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Agonist Induced Release of Intracellular Ca^{2+} in the Rabbit Aorta

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Summary. The effects of hormonal agonists (norepinephrine, angiotensin, and histamine) on ^{45}Ca efflux from the rabbit aorta were studied using a Ca-EGTA buffered efflux medium. Each caused a transient stimulation of efflux rate which probably reflected the release of an intracellular ^{45}Ca store. The size of the stimulation of efflux correlated with the size of the initial rapid phase of contraction. The norepinephrine-sensitive intracellular Ca fraction was estimated to be greater than 21 μmoles/Kg wet tissue weight. This fraction is separate from intracellular Ca which is accumulated during relaxation. Evidence is presented for the lack of cyclic nucleotide involvement in the release of Ca^{2+}, and possible alternative modes of coupling are discussed.

The calcium movements which are responsible for norepinephrine (NE) induced contraction of vascular smooth muscle remain uncertain. Earlier studies by Bohr (1963) described the different sensitivity of the early phasic and latter tonic portions of the contractile response to changes in extracellular Ca. The initial rapid increase in tension was augmented in Ca-free solutions, and reduced in higher than normal Ca (10 mM), while the latter slow increase in tension was reduced in Ca-free solutions and increased in the presence of higher Ca. Hinke, Wilson and Burnham (1964), and later Hudgins (1969) and van Breemen (1969), noted that the NE response was more resistant to Ca removal than was high K induced contraction, and that NE responsiveness returned at lower Ca levels than high K contractions. Since Briggs (1962) had reported that both NE and high K increased Ca influx, Hinke was led to conclude that NE initially utilizes a more tightly bound Ca fraction, probably located on the outside of the plasma membrane. A number of recent studies have concluded that NE activation involves a combination of cellular and extracellular Ca sources (Steinsland, Furchgott & Kirpekar, 1973; Deth & van Breemen, 1974; Bose & Innes, 1975).

By the use of the La-method to improve the accuracy of intracellular Ca measurements, we showed that high K caused a large gain in cellular Ca, while NE induced only a small increase which was associated with the slow phase of contraction (Deth & van Breemen, 1974). Similarly, blockade of Ca entry with La, which should also displace any Ca bound to the extracellular face of the cell, resulted in total inhibition of high K^+ contraction, but not the initial rapid phase of NE response (van Breemen, Farinas, Gerba, McNaughton, 1972). Thus the Ca store responsible for the initial rapid phase of NE contraction appeared to be of intracellular origin.

The properties of such intracellularly bound Ca can be studied through ^{45}Ca efflux measurements; however, previous investigations have yielded conflicting results (Hudgins & Weiss, 1969; Hudgins, 1969; Seidel & Bohr, 1971; Keatinge, 1972). The present work considers some sources of difficulty in carrying out ^{45}Ca efflux experiments using vascular smooth muscle, and provides more direct evidence for the release of intracellular Ca by NE. The source of the released Ca appears to be separate from intracellular organelles which accumulate Ca during relaxation. The ability to release a single intracellular Ca fraction is shared by several arterial agonists.

Materials and Methods

Rabbits were killed by a blow to the neck and their aortas rapidly excised. While being maintained in an oxygenated physiologic buffer, the tissue was cleaned and cut into rings of about 5 mm. Contraction experiments were carried out using these rings, in parallel to efflux studies as previously described (Deth & van Breemen, 1974). For efflux studies, rings were cut open into strips. The tissues were first placed in a low Ca^{2+} solution (0.2 mM Ca^{2+} PSS) for 20 min before being loaded for 20 min in an identical solution containing approximately 2 μcuries ^{45}Ca/ml. After loading, the tissues were mounted on aerators, briefly rinsed in a large volume of efflux medium (Ca-EGTA buffered unless specified), and then sequentially passed through a series of test tubes containing 3 ml of efflux medium. The 3 ml was later dissolved in a Triton X-100 containing scintillation fluid and counted for ^{45}Ca.

At the conclusion of each efflux experiment, the ^{45}Ca remaining in the tissue was determined. Tissues were blotted and weighed before being ashed in a muffle furnace at 500 °C. The ash was then dissolved in 1 ml of 0.1 N HCl containing 1 mM $LaCl_3$, and counted for ^{45}Ca.

^{45}Ca efflux is expressed as μmoles Ca/kg/min, as determined from the specific activity of the loading solution, and tissue wet weights.

Contraction experiments were performed as previously described (Deth & van Breemen, 1974). All tissue preparation and experiments were carried out at 37 °C in solutions bubbled with 100% O_2.

For C-AMP determination, aortas were prepared as above, and divided into longitudinal halves, one half serving as the control for the other. After the experimental treatment,

tissues were plunged into 2 ml of acidified ethanol (cooled in an acetone dry ice bath), and homogenized with a Polytron apparatus for 20 sec at high rpm. After centrifugation and a second ethanolic extraction, the solvent was removed from the combined extracts and the residue assayed for C-AMP by the method of Brown *et al.* (1971).

Solutions

Physiologic salt solution (PSS): 160 mM NaCl, 10 mM Glucose, 5 mM Hepes, 4.6 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, pH=7.2.

Ca-free PSS: Same as *PSS*, deleting $CaCl_2$.

CaEGTA PSS: 20.5 mM $CaCl_2$, 20 mM EGTA, 135 mM NaCl, 10 mM D-glucose 5 mM Hepes, 4.6 mM KCl, 1 mM $MgCl_2$, pH=7.2.

Drugs

Norepinephrine hydrochloride (Sigma), angiotensin amide (CIBA Geigy), histamine hydrochloride (Fisher), 2-diethylaminoethyl-2, 2-dephenylvalerate hydrochloride (SKF 525A) (Smith Kline and French), propranolol (Ayerst), isoproterenol (Sigma), phenoxybenzamine (Smith, Kline and French), phenylephrine (Winthrop), Cyclic-GMP (free acid, dibutyryl, monobutyryl) (Sigma). ^{45}Ca (specific activity=10.5 mCi/mg) was obtained from New England Nuclear.

Results

Previous experiments using agents which block transmembrane Ca fluxes have shown that the initial rapid phase of NE contractile response is not dependent on access of extracellular Ca (Ca_{ext}) (van Breemen *et al.*, 1972; Steinsland *et al.*, 1973; Deth & van Breemen, 1974), in contrast to the requirement for Ca_{ext} of the latter slow phase. The complete removal of Ca_{ext} should affect NE response in a similar manner. An experiment which shows this to be the case is illustrated in Fig. 1. Aortic rings from the same rabbit initially were contracted by NE to determine control responsiveness. After the addition of either 2 mM La (upper tracings), or 10 mM EGTA (lower tracings), only a single response could be obtained, in which the slow phase of contraction was absent. As shown by the second contraction sequence for each agent, the lack of contraction at the second NE addition is not due to the length of exposure to La or EGTA. Thus access of Ca_{ext} is a strict requirement for regeneration of the initial contractile response, which is apparently caused by the release of intracellular Ca.

If ^{45}Ca efflux is sensitive to the free cytoplasmic ^{45}Ca level, as is true for the squid giant axon (Blaustein & Hodgkin, 1969), an intracellu-

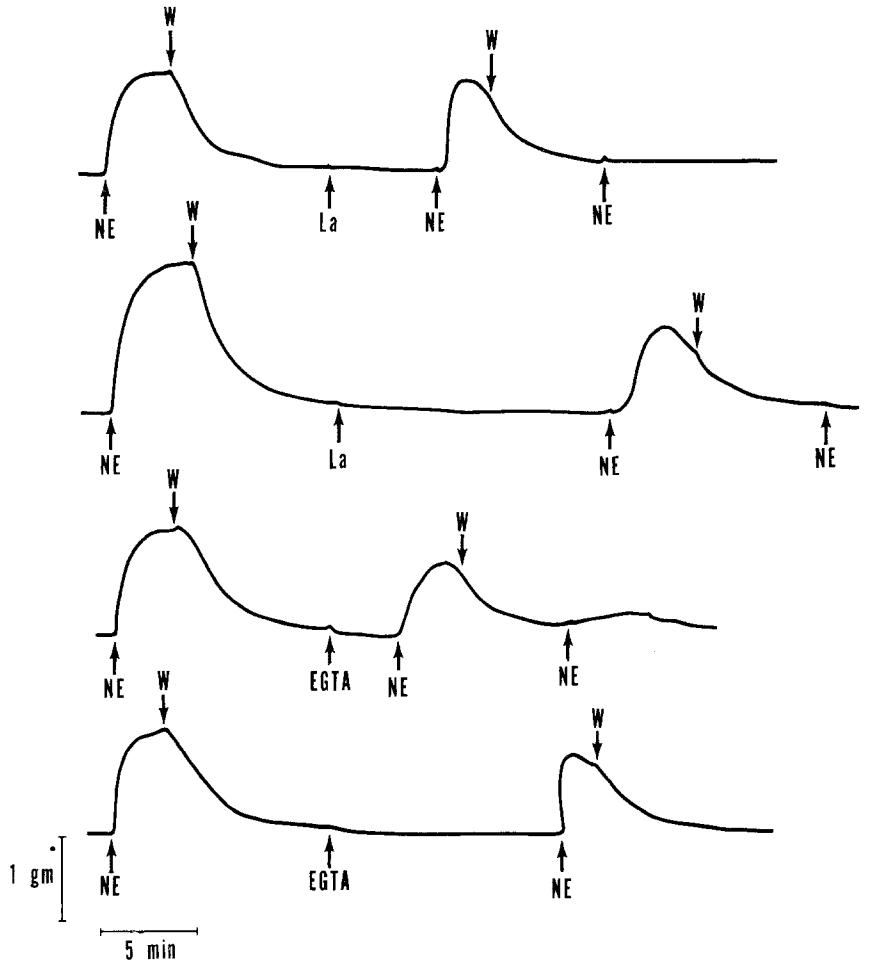

Fig. 1. *Upper two tracings:* Effect of 2 mM La^{3+} on first and second NE (10^{-5} M) responses. Second series shows first response after the longer La^{3+} exposure. *Lower two tracings:* Effect of 10 mM EGTA on first and second NE (10^{-5} M) responses. Second series shows first response after longer EGTA exposure

lar release should be reflected as a stimulation of ^{45}Ca efflux rate. However, previous studies have yielded conflicting results with regard to the effect of NE on ^{45}Ca efflux (Hudgins & Weiss, 1969; Seidel & Bohr, 1971; Keatinge, 1972). During efflux into a Ca-free medium, large amounts of ^{45}Ca remain bound in the extracellular space (*ECS*) (van Breemen & Casteels, 1974). This binding reduces the visualization of true cellular efflux, and increases the potential for significant backflux of ^{45}Ca into the cells. The use of a Ca-EGTA buffered efflux medium can minimize these conditions by chelating most of the effluxing label (>95%), while maintaining a constant free Ca level in the *ECS*. The

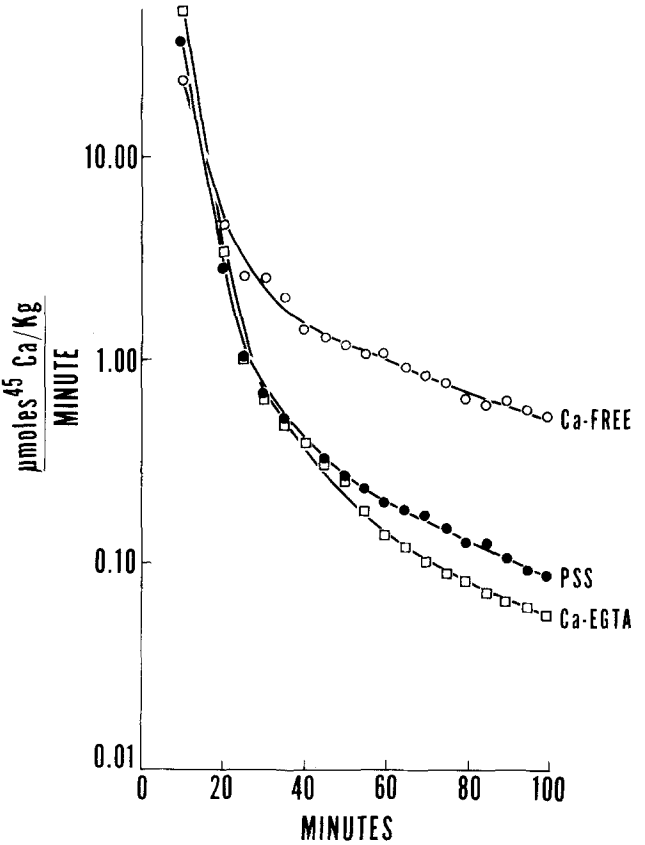

Fig. 2. ^{45}Ca efflux from rabbit aorta strips into (1) Ca-free PSS (open circles). (2) PSS (closed circles), and (3) 20 mM Ca-EGTA (squares). Each point is the average of 4 observations. *Ordinate*=rate of ^{45}Ca loss as μmoles ^{45}Ca/kg min. *Abscissa*=time in min

inclusion of Ca in the efflux medium also avoids the deleterious effects of prolonged Ca-free exposure on membrane permeability. In addition, we adopted a short loading procedure in relatively low Ca solutions (0.2 mM) with the intention of further reducing nonspecific Ca binding in the *ECS* and limiting ^{45}Ca labeling of the more slowly exchanging cellular compartments.

Fig. 2 shows the efflux pattern using either Ca-free, Ca-EGTA buffered, or normal 1.5 mM Ca (PSS) efflux medium. The presence of Ca-EGTA or Ca in the efflux medium facilitated the loss of ^{45}Ca during the earliest stages of efflux. The ability of Ca_{ext} to increase washout of extracellular ^{45}Ca is in agreement with previous observations on a variety of tissues (Shanes & Bianchi, 1960; van Breemen, Daniel & van Breemen, 1965; Chen, 1974). After about 15 min, this pattern is reversed,

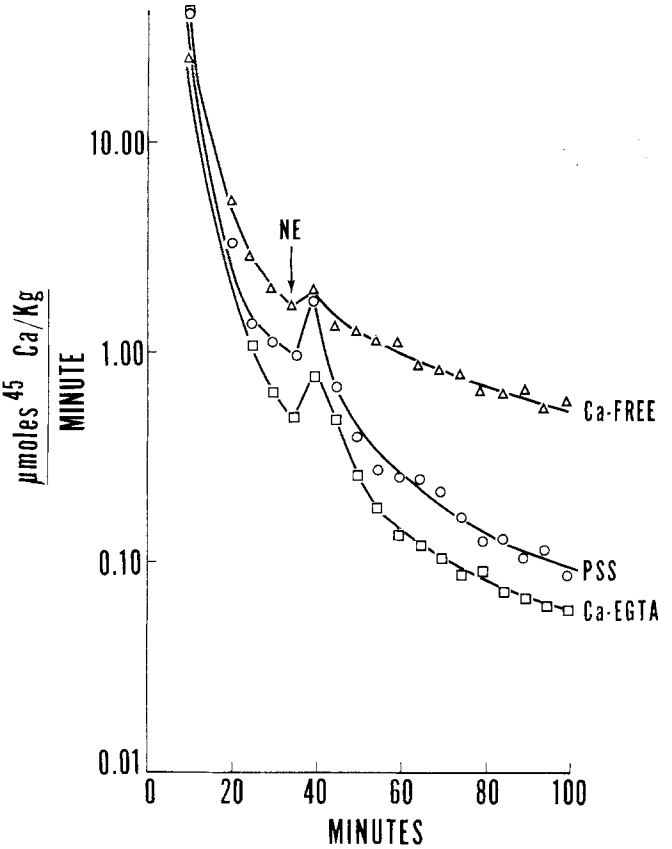

Fig. 3. NE stimulation of ^{45}Ca efflux into (1) Ca-free PSS (triangles), (2) PSS (circles) and (3) 20 mM Ca-EGTA buffer (squares) by NE. NE (10^{-5} M) added 10 min after 36 min at efflux. Each point is the average of 4 determinations. *Ordinate*=rate of ^{45}Ca efflux as µmoles ^{45}Ca/kg min. *Abscissa*=time in min

and for the remainder of efflux the rate in Ca-free medium is about 10 times that of the Ca-containing series. However, after 100 min of efflux at this higher rate, Ca-free tissues had 4–5 times the amount of ^{45}Ca remaining. Thus the increased rate does not reflect a true higher rate of cellular efflux, but is the result of a prolonged washout of *ECS* label which was not lost during earlier stages of efflux.

Fig. 3 shows that the addition of 10^{-5} M NE at 35 min of efflux causes an increased rate of efflux in all three washout media. It is clear, however, that the event is much more distinctly visible in the Ca-EGTA and 1.5 mM Ca media. This stimulation of efflux may reflect the intracellular release of Ca which initiates the NE contractile response.

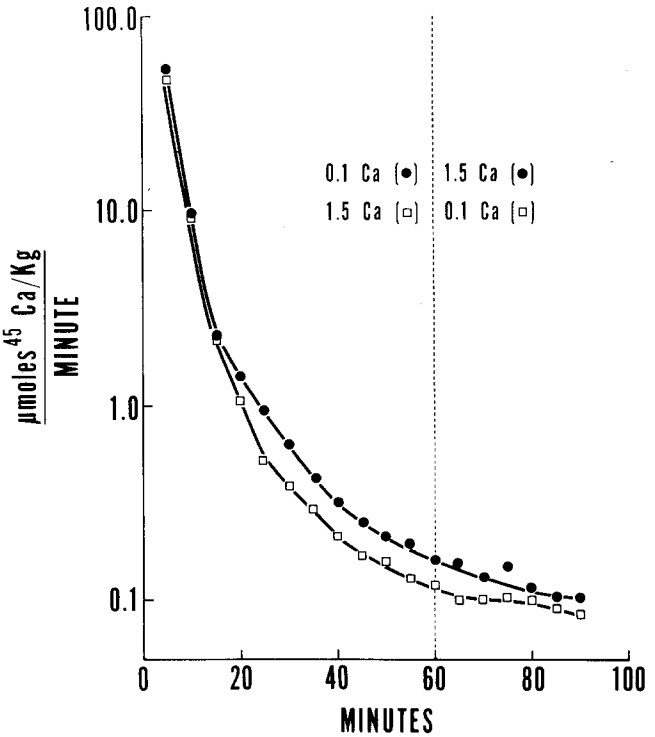

Fig. 4. ^{45}Ca efflux from rabbit aorta into (1) 20 mM Ca-EGTA plus 0.1 mM Ca for 60 min, followed by 20 mM Ca-EGTA plus 1.5 mM Ca for the remaining 30 min (dots) and (2) 20 mM Ca-EGTA plus 1.5 mM Ca for 60 min, followed by 20 mM Ca-EGTA plus 0.1 mM Ca for the remaining 30 min. Each curve is the average of three experiments

The precise mechanism of ^{45}Ca efflux from smooth muscle is poorly understood, and we sought to examine the possible importance of Ca-Ca exchange in this process. Ca-EGTA buffers were prepared with free Ca concentrations of 0.1 and 1.5 mM. Half of the tissues were effluxed in each medium, and after 60 min the free Ca^{2+} concentrations were reversed (Fig. 4). This 15-fold difference in Ca_{ext} had no effect on the efflux rate. Thus in the presence of Ca-EGTA buffer, there was no evidence of a Ca-Ca exchange diffusion mechanism. A similar situation has been reported for guinea pig taenia coli (van Breemen, Wuytack & Casteels, 1975). It should be emphasized that since a ^{40}Ca containing efflux medium is used in these experiments, the loss of ^{45}Ca reflects an overall exchange of cellular ^{45}Ca for *ECS* ^{40}Ca, as opposed to a net Ca loss.

If refilling of NE sensitive intracellular Ca stores from Ca_{ext} is required for subsequent contractions, the quantity of releasable intracellular ^{45}Ca

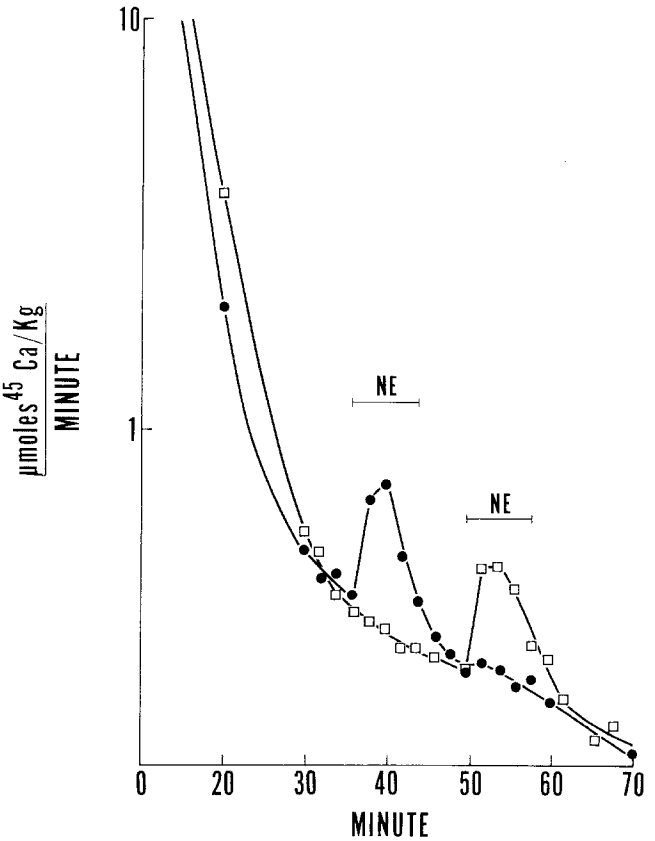

Fig. 5. Effect of sequential NE exposures on ^{45}Ca efflux from rabbit aorta. (Dots) $= 10^{-5}$ M NE for 8 min at 36 min and again at 50 min for 8 min. Control (squares) exposed to 10^{-5} M NE for the first time at 50 min. *Ordinate* = rate of ^{45}Ca loss as μmoles ^{45}Ca/kg min. *Abscissa* = time in min. Each point is the average of 4 observations

should be limited. It was found that the same stimulation of efflux ensued whether NE was present for only two min, or for the remainder of efflux. Thus we assume that the transient stimulation of ^{45}Ca efflux by NE is due to the release of a distinct Ca fraction of limited size. Further evidence for the limited size of the released ^{45}Ca is provided by the experiments of Fig. 5, in which half of the tissues (solid line) were stimulated a second time, after a short washout of the first application of NE. In a parallel contraction experiment a full contraction occurred at the second exposure, but there was no accompanying stimulation of ^{45}Ca efflux, indicating that this second contraction utilized ^{40}Ca rather than recycled ^{45}Ca. For comparison, a first NE stimulation at the later time interval of efflux is shown (open squares).

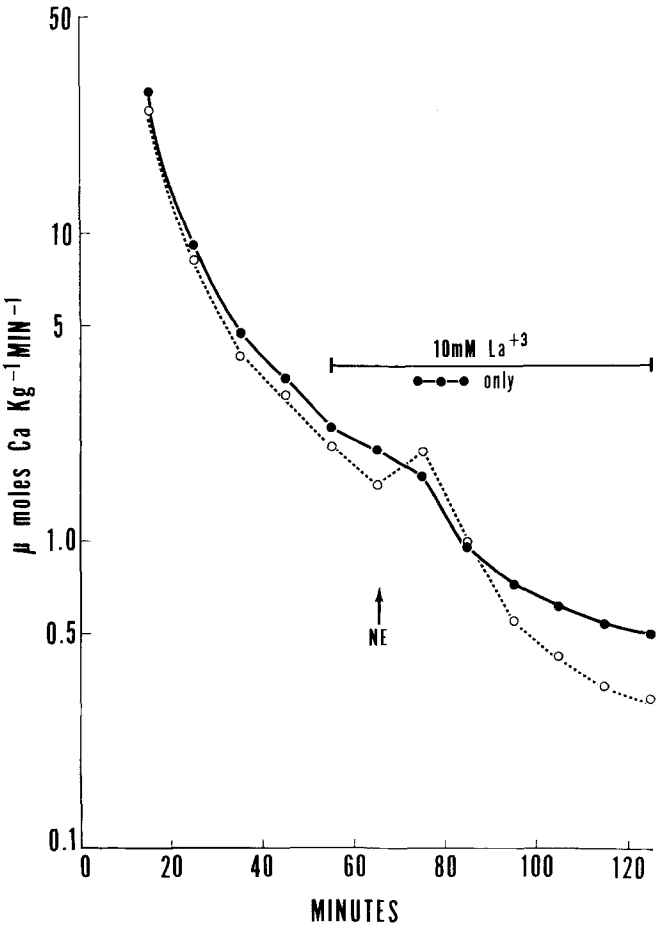

Fig. 6. Inhibition by La^{3+} of NE stimulation of ^{45}Ca efflux. 10 mM La added to half of tissues at 60 min of efflux (closed circle), NE (10^{-5} M) added to all tissues at 70 min of efflux. Efflux medium is 1.5 mM Ca^{2+} PSS. Tissues loaded in 1.5 mM ^{45}Ca for 3 hr

By virtue of its ability to block transmembrane Ca movements, La has been shown to inhibit ^{45}Ca efflux from squid axon (van Breemen & DeWeer, 1970), cultured heart cells (Langer & Frank, 1972), guinea pig taenia coli (van Breemen *et al.*, 1975), as well as rabbit aorta (van Breemen *et al.*, 1972). Stimulation of ^{45}Ca efflux by NE is eliminated by exposure to 10 mM La (Fig. 6), further indicating the intracellular source of the ^{45}Ca. However, it was found that lower concentrations of La (2 mM) were less effective. A subsequent report will provide a more complete description of the ability of La to block stimulation of ^{45}Ca efflux.

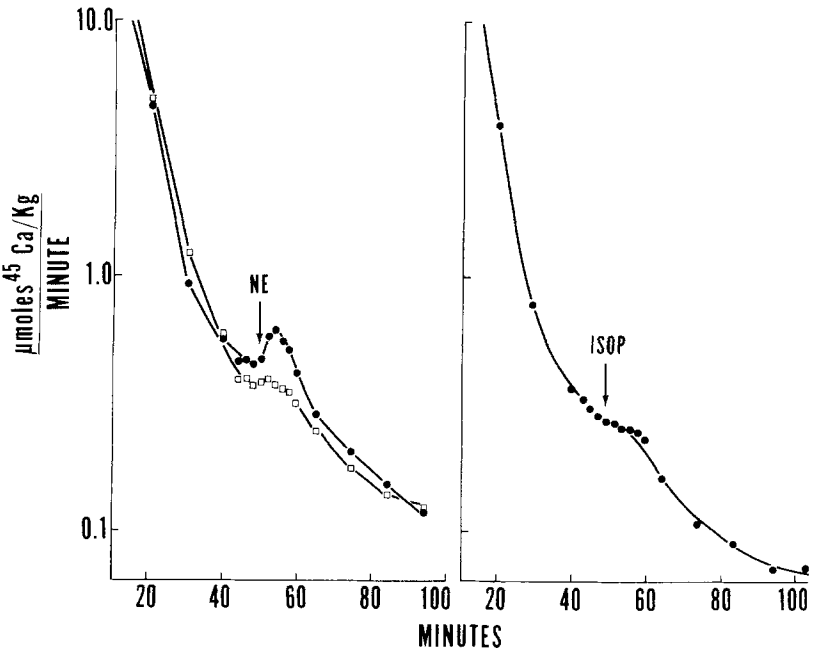

Fig. 7. *Left:* Phenoxybenzamine inhibition of NE stimulation of ^{45}Ca efflux. 10^{-5} M NE added at 50 min for both the control (dots) and experimental (squares) tissues. 2×10^{-5} M phenoxybenzamine added to the experimental tissues starting at 50 min. Each data point is the average of 4 determinations. *Ordinate*=^{45}Ca efflux μmoles ^{45}Ca/kg min. *Abscissa*= time in min. *Right:* Effect of isoproterenol on ^{45}Ca efflux from rabbit aorta. 10^{-5} M isoproterenol added starting at 50 min of efflux. Each data point is the average of 6 determinations. *Ordinate*=^{45}Ca efflux as μM Ca/kg min. *Abscissa*=time in min

The specificity of the stimulation of efflux is tested in the experiments of Fig. 7. The alpha adrenergic antagonist phenoxybenzamine blocks the stimulation of efflux by NE (left), while the Beta receptor agonist isoproterenol is without effect (right). Although not a completely satisfactory analysis, these observations indicate that stimulation of ^{45}Ca efflux is associated with NE occupation of alpha adrenergic receptors.

There has been considerable speculation that alpha receptor activation may involve primary changes in cyclic nucleotide levels as its means of excitation-contraction coupling. Specifically, increased cGMP formation (Goldberg, O'Dea & Haddox, 1973) and decreased cAMP (Anderson, 1973) formation have been suggested.

The effects of the free acid, dibutyryl and monobutyryl forms of cGMP on ^{45}Ca efflux were monitored, in the expectation that if cGMP did mediate NE stimulated Ca release, addition of the nucleotide should influence ^{45}Ca movements, and/or modify the effects of NE. As shown

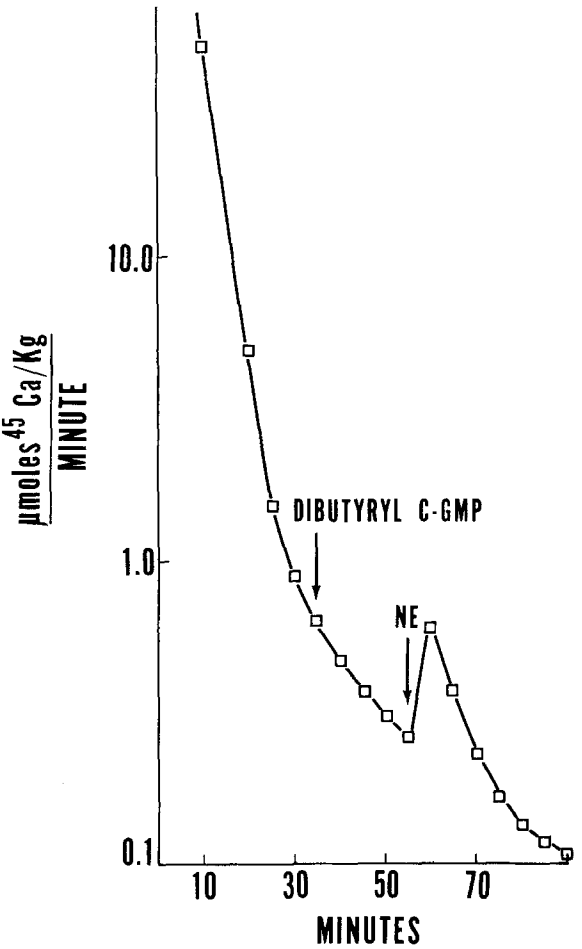

Fig. 8. Effect of dibutyryl cGMP (10^{-4} M) on ^{45}Ca efflux and subsequent NE stimulation of efflux. Monobutyryl and free acid forms gave an identical result. A NE control not exposed to cyclic nucleotide is not shown, but was superimposable on this result. Cyclic nucleotide is present from 35 to 60 min of efflux. Efflux medium = Ca-EGTA. Each point is the average of 3 determinations

in Fig. 8 (for the dibutyryl derivative) no effect of the cyclic nucleotide was observed either by itself, or on subsequent NE stimulation (control not shown since it was essentially superimposable for all three nucleotides). Preliminary experiments with dibutyryl cAMP (0.5 mM) suggest that it may increase ^{45}Ca efflux, however its effects were inconsistently observed.

cAMP levels of the rabbit aorta were measured in an attempt to find a correlation with NE induced Ca release. Control tissues contained 0.885 ± 0.109 pmoles cAMP/mg protein, a level comparable to previously

Table 1. Changes in rabbit aorta CAMP levels caused by NE (10^{-5} M) in the presence and absence of propranolol (10^{-4} M)

	% change[a] after		
	1 min	5 min	15 min
NE	+21.5 ±19.6	+78.8[c] ±18.0	+92.4[c] ±36.4
NE + Propranolol[b]	− 1.8 ± 4.5	−14.0 ±12.3	−30.1[c] ± 8.4

[a] mean of 4 observations SEM.
[b] propranolol added 10 min prior to NE.
[c] statistically significant at 0.05 level.

examined smooth muscles (Anderson, 1973; Kroeger & Marshall, 1974). Exposure to NE (10^{-5} M) resulted in a time dependent increase in cAMP (Table 1) which was statistically significant at 5 and 15 min intervals ($p < 0.05$). However, in the presence of Beta receptor blockade by propranolol, a gradual decline in cAMP was found. Under these conditions contractile response reaches 90% of its normal tension, following a time course similar to control response.

Arterial contractility is subject to the influence of several hormonal regulators, which in addition to NE include angiotensin (ANG) and histamine (HIS). Their effects are characterized by unequal maximal contractions (Somlyo & Somlyo, 1968), indicating a varied ability to provide Ca to the cytoplasm. Prior contraction experiments showed basic similarities in the sensitivity of these agonists to Ca-deprivation and suggested that they utilize a common intracellular Ca pool (Deth & van Breemen, 1974). Each of these agents causes a stimulation of ^{45}Ca efflux (Fig. 9), although not of equal magnitude. Plotting efflux rate (mmoles Ca/Kg/min) *vs.* time on linear coordinates, and extrapolating the control segment of the curve to the post stimulation segment allowed an estimation of the size of the stimulation from the enclosed area, which has units of mmoles Ca/kg. NE and ANG (as well as the full alpha agonist phenylephrine) caused essentially equal-sized stimulations, while that due to HIS was about 1/2 the others. The relationship between the size of the initial 2 min of contractile response and the size of ^{45}Ca release at 46 min of efflux was further examined in the experiments of Table 2. In each case a close parallel was found between release size and developed tension. The correspondence between these two events

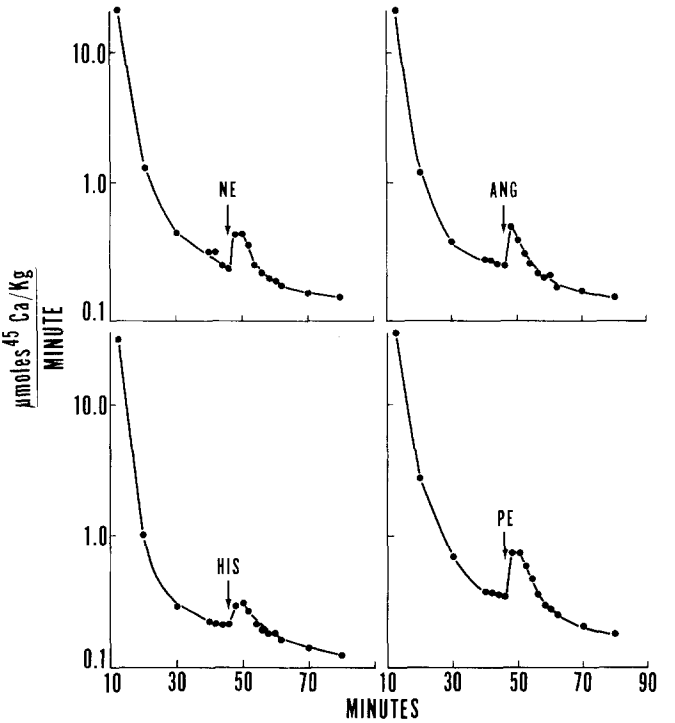

Fig. 9. The stimulation by NE, ANG, HIS and phenylephrine (PE) of ^{45}Ca efflux from rabbit aorta. Efflux medium = Ca-EGTA. Each agonist was present at 10^{-5} M throughout the remainder of efflux, beginning at 46 min. *Ordinate* = rate of Ca loss as μmoles ^{45}Ca/kg min. *Abscissa* = time in min. Each point is the average of 6 observations

Table 2

exp.	^{45}Ca release (μmoles/kg)[a]			Contractile response (G)[b]		
	NE	ANG	HIS	NE	ANG	HIS
1	1.28	1.03	0.63	3.75	2.20	1.70
2	3.91	3.80	1.19	3.30	2.88	1.45
3	4.76	2.96	1.33	3.70	2.80	1.75
% NE	100	79.9±10.0	35.8±6.7	100	73.8±8.2	45.4±1.0

[a] Each value is the mean of 3 determinations.
[b] Each value is the mean of 2 determinations.

indicates that the observed Ca release is responsible for the early rapid phase of contraction by these agonists.

In experiments analagous to those of Fig. 5, different agonists were added sequentially after an intervening period of washout (e.g., first NE, then ANG). Each agent greatly reduced the amount of ^{45}Ca subse-

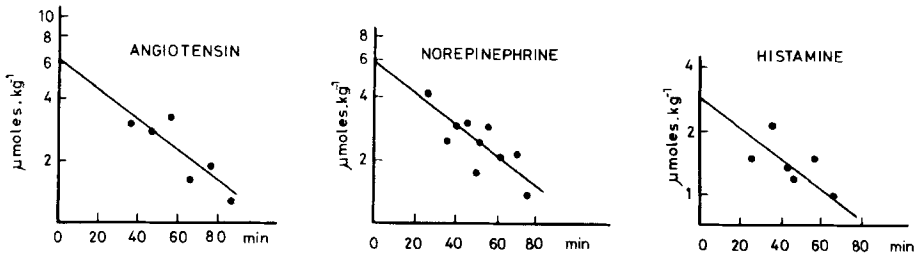

Fig. 10. Rate of washout of agonist controlled Ca pools. The size of release (*ordinate*) is plotted on semi-logs coordinates against the point in efflux at which the observation was made (*abscissa*). The calculated best fit straight line (least squares) is shown. Each point is the average of at least 3 determinations at that time. Efflux medium for all determinations is Ca-EGTA

quently releasable by the other agonists. Consistent with its smaller release size, HIS was somewhat less effective in exhausting the NE or ANG sensitive ^{45}Ca pools. Although there was a residual release by NE after HIS (1.25 μmoles/Kg at 64 min of efflux), a second HIS exposure gave no stimulation of efflux.

To better estimate the actual amount of Ca represented by the ^{45}Ca release, it was necessary to define the extent of labelling of the release fraction at the beginning of efflux. This is accomplished by measuring the gradual decline in the amount of ^{45}Ca released as a function of the length of efflux time before stimulation (van Breemen & Casteels, 1974). Using this technique, the size of the ^{45}Ca release at $t=0$ can be estimated by extrapolation (Fig. 10). For the three different receptor-linked Ca releases, essentially identical rates of label exchange were observed ($t^1/_2=36$–40 min). The relative size of the releases at $t=0$ (by extrapolation) was comparable to the relation found at 46 min of efflux in Table 2. Thus the HIS release was observed to be smaller than NE ($p<0.05$), but the two have similar exchange characteristics.

Discussion

The contraction of vascular smooth muscle by NE, ANG and HIS reflects the ability of these hormones to provide Ca to the cytoplasmic contractile elements. Experiments from a number of earlier investigators (Hinke *et al.*, 1964; Steinsland *et al.*, 1973) and from our laboratory (van Breemen *et al.*, 1972; Deth & van Breemen, 1974) suggested that the source of the Ca might be intracellular. This conclusion is firmly established by the present results.

The initial rapid phase of NE contractile response is essentially unaffected by blockade of Ca entry or removal of extracellular Ca (Fig. 1),

indicating its intracellular source. In contrast, the later slow phase is eliminated by these procedures. A similar pattern has been established for alpha adrenergic responses of the rabbit ear artery (Steinsland *et al.*, 1973), and spleen capsule (Bose & Innes, 1975). In the absence of the slow phase, subsequent NE exposure does not induce contraction, indicating that access of Ca_{ext} is essential for refilling of the intracellular release sites.

A number of studies have described the ability of intracellular organelles such as sarcoplasmic reticulum (*SR*) and mitochondria to accumulate Ca (Carsten, 1969; Somlyo & Somyo, 1971; Somlyo *et al.*, 1974; Batra, 1975; Debbas *et al.*, 1975). This ability is thought to illustrate their role in the reduction of cytoplasmic $[Ca^{2+}]$ (Ca_{cyt}) during relaxation. The results of this study show that the NE sensitive intracellular Ca fraction is separate from intracellular fractions bound during relaxation. As mentioned above, when access of Ca_{ext} is blocked, only a single transient contraction can be elicited, indicating that previously released Ca does not return to release sites via an intracellular route. In a similar manner, ^{45}Ca released during the first NE response does not return to release sites, as evidenced by the lack of increased efflux upon a second exposure (Fig. 5). Thus the NE-releasable intracellular Ca is apparently not located in organelles which accumulate Ca during relaxation (i.e., *SR* and mitochondria). This is in distinction from skeletal muscle, where intracellular recycling of Ca is believed to occur from longitudinal reticulum to regions from which it can again be released (lateral sacs) (Winegrad, 1970). These results do not exclude the possibility of a similar functional separation in *SR* of smooth muscle.

Guided by these findings, we feel that an alternative intracellular source for the NE-sensitive Ca fraction is the inner surface of the plasma membrane. Fig. 11 schematically illustrates a working model, which serves as a testable description of pertinent Ca movements. The location of release sites on the plasma membrane has been considered previously (Triggle, 1972). This locus provides an easy means of refilling exclusively from *ECS* calcium. As mentioned, the period of increased permeability associated with the slow contractile phase may represent the activation of the refilling process. In addition, some entering Ca moves into the cytoplasm to give the maintained contractile response.

Activation of arterial smooth muscle by hormonal agonists such as NE is not associated with either action potential generation, or consistent changes in membrane potential (Shibata & Briggs, 1966; Haeusler, 1972; Droogmans, *personal communication*). It has been suggested that the

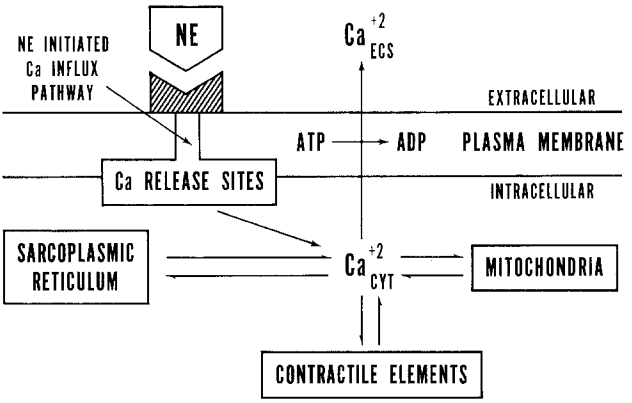

Fig. 11. Schematic diagram of Ca movements in aterial smooth muscle. Ca released by NE is subject to intracellular binding and/or Ca extrusion processes. Refilling of release sites is accomplished by influx of Ca_{ECS}

coupling event after alpha receptor activation might be either a decrease in cAMP, or an increase in cGMP levels. The results found in Table 1, and similar experiments by others (Daniel & Crankshaw, 1974; Seidel, Schnarr & Sparks, 1975) argue against an involvement of cAMP. Exogenous c-GMP was found to be without effect on ^{45}Ca efflux, and failed to modify NE response (Fig. 8), although it is possible that even the more lipid soluble butyryl derivative failed to enter the cell. Recently Schultz and Hardman (1975) showed that the changes in cGMP, which can be found after agonist induced contraction, are due to the ability of Ca to regulate cyclic nucleotide metabolism. Thus an intracellular Ca release remains as the primary event, intimately associated with receptor occupation. We feel that in the absence of classical coupling events, the consideration of a directly linked Ca release is warranted. Due to its compact nature, the initiating signal would only have to be efficiently transmitted over the 100 Å transmembrane width.

Another important question is: What is the size of the agonist-sensitive release fraction? From the experiments of Fig. 10 it is possible to estimate the amount of intracellular Ca released. The observed exchange rate (reflected in the decreasing ^{45}Ca content of the released Ca) had a t 1/2 of approximately 36 min, so that 20 min of ^{45}Ca loading would achieve 29% of exchange. The size at $t=0$ if fully exchanged would be about 21 μmoles/kg wet weight. Since a portion of the released ^{45}Ca is likely to be retained within intracellular organelles, this value should be an underestimate. This quantity should be adequate to account for the initial NE contractile response, although there is a considerable amount of variation in estimates of the amount of Ca which would

be required (van Breemen & Deth, 1976). The estimated release size for HIS is substantially lower ($\sim$10 μmoles/kg), reflecting its reduced efficiency.

Three lines of evidence suggest that NE, ANG and HIS utilize the same intracellular Ca source: (1) after La, each reduces the contraction elicited by another (Deth & van Breemen, 1974); (2) stimulation of ^{45}Ca efflux by each is reduced (or eliminated) by prior stimulation with another (*see* text); (3) the released ^{45}Ca exchanges with ^{40}Ca at a similar rate for all three agonists (Fig. 10). It is beyond the scope of this paper to speculate on how common a Ca source could be released by different receptor interactions. Further work is required to establish the basis for this intriguing relationship.

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