Excitation-Contraction Coupling in Rabbit Aorta Studied by the Lanthanum Method for Measuring Cellular Calcium Influx

By Cornelis van Breemen, Blanca R. Farinas, Peggy Gerba, and Elizabeth D. McNaughton

ABSTRACT
Lanthanum inhibits 45Ca efflux from internally injected squid axons. Evidence for La3+ blockade of Ca2+ fluxes across the cell membrane was also obtained for aortic smooth muscle: 1mM La3+ completely inhibited contractions which otherwise resulted from adding Ca2+ to calcium-free depolarizing solutions, and 2 mM La3+ caused a 50% inhibition of the late 45Ca efflux. Ca2+ bound extracellularly could be displaced by La3+, and the binding sites preferred La3+ over Ca2+. We could thus use La3+ to eliminate extracellular bound Ca2+ from our calcium-influx measurements as follows: after exposure of the aortic strips to experimental solutions labeled with 45Ca, the extracellular Ca2+ label was displaced by putting the strips in a calcium-free solution containing 2 mM La3+ for 60 minutes. The loss of intracellular Ca2+ was minimized during this hour by the La3+ blockade of Ca2+ membrane fluxes. Tissue weight, 45Ca, and total Ca2+ were then measured using standard techniques. Studies employing this new method showed that depolarization by high K+, Na+ replacement, and high pH activate smooth muscle contraction by stimulating Ca2+ influx. Low pH and La3+ block Ca2+ influx. Norepinephrine initiates aortic contractions by release of intracellularly sequestered Ca2+. Angiotensin and histamine appear to release Ca2+ from this same fraction.

KEY WORDS arterial smooth muscle lanthanum inhibition norepinephrine histamine angiotensin squid axon potassium-induced depolarization

Peripheral resistance is a function of the contractile state of arterial smooth muscle cells. Since motor innervation to arterial muscle is mainly by norepinephrine-secreting sympathetic neurons, antihypertensive therapy and research have focused on inhibition of the sympathetic nervous system. However, it has thus far been impossible to establish a cause and effect relationship between malfunction of the autonomic nervous system and essential hypertension (1). Only part of the activation of arterial smooth muscle is neurogenic, the remainder being myogenic. A promising approach to the alleviation of hypertension is thus to understand and then modify the cellular control system which regulates the contractile state. For the work reported here, it suffices to state that the system which regulates the cytoplasmic Ca2+ controls the contractile state of arterial smooth muscle. The threshold for activation of the contractile proteins is 10^-7M Ca2+ and maximum activation occurs at 10^-6M Ca2+. Examination of electron micrographs of arterial smooth muscle (2) reveals that the membrane in closest association with the myofibrils is the cell membrane. The following physical characteristics of the cell membrane
are particularly suited for the regulation of cytoplasmic \([\text{Ca}^{2+}]\). (1) There is a very large \(\text{Ca}^{2+}\) gradient across the membrane allowing a rapid influx of \(\text{Ca}^{2+}\) if permeability is increased. (2) The surface area of the membrane is greatly increased by numerous inpocketings or "micropinocytotic vesicles" which could provide binding sites for both release into and reuptake from the cytoplasm of \(\text{Ca}^{2+}\) (2). (3) It may be calculated that there is ample time for \(\text{Ca}^{2+}\) to diffuse from the surface to the myofibrils to account for the time course of force development by arterial smooth muscle. Many unsuccessful attempts have been made to measure \(\text{Ca}^{2+}\) fluxes across the cell membranes of smooth muscles (review by Lüllmann [3]). A probable reason for this failure is that the very large degree of extracellular \(\text{Ca}^{2+}\) binding has obscured the relatively small membrane \(\text{Ca}^{2+}\) fluxes. This report describes how the problem was solved using the trivalent cation, lanthanum, to isolate membrane \(\text{Ca}^{2+}\) exchange.

**Methods**

\(^{45}\text{Ca}\) Efflux from Squid Axon.—Giant axons were dissected from live specimens of Loligo pealeii, cleaned, and either used immediately or stored in ice-cold artificial seawater for up to 4 hours before injection. A solution (0.3 \(\mu\)l) of \(^{45}\text{Ca}\) (0.5 mc/ml in 0.5x KCl) was injected into squid axons longitudinally according to the method of Caldwell et al. (4). The final concentration of the injected \(\text{Ca}^{2+}\) was 0.3-0.5 mM. After the injection, the volume of solution in the chamber in which the axon was suspended was reduced to 1 ml, and the axon was perfused with the experimental solutions at the rate of 0.8 ml/min. The perfusion solutions were collected every 3 minutes, dissolved in Bray's scintillation fluid, and counted in a Packard liquid scintillation counter. For total \(\text{Ca}^{2+}\) determinations, the aortas were cleaned and cut open in two longitudinal halves, so that control and experimental variables could be obtained from the same rabbit. Tissue samples of at least 70 mg were used, and the ash was dissolved in 2 ml of 1 mM \(\text{LaCl}_3\) for 10 ml of Bray's solution was added, and the solution was counted in a Packard liquid scintillation counter. For total \(\text{Ca}^{2+}\) content, the aortas were cleaned and cut open in two longitudinal halves, so that control and experimental variables could be obtained from the same rabbit. Tissue samples of at least 70 mg were used, and the ash was dissolved in 2 ml of 1 mM \(\text{LaCl}_3\) in 0.1N HCl; the total \(\text{Ca}^{2+}\) content was measured against a proper standard curve with a Perkin Elmer 512-303 atomic absorption spectrophotometer.

\(^{45}\text{Ca}\) Influx.—After exposure to \(^{45}\text{Ca}\), aortic strips were placed in calcium-free TS containing \(2 \text{mM} \text{LaCl}_3\) for 1 hour. The strips were then blotted, weighed, and ashed. The ash was dissolved in 1 ml of 1 mM \(\text{LaCl}_3\) for 10 ml of Bray's solution was added, and the solution was counted in a Packard liquid scintillation counter. Aortic strips were allowed to take up \(^{45}\text{Ca}\) from labeled TS for 20 minutes before being placed in either calcium-free TS or calcium-free TS with 2 mM \(\text{LaCl}_3\). They were then removed at varying times, blotted, weighed, and prepared for counting as above.

**Results**

We have previously shown that transport of \(\text{Ca}^{2+}\) across a phospholipid-cholesterol artificial membrane of the Tobias type involves three steps (5): (1) association on one side, (2) exchange between fixed negative sites inside the membrane, and (3) dissociation from the other side. The experimental evidence suggested that there exists an optimum affinity for the negative binding sites for the...
Figure 1 shows the concentration dependence of \( \text{La}^{3+} \) inhibition. These results can be plotted according to the equation:

\[
\frac{\text{inhibition}}{\text{inhibition}_{\text{max}}} = \frac{[\text{La}^{3+}]}{[\text{La}^{3+}] + K_d}
\]

where \( K_d = 0.23 \) mM and the maximum inhibition is 87%. Thus in the presence of 10 mM external \( \text{Ca}^{2+} \), the half saturation value for \( \text{La}^{3+} \) inhibition of \( \text{Ca}^{2+} \) efflux (\( K_d \)) is 0.23 mM. Removal of external \( \text{Ca}^{2+} \) reduced this value to about 0.1 mM.

Using the value of 0.41 mmole \( \text{Ca}^{2+} \)/kg axoplasm (10), the average efflux from axons of \( \text{Loligo pealeii} \) (average diameter 450 mm) was \( 0.25 \pm 0.02 \) pmole/cm² sec⁻¹, in good agreement with values in the literature (10, 11). \( \text{La}^{3+} \), which appeared to compete with external \( \text{Ca}^{2+} \) for binding sites on the cell membrane, could inhibit 87% of this efflux. In some experiments, the t½ for \( ^{45}\text{Ca} \) loss after \( \text{La}^{3+} \) was 14 days. Such a slow rate of loss could possibly be caused by physical damage due to dissection. The 87% blockade should thus be regarded as the lowest estimate for \( \text{La}^{3+} \) blockade of the physiological mechanism for transport of \( \text{Ca}^{2+} \) across the cell membrane.

The remainder of the results are from experiments with rabbit aorta; these studies are conceptually divided into three parts. The first part deals with the failure of the...
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In Figure 3, we compare the conventionally measured 45Ca uptake from solutions containing either Na+, Li+, or K+, as the major monovalent cation with the rate of force development in the same solutions. No correlation is seen between the uptake of labeled Ca2+ by the whole aortic strip and its force development. However, since only 7 µmole Ca2+/kg aorta is needed for full activation (see discussion), it is probable that a large portion of the total Ca2+ exchange is unrelated to contraction.

In the discussion, we have provided documentation for both the extracellular distribution of La3+ and the extensive binding of Ca2+ to sites in direct contact with the extracellular space. La3+ should be able to displace this extracellular Ca2+ (including Ca2+ bound to outer surfaces of cell membranes). The upper curve of Figure 4 shows the loss of labeled Ca2+ from aortic strips loaded with 45Ca conventional calcium-exchange method to reveal Ca2+ fluxes responsible for activation of the contractile proteins. The second part presents evidence, most of which is indirect, demonstrating that La3+ blocks transport of Ca2+ across smooth muscle cell membranes and displaces extracellular bound Ca2+. The final part uses the principles and evidence of the preceding portion of the paper to elucidate cellular Ca2+ movements associated with variations in tension of the arterial wall.

Figure 2 illustrates total Ca2+ exchange in the aorta. The lower curve shows the rate of 45Ca uptake in tissues whose total Ca2+ content is given by the upper curve. Thus isotope exchange measured conventionally is rapid initially. The slow exchange after 10 minutes is difficult to quantify because of the initially large Ca2+ exchange.

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Indirect evidence for La$^{3+}$ blockade of membrane Ca$^{2+}$ flux. A: The solid curve shows a control contraction induced by depolarization with high K$^+$. The dotted line indicates that the rapid phase of the K$^+$ contraction is lost after 10 minutes in calcium-free solution. Adding 1.5 mM Ca$^{2+}$ to the solution gives a large contraction. B: The experiment is repeated, except 0.5 mM La$^{3+}$ is added before adding the Ca$^{2+}$. The absence of force development demonstrates that La$^{3+}$ is preventing transfer of Ca$^{2+}$ across the membrane into the cytoplasm.

for only 20 minutes. The lower curve demonstrates that when 2 mM LaCl$_3$ is added to the calcium-free TS, almost 1 mmole bound Ca$^{2+}$/kg aorta is displaced. The displaced Ca$^{2+}$ is probably all extracellular since: (1) La$^{3+}$ is thought to be distributed extracellularly; (2) La$^{3+}$ blocks Ca$^{2+}$ efflux; and (3) the effect of La$^{3+}$ is fairly rapid. This La$^{3+}$:Ca$^{2+}$ competition was measured quantitatively as follows. Aortic strips were exposed for 20 minutes to either a solution containing 290 mM sucrose, 10 mM glucose, and 1 mM $^{45}$Ca-labeled CaCl$_2$ or the same solution with 1 mM LaCl$_3$ added. The pH of both solutions was continually recorded and maintained at 7.3. Since Ca$^{2+}$ and La$^{3+}$ were the only cations in the extracellular space, the total number of negative binding sites and the specificity coefficient La$^2$/Ca$^3$ for the exchange reaction 2 La$^{3+}$ + Ca$_3$ membrane $\rightleftharpoons$ 3 Ca$^{2+}$ + La$_2$ membrane could be determined according to the method of Sparrow (12). The concentration of the calcium-binding sites was 8.1 ± 0.3 mmole/kg wet weight, and the selectivity coefficient of La$^{3+}$ over Ca$^{2+}$ was 3.1. We have also duplicated this La$^{3+}$ displacement of bound Ca$^{2+}$ in the acellular portion of rabbit tendon.

Figure 5 gives indirect evidence for La$^{3+}$ blockade of Ca$^{2+}$ flux across the membrane. The solid curve (A) indicates the control contraction caused by potassium-induced depolarization, and the dotted line indicates that the rapid phase of the potassium-induced contraction was lost after 10 minutes in a calcium-free solution. Adding Ca$^{2+}$ to the K$^+$ solution then gives a large contraction. The Ca$^{2+}$ source for this contraction is thus extracellular. For the results in B we repeated the same experiments but added 0.5 mM La$^{3+}$ before adding the Ca$^{2+}$. The absence of force development in this case demonstrates that La$^{3+}$ prevented the transfer of Ca$^{2+}$ across the membrane into the cytoplasm. The inhibition of contraction was not due to a failure of the contractile machinery, since the aorta could still contract in response to norepinephrine following a similar calcium-free La$^{3+}$ treatment. That the efflux of cellular Ca$^{2+}$ was also

\begin{figure}
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\includegraphics[width=\textwidth]{figure5}
\caption{Indirect evidence for La$^{3+}$ blockade of membrane Ca$^{2+}$ flux. A: The solid curve shows a control contraction induced by depolarization with high K$^+$. The dotted line indicates that the rapid phase of the K$^+$ contraction is lost after 10 minutes in calcium-free solution. Adding 1.5 mM Ca$^{2+}$ to the solution gives a large contraction. B: The experiment is repeated, except 0.5 mM La$^{3+}$ is added before adding the Ca$^{2+}$. The absence of force development demonstrates that La$^{3+}$ is preventing transfer of Ca$^{2+}$ across the membrane into the cytoplasm.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6}
\caption{The cellular uptake of $^{45}$Ca from solutions containing Na$^+$, Li$^+$, or K$^+$ as the major cation as measured by the La$^{3+}$ method. See text for details.}
\end{figure}

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blocked is suggested by the 50% reduction in the rate of 45Ca efflux by 2 mM La3+ seen 80 minutes after the onset of efflux. It is, however, impossible to separate cellular efflux from total efflux by this experiment in which tissues were loaded with 45Ca for 2 hours and then allowed to lose their label to nonradioactive TS.

Using the displacement of extracellular Ca2+ by La3+ and the La3+ blockade of membrane Ca2+ transport (as shown directly in the artificial membrane and squid axon and indirectly in aortic smooth muscle), we designed the following method for isolating cellular Ca2+ uptake. Aortic strips were placed in the labeled experimental solution for specific times; this was followed by a 60-minute exposure to La3+ -TS to remove the extracellular Ca2+. The tissues were then analyzed for 45Ca or for total Ca2+.

Figure 6 shows that replacement of Na+ by Li+ stimulates Ca2+ influx. Replacement of Na+ by K+, which completely depolarizes the membrane, stimulates Ca2+ influx to a greater extent. Comparison with Figure 3B shows excellent correlation between Ca2+ influx and force development. The quantitative aspects of this comparison will be delayed until the discussion. Analysis of total calcium after 1 hour in K+-TS showed that the aortic cells had gained 0.12 mmole/kg hour⁻¹ (0.32 ± 0.029 mmole/kg to 0.440 ± 0.038 mmole/kg) over tissues in Na+-TS. The possibility that the increased [Ca2+] in K+-TS might have been due to the extrusion of extracellular water on contraction was discarded by measuring the sucrose space of the tissue under both relaxed and contracted conditions. The sucrose space in Na+-TS was 454 ± 26 ml/kg, and in K+-TS it was 471 ± 15 ml/kg. Thus high-K+ solutions stimulate a net Ca2+ influx into aortic smooth muscle cells.

The agreement between force development and measured Ca2+ influx is evidence confirming the validity of the La3+ method. Further agreement is seen in Figure 7. La3+ inhibits both the K+- and Li+-induced Ca2+ influx which correlates with our observation that 2 mM La3+ completely blocks both K+- and Li+-induced contractions.

To obtain a measure of the affinity of the Ca2+ transport sites for La3+, we measured the inhibitory effect of La3+ on the rate of the contraction induced by high K+ as a function of [La3+]. After a control contraction, the aortic rings were incubated in Na+-TS with La3+ for 30 minutes. They were then exposed

\[ \text{Inhibition of } \frac{\text{d}^2 \text{Ca influx by } 2 \text{ mM La}^{3+} \text{ in Li}^+-\text{TS and K}^+-\text{TS. The distance between the horizontal bars equals two times the standard error for at least four determinations.}} \]

\[ \text{FIGURE 7} \]

\[ \text{FIGURE 8} \]

\[ \text{The rate of contraction induced by } K^+ \text{ after addition of LaCl}_3 \text{ relative to the rate of contraction in the controls is plotted as a function of [LaCl}_3]. \]
to K\(^+\)-TS with the same [La\(^{3+}\)]. The initial approximately linear rates of contraction before and after La\(^{3+}\) were then compared. Figure 8 shows that the half maximal concentration for the La\(^{3+}\) blockade was 0.1 mM.

If, as we have assumed, Ca\(^{2+}\) is transported across the membrane by negative sites (be they fixed or mobile), then the addition of protons to these sites should inhibit cellular Ca\(^{2+}\) uptake. In Figure 9, A provides supporting data for this assumption. Lowering of the pH causes a two-step reduction in Ca\(^{2+}\) uptake as if two different transport sites were involved. Increasing the pH greatly stimulates Ca\(^{2+}\) influx. Again we find excellent agreement between influx data and force development; B shows that low pH relaxes a Li\(^+\)-induced contraction, and that high pH gives rise to a maximum contraction. Short exposures (up to 20 minutes) to these large pH changes are reversible.

The above experiments suggest that negative membrane sites are involved in inward Ca\(^{2+}\) transport, that Na\(^+\) and Ca\(^{2+}\) compete for these sites, and that depolarization by high K\(^+\) further increases Ca\(^{2+}\) entry. Although these are important concepts in understanding the system controlling [Ca\(^{2+}\)], none of these stimuli are physiological.

An important remaining question is how norepinephrine activates arterial smooth muscle. Does it increase Ca\(^{2+}\) influx or release Ca\(^{2+}\) from binding sites inside the cell? Figure 10 supports the latter contention.

Figure 10B shows that 10\(^{-5}\)M norepinephrine does not increase Ca\(^{2+}\) influx over the control values, whereas high K\(^+\) does stimulate it in the same rabbit aortas. From this result we would predict that La\(^{3+}\) would not inhibit the norepinephrine-induced contraction. Figure 10A shows that this is the case. We do see that the slow phase of the norepinephrine-induced contraction is inhibited, and therefore the possibility that norepinephrine causes some Ca\(^{2+}\) to enter the cells cannot be excluded. This Ca\(^{2+}\) entry is, however, much smaller than in the case of the contraction induced by high K\(^+\). It seems most probable that the initial rapid phase of the norepinephrine-induced contraction is of intracellular origin, since it persists even after exposure to calcium-free solutions containing La\(^{3+}\). The experiment of Figure 10 provides additional information on the Ca\(^{2+}\) source for norepinephrine stimulation; namely, after La\(^{3+}\) is added to the bath, norepinephrine can cause only one contraction.

The same phenomenon shown in Figure 10A is true for histamine and angiotensin II.
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Figure 10

A: In the experiment illustrated at the top, the force development of an aortic ring is measured when the ring is exposed to K\textsuperscript+ -TS for 2 minutes, Na\textsuperscript+ -TS for 5 minutes, Na\textsuperscript+ -TS + 0.5 mM LaCl\textsubscript{3} for 33 minutes, and finally Na\textsuperscript+ -TS + 0.5 mM LaCl\textsubscript{3} + 10\textsuperscript{-5}M norepinephrine for 4 minutes. The aortic ring in the bottom experiment was exposed to the same solutions, except that between 12 and 16 minutes it was stimulated to contract with 10\textsuperscript{-5}M norepinephrine.

B: Uptake of labeled Ca\textsuperscript{2+} into smooth muscle cells of rabbit aorta from the control physiologic solution (CONT), the same solution containing 10\textsuperscript{-5}M norepinephrine (NEP), and a solution containing 160 mM KCl (K). See text for solutions. Each point is the average of at least four determinations. Standard errors are indicated by the distances between the bars and the nearest point.

Discussion

The two prominent features of this paper which merit further consideration are: (1) the properties of La\textsuperscript{3+} which make it so useful in the study of excitation-contraction coupling of arterial smooth muscle and (2) the striking correlations between the Ca\textsuperscript{2+} influx measured by the La\textsuperscript{3+} method and force development in the aorta. Lettvin et al. (7) pointed out that La\textsuperscript{3+} and Ca\textsuperscript{2+} have roughly similar hydrated radii (3.1 Å and 2.8 Å, respectively) and that the higher valence of La\textsuperscript{3+} will cause it to bind more strongly to calcium-binding sites. Chemical studies on monolayers, millipore filters impregnated with phospholipids, proteins, and mucopolysaccharides. La\textsuperscript{3+} could thus exert the following effects on biological membranes: (1) stabilize the bilayer portion of the membrane, (2) bind to the Ca\textsuperscript{2+} sites which control the excitable Na\textsuperscript+ channel (where it may substitute at low concentrations and block at high concentrations) (7) and (3) bind to sites in the membrane which are involved in the transport of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} and inhibit these processes. All these effects have indeed been observed. Pressman (personal communication) noted that passive cation leaks are reduced when La\textsuperscript{3+} is present during the preparation of red cell ghosts. Takata et al. (8) found a depressing effect of high [La\textsuperscript{3+}] on the action potential of the lobster giant axon. La\textsuperscript{3+} at low concentrations appears to be able to substitute for Ca\textsuperscript{2+} in excitation. We have shown that La\textsuperscript{3+} blocks Ca\textsuperscript{2+} efflux from the squid axon, and Mela (19) demonstrated a specific blockade of systems (13-18) have amply proven the high affinity of La\textsuperscript{3+} for carboxyl and phosphate groups on phospholipids, proteins, and mucopolysaccharides. La\textsuperscript{3+} could thus exert the following effects on biological membranes: (1) stabilize the bilayer portion of the membrane, (2) bind to the Ca\textsuperscript{2+} sites which control the excitable Na\textsuperscript+ channel (where it may substitute at low concentrations and block at high concentrations) (7) and (3) bind to sites in the membrane which are involved in the transport of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} and inhibit these processes. All these effects have indeed been observed. Pressman (personal communication) noted that passive cation leaks are reduced when La\textsuperscript{3+} is present during the preparation of red cell ghosts. Takata et al. (8) found a depressing effect of high [La\textsuperscript{3+}] on the action potential of the lobster giant axon. La\textsuperscript{3+} at low concentrations appears to be able to substitute for Ca\textsuperscript{2+} in excitation. We have shown that La\textsuperscript{3+} blocks Ca\textsuperscript{2+} efflux from the squid axon, and Mela (19) demonstrated a specific blockade of

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mitochondrial Ca\(^{2+}\) transport by very low [La\(^{3+}\)] (10\(^{-8}\)M).

If negative membrane groups mediated multivalent cation transport, then La\(^{3+}\) with its high positive charge density should dissociate from these groups much more slowly than Ca\(^{2+}\) and thus be poorly transported across the cell membrane. Many electron microscopic studies have borne this out. For example, Lesseps (20), using a suspension of single cells, found that the La\(^{3+}\) stain does not appear on any membranous structures within the cells.

Caution should be used in interpreting these results to mean complete impermeability of the cell membranes to La\(^{3+}\), since the electron microscope cannot detect low [La\(^{3+}\)]. Significant La\(^{3+}\) displacement of intracellular Ca\(^{2+}\) is unlikely, since it inhibits the efflux of cellular Ca\(^{2+}\). Recent unpublished experiments in our laboratory showed that 10\(^{-4}\)M 2, 4-dinitrophenol plus 10\(^{-3}\)M iodoacetic acid causes the aorta to gain Ca\(^{2+}\). The Ca\(^{2+}\) so gained should be intracellular, and efflux of \(^{45}\)Ca gained under these conditions is completely blocked by La\(^{3+}\). The extracellular distribution of La\(^{3+}\) and its greater affinity for phosphate and carboxyl groups than that of Ca\(^{2+}\) are ideal for the displacement of extracellular bound Ca\(^{2+}\). That such extracellular Ca\(^{2+}\) binding is extensive has been clearly demonstrated by Borle (21) in his study on HeLa cell suspensions. It thus seems reasonable that La\(^{3+}\) displaces mainly extracellular bound Ca\(^{2+}\) in the aortic smooth muscle (22). Weiss and Goodman have also shown such La\(^{3+}\) displacement of Ca\(^{2+}\) in intestinal smooth muscle (23).

Contractions induced by depolarization with high K\(^{+}\) are readily abolished by removal of Ca\(^{2+}\) from the extracellular space (24-26). When Ca\(^{2+}\) is returned to the high K\(^{+}\) bathing solution, the aorta contracts immediately, as seen in Figure 5. These experiments strongly suggest that the activating Ca\(^{2+}\) is supplied to the contractile proteins by an increased Ca\(^{2+}\) influx. By comparing Figures 3 and 6, it becomes clear that this increased Ca\(^{2+}\) influx can be demonstrated only after displacing extracellular \(^{45}\)Ca with La\(^{3+}\). The analytical data proved this to be a net influx. Figure 6 also shows direct evidence of Na\(^{+}\)-Ca\(^{2+}\) competition for transport into smooth muscle. When Li\(^{+}\) is substituted for Na\(^{+}\) in the bathing solutions, the slow but sustained contraction is rapidly abolished in calcium-free solution. These data are in agreement with the studies of Bohr et al. (27), which in addition provided evidence for coupling of Ca\(^{2+}\) influx to Na\(^{+}\) efflux, as was demonstrated in heart by Reuter and Seitz (28) and in squid axon by Baker et al. (29).

The fluxes observed using the La\(^{3+}\) method are about 5-10% of those seen before removal of extracellular Ca\(^{2+}\) and are of the right order of magnitude to explain the rates of smooth muscle activation. Thus, if we use the value of 5 mg actomyosin/g artery (30) and the values of \([\text{Ca}^{2+}]\) required for coupling given by Bianchi (31), we find that the aorta would require no more than 7 \(\mu\)mole Ca\(^{2+}\)/kg for complete activation of the contractile mechanism. The initial increase in the rate of Ca\(^{2+}\) influx from the experimental solutions over the control influx is 5.6 \(\mu\)moles/kg min\(^{-1}\) for K\(^{+}\)-TS and 2.5 \(\mu\)moles/kg min\(^{-1}\) for Li\(^{+}\)-TS. The most rapid phases of force development are 65% of maximum/min in K\(^{+}\)-TS and 10% of maximum/min in Li\(^{+}\)-TS. These observed rates of contraction are only slightly less than those calculated from the fluxes and could be readily explained if some of the Ca\(^{2+}\) penetrating the surface membrane were bound to structures other than troponin in the interior of the smooth muscle cell.

Using the La\(^{3+}\) method we were able to demonstrate that La\(^{3+}\) blocks Ca\(^{2+}\) influx. The concentration at which La\(^{3+}\) half maximally inhibits contractures is 10\(^{-4}\)M. The Ca\(^{2+}\) transport system of the squid axolemma has the same order of sensitivity to La\(^{3+}\) blockade, but Ca\(^{2+}\) transport by mitochondrial membranes is blocked by only 10\(^{-6}\)M La\(^{3+}\). On the other hand, Ca\(^{2+}\) accumulation by cardiac sarcoplasmic reticulum (32, 33) is rather insensitive to La\(^{3+}\). Lanthanum is thus able to bring out the remarkable individuality of the
various Ca\textsuperscript{2+} transport systems of cellular membranes.

The inhibition of Ca\textsuperscript{2+} influx at low pH and stimulation at high pH seems to implicate the involvement of negatively charged transport sites. However, transient conformational changes cannot at this point be ruled out.

We have seen that whenever Ca\textsuperscript{2+} influx was stimulated, it was accompanied by the predicted contraction, and when it was blocked, contractile force fell as expected. We consider this as strongly indicative that we are measuring cellular Ca\textsuperscript{2+} influx after having eliminated the extracellular Ca\textsuperscript{2+} by calcium-free La\textsuperscript{3+} solutions. Consequently, we assume that the lack of stimulation of Ca\textsuperscript{2+} influx by norepinephrine indicates that the major portion of the activating Ca\textsuperscript{2+} during this mode of stimulation has an intracellular origin. In effect this finding is in perfect agreement with the conclusion reached earlier by Hinke (24) on the basis of his norepinephrine-induced contraction studies on the rat tail artery. He found that, on removal of extracellular Ca\textsuperscript{2+}, contractile responses to norepinephrine were still present when the tissue had become unresponsive to high K\textsuperscript{+}. This has been confirmed for rabbit aorta by Hudgkins and Weiss (25) and van Breemen (26). The rabbit aorta even contracts after the extracellular Ca\textsuperscript{2+} has been removed and the extracellular bound Ca\textsuperscript{2+} has been displaced by La\textsuperscript{3+}. Since La\textsuperscript{3+} itself does not activate actomyosin, we feel that the only reasonable explanation is an intracellular release of Ca\textsuperscript{2+} by norepinephrine.

In the face of the paucity of smooth endoplasmic reticulum, the inside of the surface membrane with its many inpocketings is the most plausible site for the Ca\textsuperscript{2+} binding (34). This location would also be best suited for interaction with extracellular neurotransmitters. The interesting feature of this bound fraction is that it contains only sufficient Ca\textsuperscript{2+} for one maximal contraction. Thus after La\textsuperscript{3+}, one norepinephrine-induced contraction blocks subsequent contractions. Administration of norepinephrine, angiotensin II, or histamine is then unable to activate the muscle. We conclude from this that there is one common intracellular bound Ca\textsuperscript{2+} fraction which is released on activation of norepinephrine, angiotensin, or histamine receptors. La\textsuperscript{3+} probably prevents replenishment of this fraction by removing Ca\textsuperscript{2+} bound at the outer surface of the cell membrane. This is supported by our recent finding that SKF 525A, which also blocks the Ca\textsuperscript{2+} influx stimulated by depolarization with high K\textsuperscript{+} (35), does not prevent the replenishment of the drug-sensitive intracellular Ca\textsuperscript{2+} fraction (unpublished results). SKF 525A, a tertiary amine with a pK of 5.7 (36), is uncharged at pH 7.4 and is therefore unable to displace bound Ca\textsuperscript{2+} from the cell membranes.

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