PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Factors Controlling Cytoplasmic Ca<latex>$^{2+}$</latex> Concentration

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Phil. Trans. R. Soc. Lond. B 1973 **265**, 57-71
doi: 10.1098/rstb.1973.0009

Phil. Trans. R. Soc. Lond. B. **265**, 57–71 (1973) [57]
Printed in Great Britain

Factors controlling cytoplasmic Ca^{2+} concentration

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The study of cellular Ca^{2+} exchange in smooth muscle has been severely limited by extensive extracellular Ca^{2+} binding. This problem was solved by using La^{3+} to trap Ca^{2+} inside the cells while displacing extracellular bound Ca^{2+}. It was then shown that cytoplasmic Ca^{2+} could be raised by Ca^{2+} influx during high K^+ depolarization, Na^+ elimination and high pH. Ca^{2+} influx was inhibited by other multivalent cations, local anaesthetics and low pH.

In the rabbit aortae norepinephrine and angiotensin increase cytoplasmic Ca^{2+} by release from intracellular membrane surfaces. In this same smooth muscle relaxation is brought about by intracellular Ca^{2+} binding. Evidence from vascular, uterine and intestinal smooth muscle demonstrates that the large transmembrane Ca^{2+} gradient depends on cellular [ATP] but not on the Na^+ gradient. ATP depletion abolishes the Ca^{2+} gradient by increasing Ca^{2+} influx.

Introduction

Under physiological conditions contractile activity is directly controlled by the cytoplasmic Ca^{2+} concentration. Our approach to studying the mechanism which regulates the cytoplasmic Ca^{2+} concentration is to measure net and tracer Ca^{2+} fluxes between experimental solutions and intact smooth muscle cells of the rabbit aorta and guinea-pig taenia coli. The main questions upon which we have focused are:

(1) Does activator Ca^{2+} enter smooth muscle cells or is it released from intracellular stores such as the sarcoplasmic reticulum and the inner surface of the plasmalemma?

(2) Can we identify an 'active' Ca^{2+} transport mechanism which maintains a low cytoplasmic Ca^{2+} concentration in the face of an inward electrochemical Ca^{2+} gradient of approximately 38 kJ (9 kcal)/mol Ca^{2+}?

(3) Is relaxation brought about by extrusion of Ca^{2+} or by intracellular Ca^{2+} sequestration?

(4) Do some drugs or inorganic chemicals modify smooth muscle activity by affecting membrane Ca^{2+} transport?

In the past decade many attempts have been made to measure transmembrane Ca^{2+} fluxes and correlate them with smooth muscle activity. However, even under experimental conditions where the contractions depend directly on extracellular Ca^{2+}, analyses of Ca^{2+} flux data yielded contradictory or negative results (van Breemen & Daniel 1966; Lüllmann 1970, Krejci & Daniel 1970; Lammel & Golenhofen 1971).

The main obstacles to the study of Ca^{2+} membranes fluxes in smooth muscle are:

(1) The extracellular space is large and contains substances such as elastin, collagen and mucoproteins which bind large quantities of Ca^{2+}.

(2) Studies on the acellular portion of tendon show that extracellular Ca^{2+} exchanges at varying rates so that extracellular Ca^{2+} exchange does not precede cellular Ca^{2+} exchange.

(3) Semilogarithmic Ca^{2+} exchange curves obtained from smooth muscle tracer studies can not be fitted to unique sums of first order processes.

(4) The quantity of Ca^{2+} needed to activate a kilogram of smooth muscle tissue is of order of 10 to 50 μmol/l (Bianchi 1969), whereas the total exchangeable Ca^{2+} is two orders of magnitude greater.

(5) Ca^{2+} uptake is very sensitive to tissue dammage.

To circumvent at least the problem of extracellular Ca^{2+} exchange we developed a new method. We will first discuss the validity of this method and present experimental results related to the questions posed above.

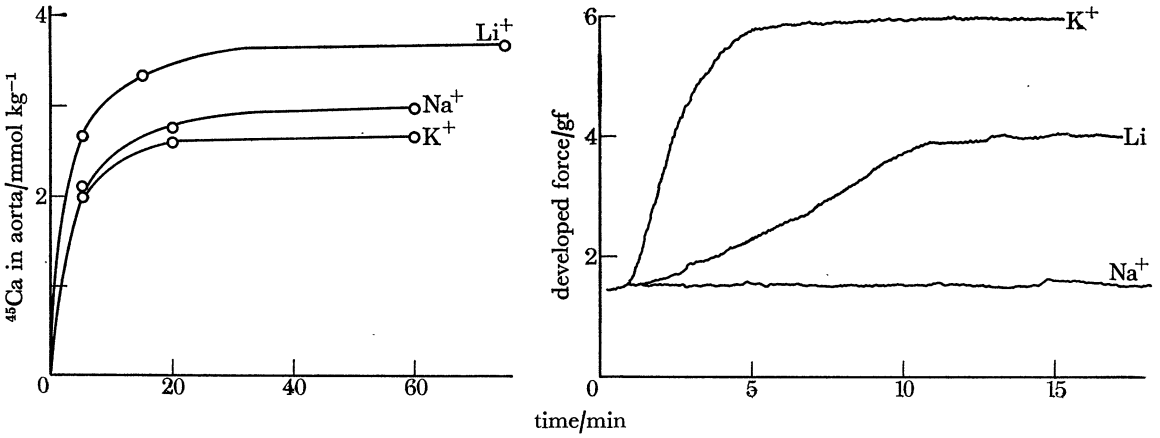

FIGURE 1. (*a*) ^{45}Ca uptake by aortic rings from solution containing either Na^+, Li^+, or K^+ as major monovalent cation. (*b*) Rate of force development in aortic rings perfused with the same solutions used in (*a*). No correlation is seen between the uptake of ^{45}Ca and force development. Each point in (*a*) is the average of at least four observations (from van Breemen *et al.* 1972).

THE LANTHANUM METHOD

In figure 1 we compare the ^{45}Ca uptake measured with conventional methods from solutions containing either Na^+, Li^+ or K^+ as the major monovalent cation with the rate of force development in the same solutions. In spite of the fact that both the K^+ depolarization contraction and the Na^+-free contraction are rapidly abolished by removal of extracellular Ca^{2+} and are blocked by lanthanum (Hinke 1965; van Breemen, Farinas, Gerba & McNaughton 1972) force development does not appear to be a function of Ca^{2+} influx. These results suggest that the conventionally measured ^{45}Ca uptake does not reflect cellular ^{45}Ca uptake. Borle (1968) demonstrated that even in a suspension of HeLa cells which lacks an extracellular matrix, 90 % of the total calcium was associated with the extracellular mucoprotein coat. We measured the concentration of fixed negative sites in the extracellular space of rabbit aortae by allowing short equilibration periods in isotonic sucrose solutions containing labelled $CaCl_2$ (Sparrow 1969). The concentration of extracellular calcium binding sites was 8.1 ± 0.3 mmol/kg wet tissue. Since these binding sites are in equilibrium with a calcium concentration which is some 15000 times higher than the intracellular free Ca^{2+} ion concentration ($[Ca^{2+}]_i$ at rest is less than 10^{-7} mol/l (Schädler 1967)), we assumed that the extracellular ^{45}Ca exchange was so great as to completely obscure the cellular influx under the conditions of figure 1. We developed a simple method to eliminate the extracellular Ca^{2+} exchange from our measurements. This method which will hereafter be referred to as the La method is represented schematically in figure 2. First the isolated smooth muscle preparation is placed in a ^{45}Ca-labelled experimental solution

for an appropriate length of time to allow entry of Ca^{2+} into the cells. Then 10 mmol/l $LaCl_3$ is added to this same solution which will cause the membrane to become impermeable to Ca^{2+}. After 3 min the preparation is transferred to an isotonic Ca-free solution containing 10 mmol/l $LaCl_3$ in which it is left for 45 min. As will be shown in this section, during this time almost all the extracellular bound Ca^{2+} is displaced from the tissue while the La^{3+} membrane blockade preserves nearly all of the cellular labelled Ca^{2+}. The tissues are then blotted, weighed and analysed according to established methods. This procedure can also be used to measure net transmembrane Ca^{2+} fluxes. Physiological solutions to which La^{3+} is added should be free of carbonate and phosphate ions to prevent precipitation of inorganic La^{3+} salts. We therefore used in the control solution the following concentrations (mmol/l): NaCl 160, $MgCl_2$ 1, $CaCl_2$ 1.5, KCl 4.6, dextrose 10, tris or Hepes 5. The pH was 7.4 or 7.2 and the solution was bubbled with O_2; the temperature was 37 °C. The validation of the La-method requires experimental evidence for La^{3+} blockade of Ca^{2+} membrane fluxes and displacement of bound calcium. As early as 1910, Mines demonstrated the inhibiting effect of La^{3+} on frog heart contractions.

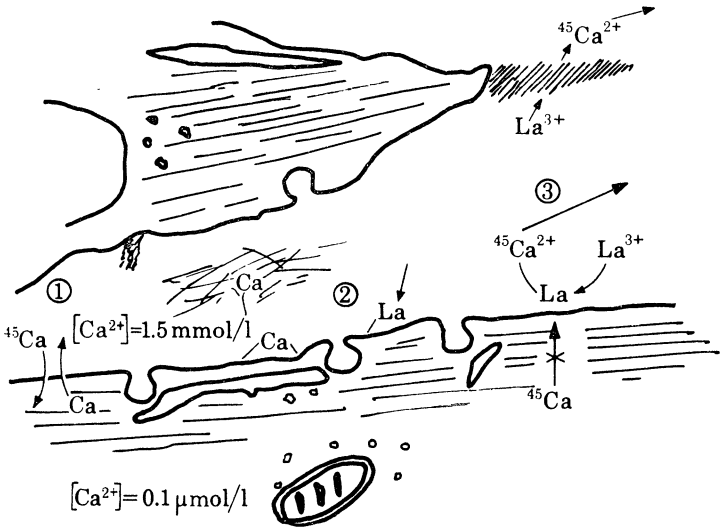

FIGURE 2. Schematic representation of the La method for measuring cellular Ca^{2+} in isolated smooth muscle. In (1) ^{45}Ca enters the cells during tissue exposure to an experimental solution. At the termination of the experimental period (2) 10 mmol/l $LaCl_3$ is added to the solution so that La^{3+} is able to bind to the membrane and block Ca^{2+} transport; 3 min later (3) the tissue is transferred to an isotonic Ca^{2+}-free solution containing 10 mmol/l $LaCl_3$ for 45 min. During this time extracellularly bound ^{45}Ca is displaced from the tissue by La^{3+} while the intracellular ^{45}Ca remains locked inside the cells. Finally the tissue is removed from the solution, blotted and analysed for ^{45}Ca and/or total Ca^{2+}.

Much later Lettvin, Pickard, McCullock & Pitts (1964) pointed out that La^{3+}, as a result of its much higher positive charge density and its similarity in hydrated radius, should have a much higher affinity for Ca^{2+} binding sites than Ca^{2+} itself. In a study of Ca^{2+} transport across an artificial phospholipid cholesterol membrane, van Breemen & van Breemen (1969) concluded that an optimum affinity of the negative charged transport sites for the polyvalent cation exists at which the transport rate reaches a maximum. At increasing affinities the transport would be limited by slower dissociation rates. Since the affinity for La^{3+} is much greater than for Ca^{2+}, La^{3+} itself would permanently occupy the transport sites and thus block Ca^{2+} transport across the membrane. This prediction was verified as is demonstrated in figure 3*a*. La^{3+} also displaced bound Ca^{2+} from the phospholipids. The next step was to demonstrate the same actions of

La^{3+} on cell membranes, most importantly on those of the tissues on which the method was to be used. The most unequivocal way to show the La^{3+} blockade of cellular Ca^{2+} efflux was to inject labelled Ca^{2+} longitudinally into squid giant axons, wait until the rate coefficient of Ca^{2+} efflux had become constant and then to add La^{3+} to the external solution (van Breemen & de Weer 1970). The effective blockade by La^{3+} is shown in figure 3*b*. The 87 % inhibition of Ca^{2+} efflux can be considered a low estimate since efflux of ^{45}Ca through mechanically damaged portions of the axolemma is not expected to be blocked by this procedure. La^{3+} blockade of Ca^{2+} fluxes has also been demonstrated in membranes of mitochondria (Mela 1968) and red cells (Schatzmann & Tschabold 1971).

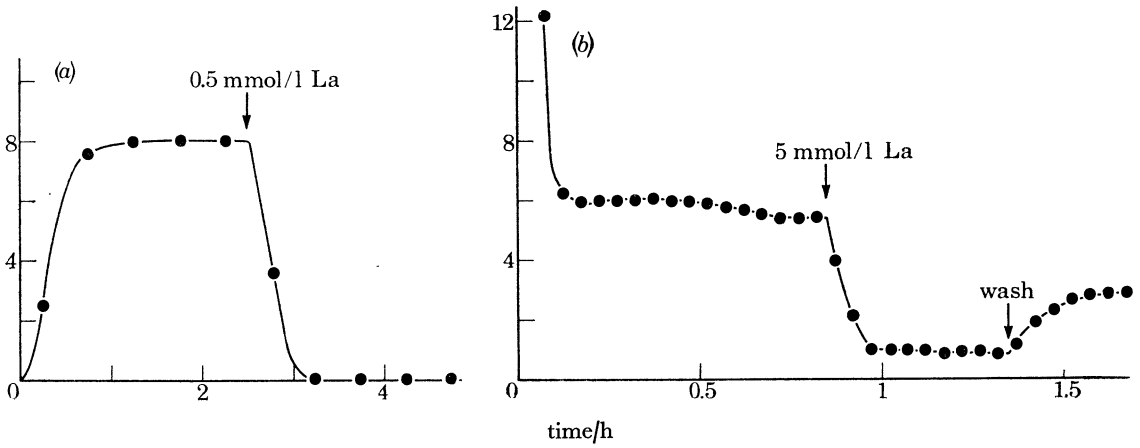

FIGURE 3. (*a*) Blockade by La^{3+} of ^{45}Ca flux across a millipore filter impregnated with an equimolar mixture of phospholipids and cholesterol. 0.5 mmol/l $LaCl_3$ is added to the aqueous solutions bathing both sides of the membrane at the time indicated by the arrow. (*b*) ^{45}Ca efflux from the squid axon bathed in phosphate and sulphate-free artificial sea water. 5 mmol/l $LaCl_3$ is added and removed from the bathing solution at the times indicated by the arrows (from van Breemen & De Weer 1970).

It is not possible to demonstrate this action of La^{3+} under control conditions in the rabbit aorta since we need the La method in the first place to reveal the cellular Ca^{2+} exchange. We see only a 50 % inhibition in the rate of ^{45}Ca washout after 3 h of exposure to efflux solution containing 2 mmol/l La^{3+}. However, the cellular Ca^{2+} content can be increased by metabolic inhibition (van Breemen, Daniel & van Breemen 1966). If we thus load the aorta with ^{45}Ca during 3 h exposure to 10^{-4} mol/l 2,4-dinitrophenol (DNP) and 10^{-3} mol/l monoiodoacetic acid (IAA) we can increase the relative contribution of the cellular efflux. In figure 4 we see that under these conditions La^{3+} clearly blocks the efflux of ^{45}Ca from aortic smooth muscle cells. Identical results were obtained with the taenia coli as will be shown later.

The combination of DNP and IAA reduces the ATP concentration irreversibly to zero so that figure 4 shows only La^{3+} blockade of passive Ca^{2+} efflux. However, in red cells La^{3+} blocks active transport (Schatzmann & Tschabold 1971) and in squid axon it blocks the Na–Ca exchange mechanism (Baker, Blaustein, Hodgkin & Steinhardt 1969). La^{3+} also blocks both the control and the serotonin stimulated Ca^{2+} efflux from the anterior byssal retractor muscle of *Mytilus edulis* (F. Baguet & C. van Breemen, unpublished results). This serotonin stimulated Ca^{2+} efflux is considered to be active (Bloomquist & Curtis 1972). We have now also shown that La^{3+} inhibits Ca^{2+} efflux after increasing the cellular ^{45}Ca content of the aorta during high K^+ exposure. The second criterion which the La method has to meet is the displacement of

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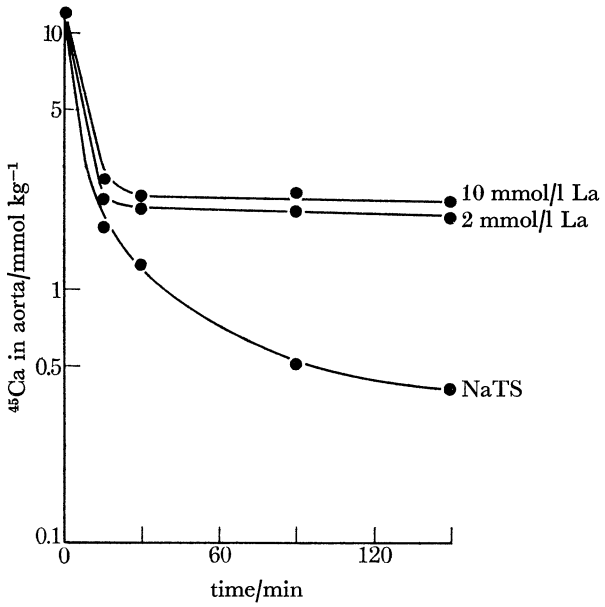

FIGURE 4. La^{3+} inhibition of ^{45}Ca efflux from aortic strips previously allowed to gain cellular ^{45}Ca by exposing them for 3 h to a solution containing 10 mmol/l ^{45}Ca, 10^{-4} mol/l 2,4-dinitrophenol and 10^{-3} mol/l monoiodoacetic acid. Efflux was measured either in normal tris buffered physiological solution (NaTS) or a Ca-free solution to which $LaCl_3$ has been added in the indicated concentrations.

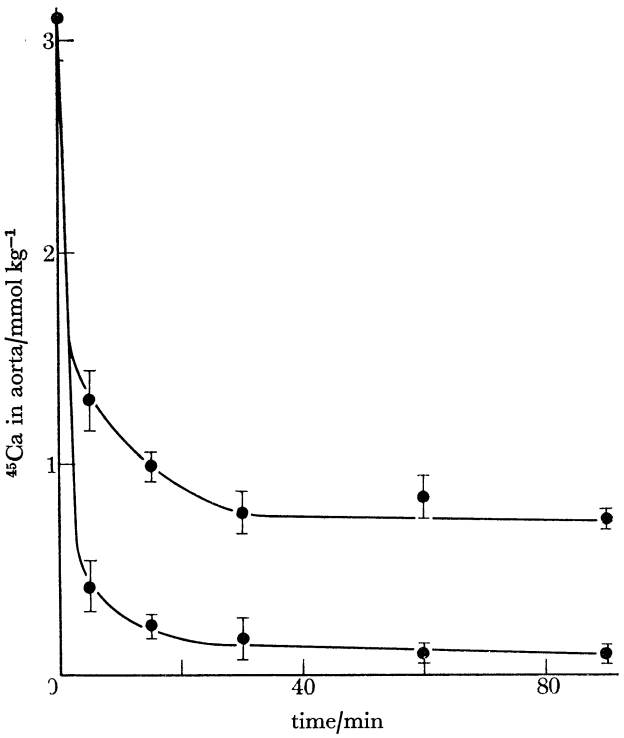

FIGURE 5. The loss of labelled Ca^{2+} from aortic strips, which had been exposed to ^{45}Ca for 20 min, to either calcium-free solution (upper curve) or calcium-free solution with 2 mmol/l $LaCl_3$ (lower curve). The difference between the horizontal bars equals twice the standard error for at least four observations (from van Breemen *et al.* 1972).

extracellular bound Ca^{2+}. This is illustrated in figure 5. Rings of isolated rabbit aorta were loaded with ^{45}Ca under control conditions for only 20 min and then effluxed in Ca-free solution or Ca-free solution with 2 mmol/l La^{3+}. It is evident that La^{3+} displaces about 0.65 mmol tightly bound ^{45}Ca/kg wet tissue. Weiss & Goodman (1969) have observed similar Ca^{2+} displacements by La^{3+} in the longitudinal smooth muscle of the ileum and this action is also readily demonstrated in the taenia coli (see figure 9). A final validation of the La method in guinea-pig taenia coli is shown in figure 6. Here we have allowed the cells to accumulate labelled Ca^{2+} during metabolic depletion (substituting N_2 for O_2 and sorbitol for glucose). The two uptake curves are seen to be parallel so that the cellular Ca^{2+} gain is the same whether measured conventionally or with the La method.

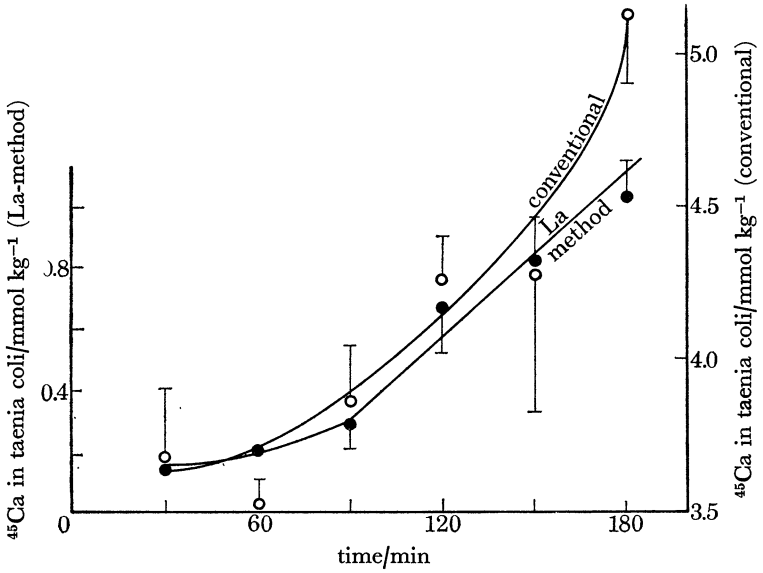

FIGURE 6. Correlation between cellular ^{45}Ca gain measured conventionally (right ordinate) and with the lanthanum method (left ordinate). The tissues were allowed to gain ^{45}Ca by metabolic deprivation with N_2 instead of O_2 and sorbitol instead of glucose. Each point is the average of four determinations. Indicated standard errors are noticeably smaller when lanthanum method is used. Note that although the left scale starts at 0 and the right scale at 3.5 mmol/kg, the units are identical.

In conclusion the La method provides a reasonably accurate measurement of cellular Ca^{2+} exchange in the rabbit aorta and guinea-pig taenia coli. It should be useful in all tissues with extensive extracellular spaces. However, one should be cautious and show, before use, that it meets the two criteria of blockade of Ca^{2+} membrane flux and displacement of bound Ca^{2+}.

MEMBRANE Ca^{2+} TRANSPORT AND SMOOTH MUSCLE ACTIVATION

Figure 7 shows the cellular ^{45}Ca uptake as measured with the La method during stimulation with high K^+ depolarization, with Li^+ substitution for Na^+, with 4×10^{-6} mol/l norepinephrine, and during inhibition with 2 mmol/l La^{3+}.

K^+ *depolarization*

K^+ depolarization stimulates a large ^{45}Ca uptake which by measuring total Ca^{2+} with atomic absorption spectrophotometry was shown to be a net influx. This Ca^{2+} influx initiates the

contraction since both removal of Ca^{2+} from the extracellular space by 10 min Ca-free treatment and blockade of Ca^{2+} influx by La^{3+} (see figure 7) block the K^+ contraction. K^+ depolarization also increases membrane permeability to other polyvalent cations. For example K^+ depolarization will cause a slow contraction in the aorta when Mn^{2+} is substituted for Ca^{2+} and in the taenia coli when either Mn^{2+} or La^{3+} are substituted for extracellular Ca^{2+}. By applying these agents to glycerinated taenia coli fibres, F. Baguet (1972, personal communication) has shown that neither La^{3+} nor Mn^{2+} directly activate the contractile proteins. We have shown using the La-method in combination with atomic absorption spectrophotometry that

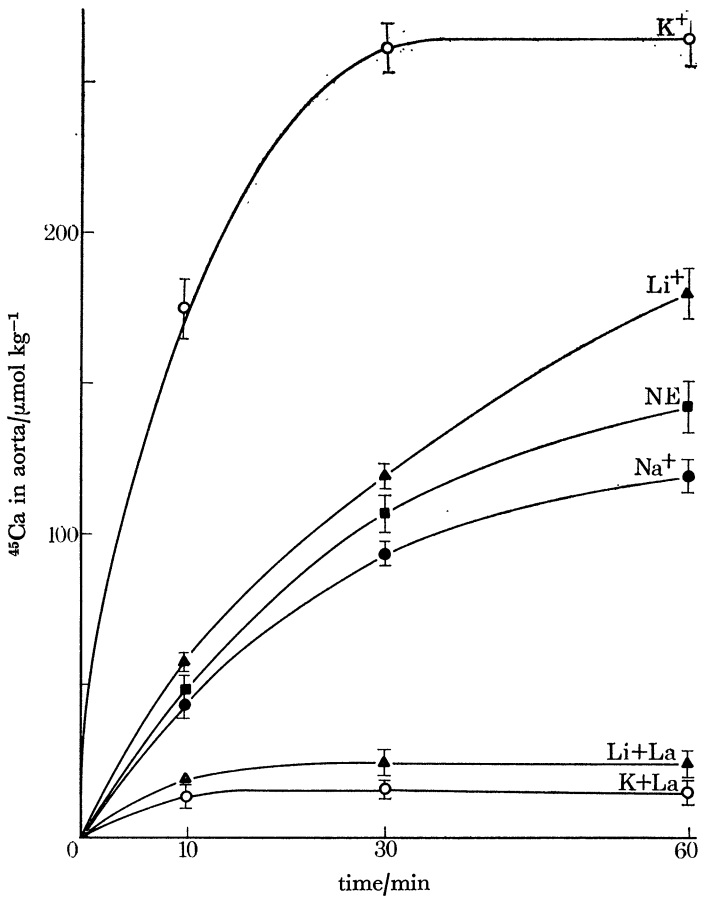

FIGURE 7. The cellular uptake of ^{45}Ca from solutions containing Na^+, Li^+ or K^+ as the major cation as measured by the La method. The effect of 4×10^{-6} mol/l norepinephrine (NE) on the ^{45}Ca uptake in Na^+ solution. Inhibition of ^{45}Ca uptake by 2 mmol/l $LaCl_3$ in Li^+ and K^+ solutions. The distance between the horizontal bars equals two times the standard error for at least four observations.

K^+ depolarization causes a net entry of Mn^{2+}, while Somlyo & Somlyo (1971) showed by electronmicroscopy that Sr^{2+} entered vascular smooth muscle cells during depolarization and was deposited in the sarcoplasmic reticulum and mitochondria. Thus we conclude that K^+-depolarization causes the influx of Mn^{2+} but not of La^{3+} into aortic smooth muscle cells and the influx of both these ions into cells of the taenia coli. They then displace intracellularly bound Ca^{2+} which binds to troponin to initiate contraction. Mn^{2+} added to a Ca^{2+} containing depolarizing solution slows the aortic contraction rate by diminishing Ca^{2+} influx. Hagiwara & Nakajima (1965) first showed Mn^{2+} suppression of Ca^{2+} permeability electrophysiologically,

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while Bülbring & Tomita (1968) demonstrated the same effect on guinea-pig taenia coli. We have studied the inhibitory effects of a series of polyvalent cations on the rate of high K^+ depolarization contractions in the presence of 1.5 mmol/l Ca^{2+}. The relative blocking efficiencies which are most likely directly related to the affinities of these ions for the membrane Ca^{2+} transport sites are: $La^{3+} > Fe^{2+} > Ni^{2+} > Co^{2+} > Zn^{2+} > Mn^{2+} > Mg^{2+}$.

Li *substitution*

Coret & Hugues (1964) have shown that Na-free solutions caused contractions of the aorta. These contractions are readily blocked by La^{3+} or by Ca-free exposure and as indicated by figure 7 are caused by Ca influx. The Li^+ stimulated Ca^{2+} uptake is much slower than in the case of high K^+ which corresponds with the much slower rate of contraction. In our hands the Li^+ contractions were variable and increased in amplitude upon successive exposures to Li^+-substituted solutions. In order to be certain that all preparations would contract, all Na^+ needed to be replaced by Li^+. These results suggest the existence of competition between Na^+ and Ca^{2+} for influx. The contraction amplitudes could not be used to obtain any quantitative data regarding this competition since Ca^{2+} influx is not the only factor involved (see last section).

Norepinephrine

Although the norepinephrine-induced contraction is at least as rapid and of slightly greater amplitude than the K^+-induced contracture the accompanying stimulated Ca^{2+} influx is much smaller and hardly significant (see figure 7). Thus the major Ca^{2+} source for the norepinephrine induced contraction is intracellular. This was suggested earlier by Hinke (1965) who found that norepinephrine-induced contractions were much more resistant to Ca^{2+} removal than high K^+ contractions. Norepinephrine contractions of rabbit and rat aortae are not blocked by agents which we have now shown to inhibit Ca^{2+} influx. These agents are La^{3+} (van Breemen 1969) SKF 525-A (Kalsner, Nickerson & Boyd 1970) and verapamil (Peiper, Griebel & Wende 1971). However, in 1963, Bohr showed that there were two components to the norepinephrine contraction, the initial fast component which was suppressed by increasing extracellular Ca^{2+} and a slow tonic component which was stimulated by the same procedure. van Breemen (1969), Godfraind & Kaba (1969) and Sitrin & Bohr (1971) subsequently showed that the slow phase depended on extracellular Ca^{2+}. In addition, Somlyo & Somlyo (1968) obtained evidence from electrophysiological observations that norepinephrine increases membrane Ca^{2+} permeability. Figure 8 provides additional data which show that the initial rapid phase is due to intracellular Ca^{2+} release and that the slow tonic phase is due to Ca^{2+} influx. Preincubation in a Ca^{2+}-free solution removes the slow phase, which reappears on addition of Ca^{2+} during the maximal height of the rapid phase. We have measured ^{45}Ca uptake under identical conditions as shown in figure 8(*b*). This labelled Ca was added after Ca-free response in the presence or absence of norepinephrine. Under these conditions norepinephrine shows a marked stimulation of ^{45}Ca uptake. Prior Ca^{2+}-free exposure thus appears to potentiate the stimulating effect of norepinephrine on Ca^{2+} permeability. This potentiation is also reflected, although to a lesser degree, in the contractions. The slow phase reaches maximum tension more rapidly after Ca-free response than under control conditions. One factor which would make this potentiation less obvious in figure 8*a* is that upon addition of Ca^{2+} during the second norepinephrine exposure the Ca^{2+} has to first diffuse through the extracellular space before it can penetrate the cells to activate the contractile proteins. The slow contraction and

the additional Ca^{2+} influx observed upon addition of labelled Ca^{2+} to a Ca-free norepinephrine containing solution are both abolished by either SKF 525-A or La^{3+}.

The intracellular Ca^{2+} fraction in aorta which is released by norepinephrine, angiotensin II or histamine appears to be limited in size since only one contraction can be obtained after La^{3+} binding to the membrane (van Breemen *et al.* 1972). SKF 525-A, which like La^{3+} blocks Ca^{2+} influx contractions but unlike La^{3+} does not displace Ca^{2+} bound to the outer surface of the membrane, does not block subsequent norepinephrine contractions. These results suggest that the intracellular Ca^{2+} release sites may be replenished by Ca^{2+} bound in the cell membrane, but are distinct from intracellular Ca^{2+} uptake sites which bind cytoplasmic Ca^{2+} to initiate relaxation (see also p. 69).

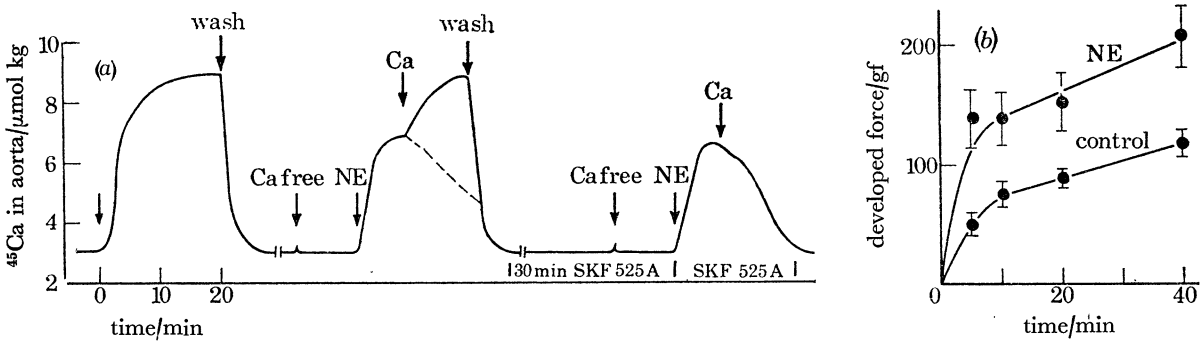

FIGURE 8. (*a*) The calcium release and the calcium influx fractions of the norepinephrine contraction. In Ca-free medium only the release fraction can be seen. Upon reintroduction of Ca^{2+}, the influx fraction appears and the sum force developed is equal to that of the control contraction. SKF-525-A, which is known to block Ca^{2+} influx, prevents the influx fraction of the norepinephrine contraction. (*b*) ^{45}Ca influx in the presence and absence of norepinephrine, after 13 min Ca^{2+}-free exposure. The norepinephrine was added 3 min before the addition of 1.5 mmol/l labelled Ca.

TABLE 1. ACTIVATING EFFECTS OF VARIOUS STIMULANTS ON RABBIT AORTA

stimulus	rate of contraction	dependence on [Ca]^e	blockade by La^{3+}	stimulated Ca^{2+} influx in first hour mmol/kg	depolarization
high K^+	very fast	complete	complete	0.15	complete
high Li^+	slow	complete	complete	0.06	23 mV†
norepinephrine	very fast then slow	only for slow phase	only for slow phase	0.02	partial
DNP	slow	none	none	none	6 mV† transient
ouabain	slow after 20 min	complete	complete	0.09 after 20 min delay	29 mV†
high pH	fast	complete	complete	1.8 at pH 10.5	?
Ba^{2+}	fast	none	complete	—	?

† Unpublished results obtained by Dr H. Hendrickx on the rabbit ear artery.

Other contractions

Table 1 summarizes the relationship between contractile response and Ca^{2+} influx in the rabbit aorta under the various experimental conditions used in our laboratory. Ouabain causes a delayed contraction which is accompanied by depolarization and Ca^{2+} influx. DNP, on the

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other hand, slightly suppresses Ca^{2+} influx and its contractile response is most likely due to release of Ca^{2+} from the mitochondria. The effects of changes in pH on Ca^{2+} influx tend to indicate that Ca^{2+} transport is mediated by negative membrane sites which are blocked when protonated and which carry Ca^{2+} when the hydrogen-ion concentration is much reduced (see van Breemen *et al.* 1972).

Agents blocking Ca^{2+} *influx*

We have shown inhibition of cellular Ca^{2+} influx by La^{3+}, Mn^{2+}, 4×10^{-5} mol/l SKF 525-A (a tertiary amine), D 600 (a close analogue of verapamil) and 10^{-3} mol/l chlorpromazine. All these agents blocked high K^+ and Li^+ contractions. The difference between La^{3+} and the organic inhibitors is that 10 mmol/l La^{3+} reduces the influx to almost zero, whereas the others prevent mainly the additional Ca^{2+} influx due to stimulation. We assume that La^{3+} is able to penetrate some distance into the membrane and prevent ^{45}Ca binding there if it is added before addition of ^{45}Ca, but locks in some ^{45}Ca bound more deeply in the membrane if it is added afterwards. The control ^{45}Ca influx measured with the La-method may thus partly consist of ^{45}Ca binding in the membrane. Accordingly it is more relevant to cytoplasmic Ca^{2+} control to consider the stimulated Ca^{2+} uptake. In this context it is of interest that D 600, which inhibits spontaneous activity in the taenia coli, reduced the control influx by only 25 μmol/kg over 1 h period (Casteels, van Breemen & Mayer 1972). During this period we counted roughly 60 spontaneous contractions. This would mean that less than 0.5 μmol Ca^{2+} enters the cells per action potential. Two explanations are possible: Either this small amount of Ca^{2+} functions as 'trigger' Ca^{2+} to release activator Ca^{2+} from the sarcoplasmic reticulum (Bianchi 1968) or a Ca^{2+} extrusion pump removes ^{45}Ca before it can mix with the bulk of cellular Ca^{2+}.

Relationship between ATP, Na^+ gradient, Ca^{2+} gradient and membrane permeability

Figure 9 illustrates the changes in Na^+, K^+, ^{45}Ca, ATP (Greengard 1965) and [^{14}C]sorbitol in guinea-pig taenia coli during exposure to 10^{-4} mol/l 2,4-dinitrophenol (DNP) and 10^{-3} mol/l monoiodoacetic acid (IAA), and increased external Ca^{2+}. Active Na^+ and K^+ transport are immediately arrested by the rapid decline of ATP concentration, but Ca^{2+} influx is negligibly small for the first hour. After this delay we see a large Ca^{2+} influx which is completely blocked by 10 mol/l La^{3+}. Parallel with the Ca^{2+} influx is an entry of [^{14}C]sorbitol into the cells, which can also be blocked by La^{3+}. Entry of sucrose during metabolic inhibition has been shown previously by Villamil, Rettori, Barajas & Kleeman (1968) and Daniel & Robinson (1971). The sorbitol which has entered the cells goes out very rapidly upon removal of the label from the bathing solution. We have demonstrated the cellular location of the [^{14}C]sorbitol gained during metabolic inhibition by preventing its efflux with La^{3+} (Casteels, van Breemen & Wuytack 1972). The principal factor allowing dissipation of the Ca^{2+} gradient upon ATP removal is thus a non-specific membrane permeability increase. We have shown the same to be true when ATP is depleted by removal of O_2 and glucose. The exact relation between [ATP] and passive membrane permeability is not clear and is an interesting subject for further investigation. The delay between the decrease in [ATP] and the onset of membrane leakiness suggests loss of a membrane constituent which is essential for maintaining membrane integrity and whose continual resynthesis depends on normal [ATP]. The physiological state of the smooth muscle membrane appears to be one of very low passive Ca^{2+} permeability. This may be deduced from

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the very slow net Ca^{2+} gain during the period that cellular ATP is already depleted but before the membrane becomes non-specifically leaky (between 20 and 60 min in figure 9). This lack of significant Ca gain is not due to the Na gradient as shown in figure 10. With reference to the first section of our discussion, figure 9 also demonstrates effective displacement of extracellular bound Ca^{2+} by La^{3+} since practically all the Ca^{2+} uptake seen in the presence of 10 mmol/l La^{3+} can be calculated to be free in the extracellular space. In figure 10 we follow total tissue concentrations of Na^+, K^+, Ca^{2+} and ATP after taenia coli samples are moved from a control solution to one containing 4.5 mmol/l Ca^{2+} while the Na^+–K^+ pump is inhibited by either

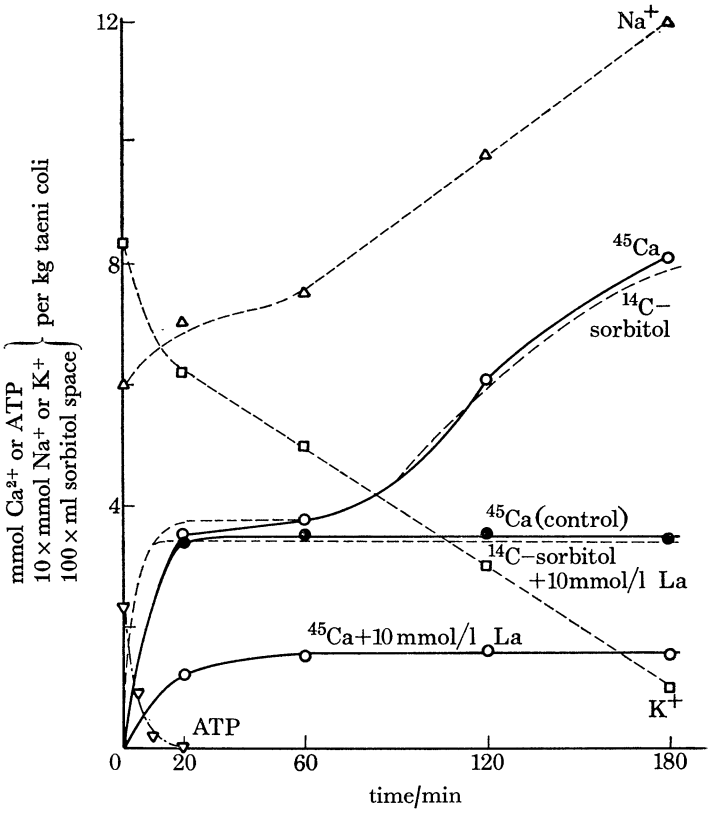

FIGURE 9. The effect of metablic inhibition by 10^{-4} mol/l 2,4-dinitrophenol and 10^{-3} mol/l monoiodoacetic acid in 4.5 mmol/l Ca^{2+} on: ^{45}Ca uptake, ^{14}C-sorbitol uptake, Na^+, K^+ and ATP levels in the taenia coli. Blockade by La^{3+} of the increased permeabilities to ^{45}Ca and ^{14}C-sorbitol. Only the curve labelled ^{45}Ca (control) was obtained in the absence of DNP and IAA.

ouabain or omission of external K^+. The total Ca^{2+} reaches a value of 4 mmol/kg wet tissue which is slightly higher than the value for ^{45}Ca in figure 9 due to the presence of inexchangeable Ca^{2+}. The Ca^{2+} content does not, however, move towards equilibrium in spite of the fact that the Na^+ gradient is abolished. The ATP levels remain at control value and there is no significant increase in [^{14}C]sorbitol space. This figure thus clearly demonstrates that in smooth muscle (we have demonstrated the same for both uterus and aorta) the Ca^{2+} gradient does not depend on the Na^+ gradient. Note that neither ouabain nor the K-free condition increase $[ATP]_1$. A further lack of correlation between Na^+ and Ca^{2+} gradients is seen in figure 11. Following removal of O_2 and glucose, the cellular ^{45}Ca as measured with the La method starts to rise after a delay which is longer than the delay seen before Na^+ gain. ATP levels do not reach zero and recovery is seen when fuel in the form of O_2 and glucose is again made available. At this time

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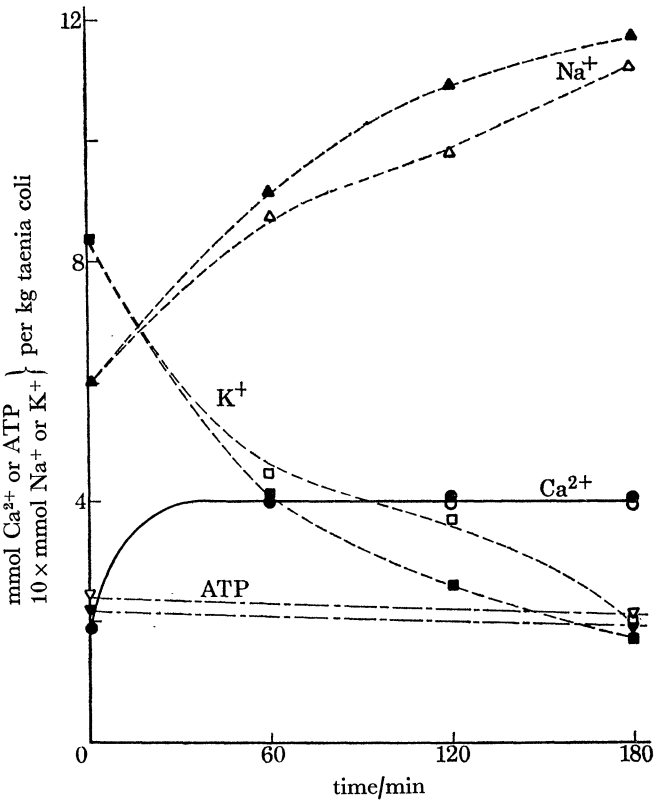

FIGURE 10. The effect of K^+-free medium (open symbols) and 2×10^{-5} mol/l ouabain (filled symbols) on total Ca^{2+}, Na^+, K^+ and ATP levels in the taenia coli. Inhibition of the Na^+-K^+ pump obviously does not alter $[Ca]_i$ or $[ATP]_i$ significantly.

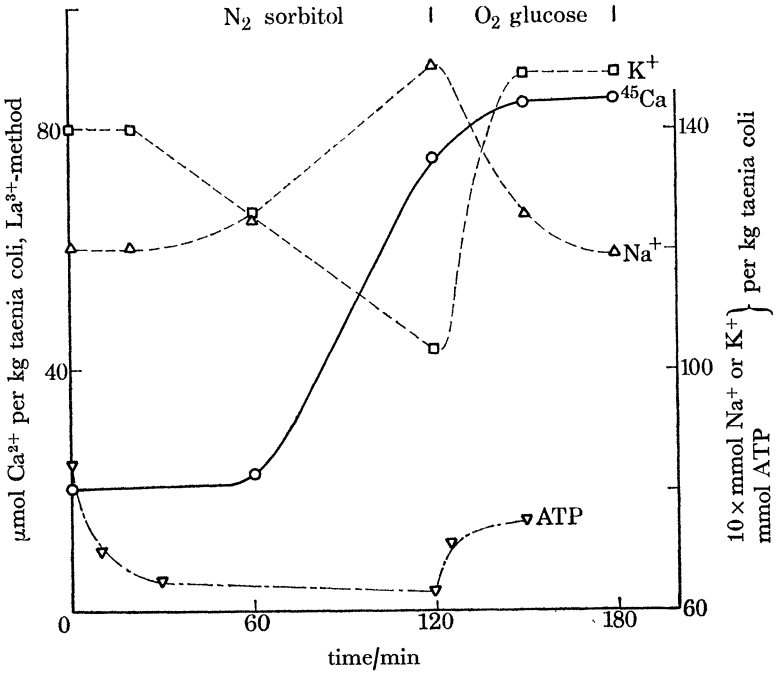

FIGURE 11. After a 120 min exposure to a solution with N_2 substituted for O_2 and sorbitol substituted for glucose pieces of taenia coli were returned to a control solution (O_2 and glucose). The labelled Ca^{2+} gained during metabolic depletion was not lost during the subsequent recovery period in spite of return to normal control values of intracellular Na, K and ATP.

both the Na^+ and K^+ gradients recover whereas the Ca^{2+} gained remains inside the cells. The membrane also becomes again impermeable to [^{14}C]sorbitol. We conclude from the experiments presented in this section that under physiological conditions the Ca^{2+} gradient depends on the presence of ATP and very low passive Ca^{2+} membrane permeability, but not on the Na-gradient. However, the mechanism whereby ATP maintains the Ca^{2+} gradient has thus far eluded us.

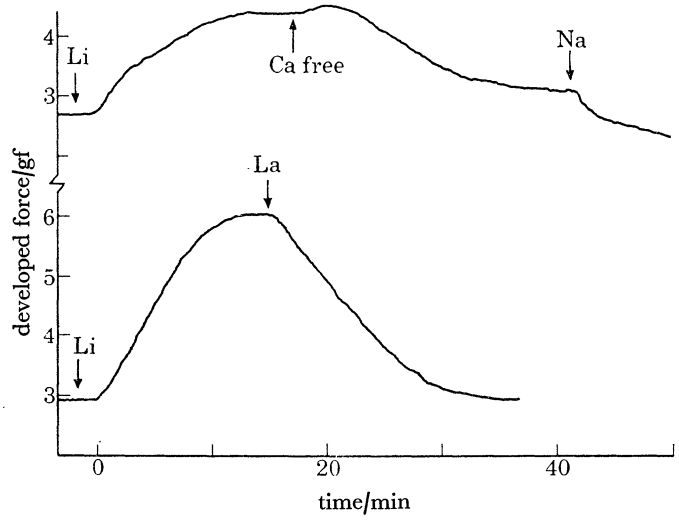

FIGURE 12. Force development by an aortic ring in Li^+ solution and its relaxation upon changing to a Ca^{2+}-free Li^+ medium or upon the addition of 2 mmol/l La^{3+}.

RELAXATION BY INTRACELLULAR Ca^{2+} BINDING

If relaxation is caused by Ca^{2+} extrusion then we should be able to prevent it by blocking Ca^{2+} efflux with La^{3+}. Instead, the opposite is true; all contractions which we have thus far recorded, i.e. due to K^+, Li^+, norepinephrine, high pH or ouabain, are relaxed by adding La^{3+} to the contracting solution. The only exception is the DNP + IAA contraction which is probably caused by Ca^{2+} leakage from the mitochondria. Figure 12*b* shows such a La^{3+} relaxation of a Li^+ contraction which is even more rapid than the relaxation induced by removal of Ca^{2+} from the Li^+ solution seen in figure 12*a*. Thus two factors seem to regulate the contractile strength during this type of contraction, the stimulated Ca^{2+} influx and intracellular binding of Ca^{2+}. When influx is blocked, intracellular Ca^{2+} binding lowers the cytoplasmic free Ca^{2+} concentration to cause relaxation. Taenia coli can gain at least 300 μmol intracellular Ca^{2+}/kg under conditions of metabolic depletion or in high $[K^+]_o$ and still completely relax in the recovery solution without losing this accumulated calcium.

Figure 13 shows that the rapid relaxation when the aorta is moved from a high K^+ to a Na^+ control solution is not accompanied by a rapid phase of Ca^{2+} extrusion. However, in the aorta we do consistently see a slow decline of the gained labelled Ca^{2+} below the plateau value reached in the high K^+ solution. Under these conditions we were also able to measure a similar net Ca^{2+} loss. For aortic smooth muscle we thus picture that Ca^{2+} which enters during depolarization to initiate contraction, probably through a 'trigger' mechanism, is then rapidly absorbed by the sarcoplasmic reticulum (Devine, Somlyo & Somlyo 1972) and perhaps the internal surface of the cell membrane. A more slowly operating active Ca^{2+} extrusion serves to maintain a steady state.

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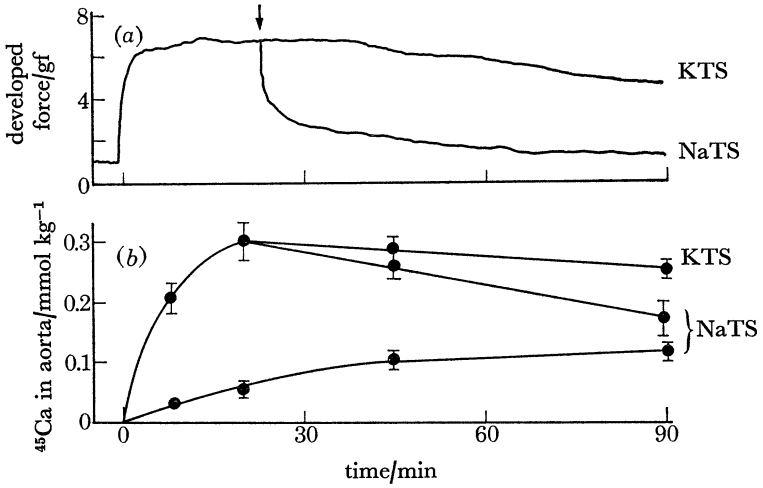

FIGURE 13. At the arrow some aortic rings are transferred from a high K^+ solution to Na control solution. Both solutions contain 1.5 mmol/l ^{45}Ca at the same specific activity. Other aortic rings are maintained in the high K^+ or Na^+ control solution for the entire period. A slow loss of labelled Ca is seen during return to Na (*b*), which, however, does not correlate with the rapid relaxation seen in (*a*). Two times the standard error of at least five determinations is equal to the distance between the horizontal bars or less than the diameter of the circles.

CONCLUSION

A method has been presented and validated for the rabbit aorta and guinea-pig taenia coli which makes it possible to study transmembrane Ca^{2+} fluxes in smooth muscle cells. A number of experimental conditions could induce contraction by sizable Ca^{2+} influxes. However, our conclusion is that under physiological conditions most of the activating Ca^{2+} is released from intracellular Ca^{2+} stores and that relaxation is brought about by intracellular Ca^{2+} binding. The large inward electrochemical Ca^{2+} gradient depends primarily on very low Ca^{2+} permeability and intracellular ATP, but not on the Na^+ gradient.

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