We have therefore to assume that a depolarization of the cells in a Ca-containing solution increases not only the size of this compartment but also its exchangeablity. The exact mechanism of this change and the reason for its rather slow return to control conditions remains obscure. Another aspect of these experiments is that the NE contraction gives no stimulation to ^{45}Ca efflux although the specific activity of the ^{45}Ca in the extracellular space is the same as that of the control tissue. Thus it is clear that the stimulation of the efflux rate by NE is not due to mechanical distortions of the extracellular space.

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