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EFFECTS OF EXTERNAL CATIONS ON CALCIUM EFFLUX FROM SINGLE CELLS OF THE GUINEA-PIG TAENIA COLI AND PORCINE CORONARY ARTERY

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SUMMARY

1. Single smooth muscle cells were prepared from the guinea-pig taenia coli and the porcine coronary artery by treatment with collagenase, in order to measure the ^{45}Ca flux with special reference to the effects of external cations.

2. In excess $[K]_o$ solution, cell suspensions prepared from both tissues showed an increased ^{45}Ca uptake within 3–5 min. In Na-free solution, the cells prepared from taenia coli, but not from coronary artery showed an increased ^{45}Ca uptake. The Ca uptake of the cells paralleled with the tension increase recorded from tissues of both species.

3. The efflux of ^{45}Ca into Ca-free EGTA containing solution was markedly increased by $[Na]_o$ in cells from the taenia coli, but not in cells from the coronary artery.

4. The $[Na]_o$-activated ^{45}Ca efflux from cells of the taenia coli was slightly larger in Ca-free solution than in the Ca-containing (10^{-4}M) solution. Depolarization of membranes produced by excess $[K]_o$ did not effect the $[Na]_o$-activated ^{45}Ca efflux.

5. Increase in $[Na]_i$ by treatment with K-free solution suppressed the $[Na]_o$-activated ^{45}Ca efflux in the taenia coli. Re-addition of $[K]_o$ reactivated the $[Na]_o$-activated ^{45}Ca efflux. This re-activation was blocked by ouabain.

6. The efflux of ^{45}Ca was slightly activated by $[Ca]_o$ in cells from the taenia coli. This $[Ca]_o$-activated ^{45}Ca efflux was larger in Na-free solution than in Na-containing solution, thus suggesting interactions between $[Na]_o$ and $[Ca]_o$ on the Ca efflux.

7. In cells from the taenia coli, ^{45}Ca efflux could still be observed in nominally Na- and Ca-free solution. This residual ^{45}Ca efflux made a large contribution to the total ^{45}Ca efflux.

8. When ^{45}Ca uptake was measured in Na-free (Tris) solution, the $[Na]_o$-activated, $[Ca]_o$-activated and residual ^{45}Ca effluxes of cells from the taenia coli were accelerated, non-selectively.

9. These results obtained with cells prepared from the guinea-pig taenia coli are comparable to the Ca^{2+} efflux mechanism seen in the squid axon. However, maintenance of low concentrations of $[Ca]_i$ seems to require not only the above three ^{45}Ca efflux mechanisms, but also Ca sequestering mechanisms in the cell.

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INTRODUCTION

In many cells, the outward transport of Ca ions is an energy requiring process due to the large electrochemical gradient. However, the energy required for Ca extrusion is derived from different sources in different tissues. In the plasma membrane of red blood cells, Ca-ATPase is involved in the active transport of Ca ions (Schatzmann, 1975). In other tissues such as L cells (Lamb & Lindsay, 1971) and liver cells (Cittadini & Van Rossum, 1978), an active Ca-pump contributes to the Ca extrusion mechanism.

In squid axon (Baker & McNaughton, 1976; Blaustein, 1977), cardiac muscle (Reuter & Seitz, 1968; Busselen & Kerkhove, 1978), barnacle muscle (Russell & Blaustein, 1974; Ashley, Ellory & Hainaut, 1974) and brain synaptosome (Blaustein & Ector, 1976), it has been proposed that necessary energy for uphill transport of the Ca ions is derived from the energy of the Na gradient across the plasma membrane. However, an active Ca-pump mechanism involved in the Ca extrusion in the above tissues cannot be ruled out (DiPolo, 1974; Baker & McNaughton, 1976; DiPolo & Beauge, 1979), and the role of ATP in the $[Na]_o$-activated Ca efflux remains unknown (Baker & Glitsch, 1973; Jundt & Reuter, 1977; Blaustein, 1977).

In smooth muscle, no consensus has been reached concerning the Ca extrusion mechanisms. Reuter, Blaustein & Haeusler (1973) demonstrated a $[Na]_o$-activated fraction of Ca efflux in the smooth muscles of the rabbit aorta and pulmonary artery, whereas Raeymaekers, Wuytack & Casteels (1974) showed that the washout of Ca from the guinea-pig taenia coli largely reflected an exchange of extracellularly bound ions on the surface of the cells. Furthermore, Casteels & Van Breemen (1975) suggested that the smooth muscle tissues possess an ATP-dependent Ca pump which does not involve the Na gradient in Ca extrusion. To minimize the interaction of ions at the extracellular binding sites, they used a relatively high concentration of Ca-EGTA buffer. Moreover, Droogmans & Casteels (1979) ruled out the possible role of the Na gradient in the Ca extrusion mechanism in the rabbit ear artery when examining the effects of Na gradient on the passive Ca flux.

In the taenia coli of the guinea-pig, Katase & Tomita (1972) observed that tissues initially contracted in high K solution remained contracted when $[K]_o$ was reduced to normal in Na-free (sucrose) solution, but would then relax on re-addition of Na, and more recently, Brading (1978) has also reported evidence suggesting some involvement of the Na gradient in transmembrane Ca movement in this tissue, and Brading & Widdicombe (1976) have demonstrated a $[Na]_o$-dependent Ca efflux and a $[Na]_i$-dependent Ca uptake in intact tissues.

We investigated whether or not the Na gradient of the taenia coli would provide sufficient energy to promote Ca extrusion. For this purpose, the single cell suspension prepared by treatment with collagenase was used in order to reduce the interaction of ions at extracellular sites.

METHODS

Preparations of single smooth muscle cells

Single smooth muscle cells from the guinea-pig taenia coli were prepared by essentially the same method as used by Small (1977), with a slight modification. Freshly dissected muscle strips from male guinea-pigs were fixed at a slightly extended length to a plastic plate in Ca-free Locke solution

(EGTA not present), equilibrated at 36 °C for 1 hr then placed in a Ca-free Locke solution containing collagenase 1 mg/ml (Sigma, type I) plus trypsin inhibitor 0·5 mg/ml (Sigma). The strips became rather loose after about 1 hr at 36 °C. These fragile strips were then cut from the plate and single cells were released into the same solution by passing the strips repeatedly through a Pasteur pipette for 1 hr. The suspension containing single cells and undigested residue was filtered through two layers of a nylon gauze and the residue was trapped on the gauze. The single cells were collected by centrifugation at 1,000 rev/min for 1 min, and were suspended in fresh solution.

Single cells from porcine coronary artery were prepared by much the same method, except that the tissues were placed in a solution containing collagenase, trypsin inhibitor and elastase 0·5 mg/ml (Sigma).

Solutions

For Ca flux experiments, modified Locke solution composed of NaCl, 154 mM; $NaHCO_3$, 8 mM; KCl, 5·6 mM; glucose, 5·5 mM; and $CaCl_2$, 2·2 mM, was used. This solution was bubbled with 97% O_2 and 3% CO_2. The pH was 7·4. The Ca-free solution was prepared by replacing $CaCl_2$ with an equivalent amount of NaCl or $MgCl_2$, and 0·5 mM-or 2 mM-ethylene glycol-bis-(β-aminoethylether)-*N*, *N*-tetra-acetic acid (EGTA) was added. The Na-free and Na-deficient solutions were prepared by replacing the NaCl and $NaHCO_3$ with Tris. Since 0·1 M-EGTA was titrated with 0.25M-NaOH, the nominally Na-free and Ca-free solution contained 1·25 mM- or 5 mM-$[Na]_o$. In some experiments, $[Ca]_o$ was varied between 10^{-8} M and 10^{-3} M using a Ca-EGTA buffer. The desired concentrations of $[Ca]_o$ were obtained by adding an appropriate amount of $CaCl_2$ (prepared with $CaCO_3$ and HCl) to a solution containing 2 mM-EGTA, assuming that the apparent affinity constant was $1{\cdot}58 \times 10^7$ M^{-1} at pH 7·4 (Saida & Nonomura, 1979). K-free solution was prepared by replacing KCl with equimolar NaCl. For tension recordings, modified Krebs solution was used (NaCl, 136·9 mM; KCl, 4·7 mM; $NaHCO_3$, 15·5 mM; KH_2PO_4, 1·2 mM; $MgCl_2$, 1·2 mM; $CaCl_2$, 2·5 mM and glucose, 11.5 mM). The Na-free solution was prepared by replacing NaCl and $NaHCO_3$ with an equimolar amount of Tris-Cl. The K-rich solution was made by replacing NaCl with an equimolar KCl (total K concentration 118 mM).

Measurement of ^{45}Ca flux

(1) *^{45}Ca influx experiments.* For ^{45}Ca uptake experiments, the cell pellets were suspended in ^{45}Ca containing solution, and incubations were carried out at 36 °C. At specific times, an aliquot of the suspension was transferred to another test tube to which EGTA (titrated with Tris) had previously been added. The final concentration used was 10 mM in order to remove the extracellular Ca^{2+}. After 2 min, this suspension was passed through a glass fibre filter (Whatman GF/C) which had been rinsed with an appropriate saline. The filter was immediately washed 5 times with 10–15 ml. of the saline. Preliminary experiments indicated that 10 mM-EGTA for 2 min washing was sufficient to remove the extracellular Ca^{2+}. The washed filter was then placed in a counting vial, and dried at 80 °C for 1 hr. A scintillation cocktail which consisted of 4 g PPO (2,5-diphenyloxazole) and 0·3 g POPOP (1,4-bis-2-(4-methyl-5-phenyloxazolol)-benzene) in 1,000 ml. toluene was added to the vial, and the sample was counted in a Packard Tri-Carb liquid scintillation counter.

(2) *^{45}Ca efflux experiments.* The cell pellet was first suspended and incubated in ^{45}Ca containing solution for various durations at 36 °C. Aliquots of the suspension were put into several test tubes to measure the ^{45}Ca efflux into various solutions, from the same tissue. The tubes were centrifuged at 1,000 rev/min for 1 min. After the sediment was separated from the supernatant, the contaminating solution on the wall of the test-tube was carefully removed with filter paper. The volume of the sediment was approximately 30–40 μl. One ml of an appropriate saline was added to the sediment and the preparation was incubated at 36 °C for 10 min. For termination of Ca efflux experiments, the suspension was treated with EGTA and filtered, and the filter was counted as described above. The ^{45}Ca efflux was calculated as the difference in the ^{45}Ca count between the freshly loaded and the washed out samples, and expressed as n-mole/mg protein . 10 min.

Mechanical recordings

For tension recording, the guinea-pig taenia coli and the porcine coronary artery were prepared in strips, 0·5 mm in width and 10 mm in length. Tension was recorded using a strain gauge, as described by Hirata & Kuriyama (1980).

RESULTS

^{45}Ca uptake by single cells

Fig. 1 shows the tension development in strips of the guinea-pig taenia coli and the porcine coronary artery in K-rich (118 mM) and Na-free solutions. In this study, we only used Tris as a Na substitute. A marked tension development in strips from both tissues was observed in K rich solution, whereas tension developed only in the

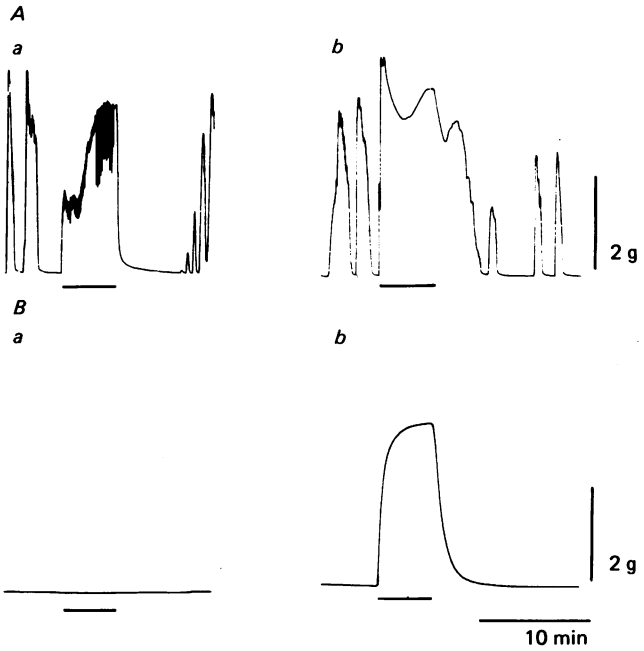

Fig. 1. Tension development in the guinea-pig taenia coli and the porcine coronary artery on exposure to Na-free (Tris) and K-rich (118 mM) solution. *A*, guinea-pig taenia coli; *B*, porcine coronary artery. *a*. Na-free (Tris) solution, *b*. K-rich (118 mM) solution. Horizontal bars indicate the duration of the exposure to the solution.

taenia coli in the Na-free (Tris) solution. Tension in the Na-free (Tris) solution developed to almost the same amplitude as in the 118 mM-K induced contracture, and fell rapidly when $[Na]_o$ was re-admitted. Other substitutes, such as sucrose, Li and Mg, give different patterns of contractures in the taenia coli (A. Brading, personal communication). After exposure to Ca-free solution, tension did not develop in Na-free (Tris) solution, implying that in the taenia coli, generation of tension in Na-free solution might require influx of Ca from the external solution. The strip prepared from the coronary artery showed no tension development in Na-free solution during the 120 min observation period.

^{45}Ca uptake by single cells of both taenia coli and coronary artery in K-rich and Na-free (Tris) solutions is shown in Fig. 2. Droogmans & Casteels (1979) could not detect any increase in ^{45}Ca uptake in the rabbit ear artery in K-rich solution, despite a marked tension development, and in the guinea-pig taenia coli only after a long

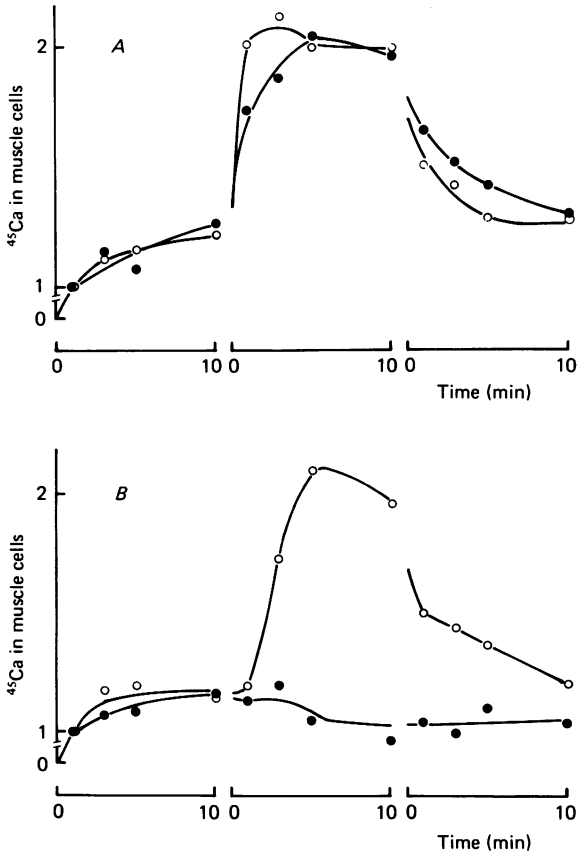

Fig. 2. ^{45}Ca uptake of the single cell suspension from the guinea-pig taenia coli and the porcine coronary artery in Na-free (Tris) and K-rich (118 mM) solution. *A*, guinea-pig taenia coli; *B*, porcine coronary artery. Cell suspensions were incubated in ^{45}Ca-containing media, and at intervals aliquots were removed, and cellular ^{45}Ca estimated as described in the 'Methods' section. For the first 10 min, incubation was in normal Locke solution, subsequently the solutions were changed to either Na-free (Tris) (●) or K-rich (118 mM) (○) solutions for 10 min, and then back to normal Locke for the final 10 min. Solution changes were made by centrifuging the suspensions for 1 min at 1,000 rev/min, removing the supernatant and adding new solution with identical specific activity of ^{45}Ca, leaving the final volume unchanged. Ordinate: ^{45}Ca counts relative to the counts in cells after 1 min in ^{45}Ca Locke solution. Each symbol is the mean of three experiments.

exposure did the muscle cells show an increased Ca content in response to K-rich and Na-free solutions (Casteels & Van Breemen, 1975; Van Breemen, Farinas, Casteels, Gerba, Wuytack & Deth, 1973; Brading, 1978). With the single cell suspensions, however, we observed an increase in ^{45}Ca uptake within 3–5 min exposure to K-rich solution in both taenia coli and coronary artery cells, whereas an increased ^{45}Ca uptake in response to Na-free (Tris) solution was not seen in the coronary artery cells, although the cells of the taenia responded with an increased uptake of ^{45}Ca comparable to that seen in K-rich solution. Readdition of $[Na]_0$ caused a rapid

decrease in ^{45}Ca content of the taenia, suggesting that net Ca efflux was evoked by readdition of $[Na]_o$. If the cells were disrupted by exposure to water (osmotic shock), ^{45}Ca uptake was not observed with the above solutions.

K-free solution, also increased the ^{45}Ca content in cell suspensions prepared from the taenia coli. In this solution, ^{45}Ca uptake began to increase after about 15 min and

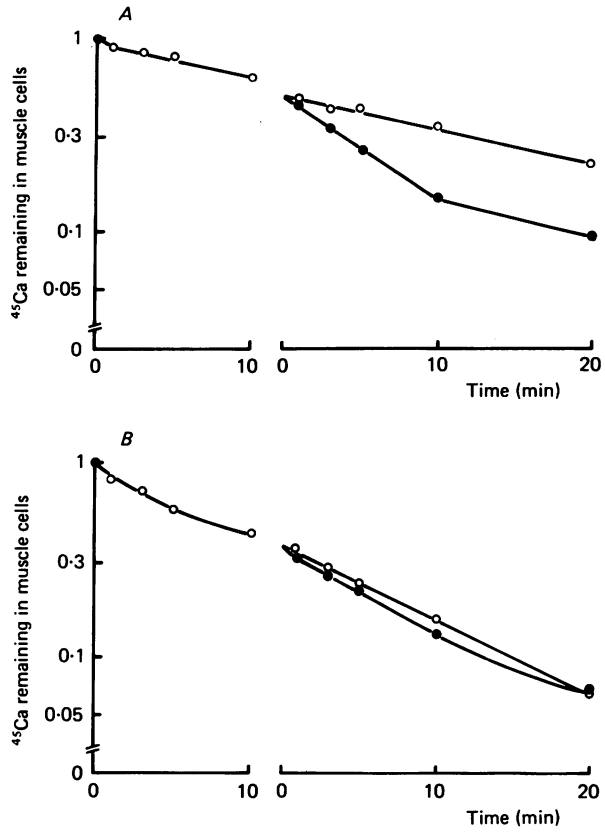

Fig. 3. Effects of $[Na]_o$ on ^{45}Ca efflux in the cells of the guinea-pig taenia coli (*A*) and the porcine coronary artery (*B*). The cell suspensions were incubated at 36 °C for 10 min in Na-free (Tris) and ^{45}Ca containing solution. After the first centrifugation at 1,000 rev/min for 1 min, Na-free (Tris), Ca-free (0·5 mM-EGTA) solution was added to the cell pellet, the ^{45}Ca efflux was measured at 36 °C for 10 min, and the suspension divided into two. After the second centrifugation at 1,000 rev/min, for 1 min, an appropriate amount of Na- and Ca-free solution (○) or Na-containing and Ca-free (0·5 mM-EGTA) solution (●) was added to the cell pellet. The second ^{45}Ca efflux was then measured for 20 min.

showed a maximum uptake after 30–40 min (data not shown). In the taenia coli, the ^{45}Ca content of the cells was $1{\cdot}90 \pm 0{\cdot}16$ n-mole/mg protein ($n = 8$) and this value increased to $3{\cdot}82 \pm 0{\cdot}67$ ($n = 9$) at 10 min after exposure to Na-free (Tris) solution and $4{\cdot}14 \pm 0{\cdot}40$ ($n = 19$) n-mole/mg protein after 60 min exposure to K-free solution.

Effects of $[Na]_o$ on ^{45}Ca efflux; $[Na]_o$-activated ^{45}Ca efflux

Reuter *et al.* (1973) were first to demonstrate $[Na]_o$-activated fraction of Ca efflux in rabbit aortic and pulmonary arterial strips. However, Raeymaekers *et al.* (1974)

showed that it is very difficult to obtain a true value for the transmembrane ^{45}Ca flux because of the large amount of extracellular Ca. To clarify this problem, the ^{45}Ca efflux from single cell suspensions, was measured. The addition of EGTA to the washout solution meant that with this technique the extracellular space, and also the non-specific binding of Ca on the surface of cells could be neglected. Fig. 3 shows

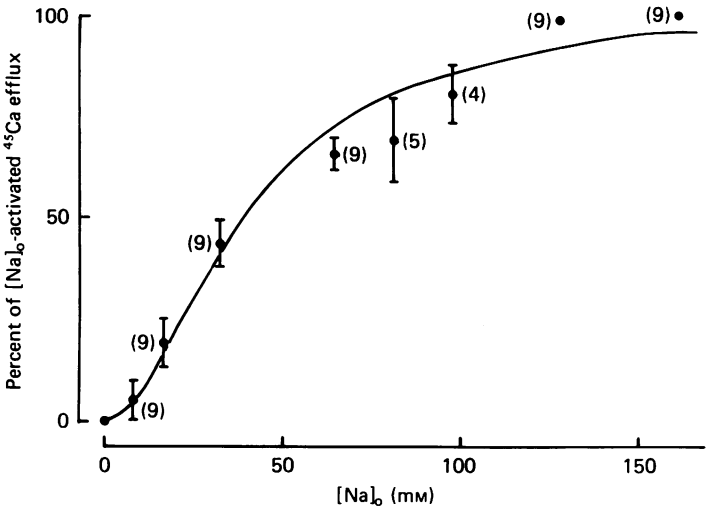

Fig. 4. $[Na]_o$-activated ^{45}Ca efflux as a function of $[Na]_o$ in the guinea-pig taenia coli. Experimental procedures were much the same as described in Fig. 3. ^{45}Ca efflux was measured at 10 min. ^{45}Ca efflux into Na- and Ca-free solution was used as a reference 0 % and ^{45}Ca efflux into normal Na, Ca-free solution was given as 100 %. The continuous line was drawn to fit the equation

$$V = \frac{V_{max}}{1+(K_{Na}/[Na]_o)^2},$$

where V is the $[Na]_o$-activated ^{45}Ca efflux at any $[Na]_o$. The V_{max} was 100 %, and the apparent half-saturation constant for $[Na]_o$, K_{Na}, was 40 mM.

the ^{45}Ca efflux from the cells of both taenia coli and coronary artery as a function of time. In the taenia coli, the ^{45}Ca content of the muscle cells decreased exponentially in the Na- and Ca-free solution. When $[Na]_o$ was re-admitted, the efflux was markedly enhanced for the first 10 min (Fig. 3*A*). On the other hand, in cells of the coronary artery, a $[Na]_o$-activated fraction of ^{45}Ca efflux was not apparent (Fig. 3*B*). Therefore, $[Na]_o$-activated ^{45}Ca efflux such as that observed in the taenia coli is not common to all visceral muscles, thereby demonstrating tissue specificities. The rest of the ^{45}Ca efflux experiments were carried out using the guinea-pig taenia coli. The relationship between the $[Na]_o$ and the ^{45}Ca efflux is shown in Fig. 4. The ^{45}Ca efflux was measured for the first ten min in Ca-free (0·5 mM-EGTA) solution with various concentrations of $[Na]_o$. The experimental results showed a sigmoidal rather than a hyperbolic curve and did not fit with simple saturation kinetics. The results could however, be fitted by a curve drawn from the square law equation, except for $[Na]_o$ of 70–100 mM, which resulted in less activation of ^{45}Ca efflux than predicted. These results are compatible with findings in the guinea-pig auricle (Jundt, Porzig, Reuter & Stucki, 1975; Jundt & Reuter, 1977). The square-law relationship of $[Na]_o$-activated ^{45}Ca efflux suggests

an electro-neutral exchange of two Na ions for one Ca ion across the membrane of the taenia coli. Brading (1979) has also considered that the Na:Ca exchange is an electro-neutral ion exchange in the taenia coli. If the coupling ratio differs from 2:1 (Na:Ca), the exchange would be electrogenic, not electro-neutral and hence a considerable change in $[Na]_o$-activated ^{45}Ca efflux would occur during changes in membrane potential (Mullins & Brinley, 1975). The effects of membrane depolarization produced by high $[K]_o$ on the $[Na]_o$-activated ^{45}Ca efflux in the taenia coli were therefore examined. When 97·2 mM-$[Na]_o$ was replaced by either K or Tris, the $[Na]_o$-activated fraction of ^{45}Ca efflux was $0{\cdot}57 \pm 0{\cdot}07$ or $0{\cdot}60 \pm 0{\cdot}09$ n-mole/mg protein . 10 min ($n=6$), respectively. This result indicates that the $[Na]_o$-activated ^{45}Ca efflux is independent of the membrane potential level. Presumably an electrogenic exchange of Na and Ca does not occur and the coupling ratio of Na to Ca is 2:1 as has been demonstrated in the guinea-pig auricle by the forementioned authors.

Effects of increased $[Na]_i$ on $[Na]_o$-activated ^{45}Ca efflux

Blaustein (1977) has demonstrated in the internally dialysed squid axon that increase in $[Na]_i$ inhibits the $[Na]_o$-activated ^{45}Ca efflux, and assumed that this inhibition was due to displacement of Ca by the $[Na]_i$ at the internal face of the carriers. To determine whether or not there is an inhibition of $[Na]_o$-activated ^{45}Ca efflux by increased $[Na]_i$ in the taenia coli, we examined the effects of increases in $[Na]_i$ on ^{45}Ca efflux. Prolonged incubation of the taenia coli in K-free solution increased the $[Na]_i$ and the membrane was markedly depolarized, as found by Casteels, Droogmans & Hendrickx (1971). As shown in Table 1, when the ^{45}Ca efflux was measured in K-free solution after 10 min, the $[Na]_o$-activated ^{45}Ca efflux was about 0·21 n-mole/mg protein . 10 min. When $[K]_o$ was re-admitted, the $[Na]_o$-activated ^{45}Ca efflux was enhanced to about 0·58 n-mole/mg protein . 10 min. This enhancement did not take place in the presence of 10^{-4} M-ouabain.

The relationship between $[Na]_o$ and the $[Na]_o$-activated ^{45}Ca efflux in $[Na]_i$ loaded cells is shown in Fig. 5. The cells were incubated for 1 hr in K-free solution to increase $[Na]_i$. The kinetic curve obtained differed somewhat from that obtained in Fig. 4, the most striking difference being a depression of the degree of activation in the $[Na]_o$-activated ^{45}Ca efflux at around 70 mM-$[Na]_o$. This suggests the existence of two different $[Na]_o$-activated ^{45}Ca effluxes; one with a high affinity for $[Na]_o$, and the other with low affinity. This phenomenon is present in tissues with low $[Na]_i$, but is more evident when the level of $[Na]_i$ has been raised. With re-addition of $[K]_o$, the kinetic curve of the $[Na]_o$-activated ^{45}Ca efflux was much the same as the control (Fig. 4), except that here, a higher degree of activation of the $[Na]_o$-activated ^{45}Ca efflux took place at less than 40 mM-$[Na]_o$.

Effects of $[Ca]_o$ and $[Ca]_i$ on the $[Na]_o$-activated ^{45}Ca efflux

To observe the possible competition between $[Na]_o$ and $[Ca]_o$ for the external activating sites of Ca-effluxes, the effects of $[Ca]_o$ on the $[Na]_o$-activated ^{45}Ca efflux were investigated. Since an extensive Ca influx occurs in the absence of $[Na]_o$ (Fig. 2), a decrease in the specific activity of the intracellular ^{45}Ca would take place, and an analysis of the effects of $[Ca]_o$ on the ^{45}Ca efflux in the absence of $[Na]_o$ is thus difficult. Therefore, we used 10^{-4} M-$[Ca]_o$ buffered with EGTA to reduce the Ca influx.

TABLE 1. Effects of treatment with K-free solution on the $[Na]_o$-activated ^{45}Ca efflux in the guinea-pig taenia coli. The cells were incubated at 36 °C for 60 min in K-free, ^{45}Ca containing solution. The suspension was put into several tubes, and centrifugation was carried out at 1,000 rev/min for 1 min. Appropriate solutions were added to the cell pellets, and ^{45}Ca was measured at 10 min at 36 °C. The same symbols indicate the ^{45}Ca efflux measured from the same specimen.

	^{45}Ca efflux into		Na-activated ^{45}Ca efflux
	Ca-free Na	Ca-free Tris	
	A. K-free		
1	2·72	2·42	0·30
2	2·31	2·09	0·22
3	1·64	1·39	0·25
△4	1·24	1·13	0·11
▽5	1·63	1·43	0·20
●6	1·79	1·74	0·05
■7	2·45	2·12	0·33
	1·97 ± 0·20	1·76 ± 0·18	0·21 ± 0·04
	B. K-readmission		
1	3·95	3·03	0·92
2	1·29	0·86	0·43
3	3·28	2·11	1·17
○4	1·87	1·51	0·36
△5	1·73	1·16	0·57
□6	1·90	1·33	0·57
▽7	1·98	1·48	0·50
●8	2·15	1·73	0·42
▲9	2·31	1·96	0·35
■10	2·72	2·08	0·64
	2·32 ± 0·25	1·73 ± 0·19	0·59 ± 0·08
	C. K-readmission + Ouabain (10^{-4} M)		
1	2·18	1·63	0·55
○2	1·64	1·52	0·12
□3	1·58	1·36	0·22
▽4	1·79	1·46	0·33
●5	1·79	1·76	0·03
▲6	1·95	1·87	0·08
	1·82 ± 0·09	1·60 ± 0·08	0·22 ± 0·08

(n-mole/mg protein . 10 min)

As shown in Table 2*A*, the ^{45}Ca efflux was measured after incubation in normal Na-containing solution. The $[Na]_o$-activated ^{45}Ca efflux was 0·33 n-mole/mg protein . 10 min. This value was obtained by subtracting the ^{45}Ca efflux measured in Na-containing, Ca-free solution from the ^{45}Ca efflux measured in Na-free, Ca-free solution. In the presence of 10^{-4} M-$[Ca]_o$, the $[Na]_o$-activated ^{45}Ca efflux was reduced to 0·21 n-mole/mg protein . 10 min ($P < 0·10$). This indicates that interactions between $[Na]_o$ and $[Ca]_o$ do take place on the external activating sites for Ca effluxes.

An increase in $[Ca]_i$ could be obtained in the cells of the taenia coli by treatment with Na-free (Tris) solution, as described previously (Figs. 1 and 2). After incubation in Na-free (Tris) solution, the ^{45}Ca efflux was measured (Table 2*B*), and the value

was compared with that obtained in tissues with a low concentration of $[Ca]_i$. The $[Na]_o$-activated ^{45}Ca efflux was increased from 0·33 n-mole/mg protein . 10 min to 0·81 n-mole/mg protein . 10 min in the absence of $[Ca]_o$. In the presence of 10^{-4} M-$[Ca]_o$, the value was significantly increased from 0·21 n-mole/mg protein . 10 min to 0·88 n-mole/mg protein . 10 min ($P < 0·001$). The inhibition of $[Na]_o$-activated ^{45}Ca efflux in the presence of 10^{-4} M-$[Ca]_o$ apparently disappeared when the level of $[Ca]_i$ was increased.

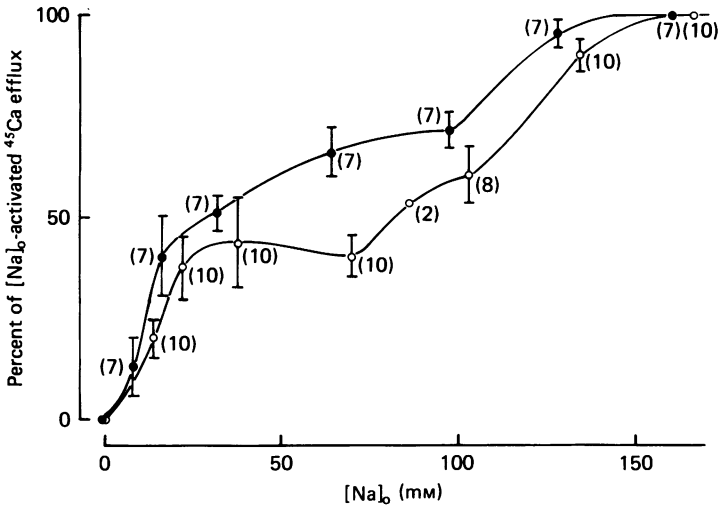

Fig. 5. $[Na]_o$-activated ^{45}Ca efflux (10 min) as a function of $[Na]_o$ in cell suspensions with raised $[Na]_i$. Cells were enriched with Na by incubation for 60 min at 36 °C in ^{45}Ca-containing K-free solution. Points represent the relative 10 min efflux into Ca-free K-free solutions (○) or Ca-free solutions with K readded (●) at different values of $[Na]_o$. Ordinate as Fig. 4.

$[Ca]_o$-activated ^{45}Ca efflux

Table 2 also shows the effects of $[Ca]_o$ on the ^{45}Ca efflux. The $[Ca]_o$-activated fraction of ^{45}Ca efflux was 0·19 n-mole/mg protein . 10 min when the ^{45}Ca efflux was measured in the absence of $[Na]_o$, while in the presence of $[Na]_o$ this fraction was significantly smaller (0·07 n-mole/mg protein . 10 min, $P<0·01$). By raising the level of $[Ca]_i$ following pretreatment with Na-free (Tris) solution, the $[Ca]_o$-activated ^{45}Ca efflux was significantly enhanced from 0·07 to 0·37 n-mole/mg protein . 10 min in the Na-containing solution ($P < 0·01$) and from 0·19 to 0·45 n-mole/mg protein . 10 min in Na-free (Tris) solution ($P<0·05$). These observations are compatible with findings in the squid axon (Baker & McNaughton, 1976; Blaustein, 1977), cardiac muscle (Reuter & Seitz, 1968; Busselen & Kerkhove, 1978), barnacle muscle (Ashley *et al.* 1974; Russel & Blaustein, 1974) and brain synaptosome (Blaustein & Ector, 1976).

Fig. 6 shows the ^{45}Ca efflux measured from the taenia coli plotted as a function of $[Ca]_o$. It was difficult to obtain the precise concentration of $[Ca]_o$, as we did not know the precise concentration of the Ca^{2+} contaminating the pellet. The volume of the pellet after centrifugation following pretreatment with 2·2 mM-$CaCl_2$ containing solution was 30–40 μl. and 1 ml. of the appropriate solution was added to this pellet.

TABLE 2. ^{45}Ca efflux from cells of the guinea-pig taenia coli in the presence or absence of $[Na]_o$ and $[Ca]_o$. In *A*, cells were incubated with ^{45}Ca at 36 °C for 30 min in normal Locke solution. In *B*, incubation was carried out in Na-free (Tris) solution at 36 °C for 10 min after incubation in normal Locke solution for 30 min. ^{45}Ca efflux was measured at 10 min at 36 °C.

	^{45}Ca efflux into Na		^{45}Ca efflux into Tris		Na-activated ^{45}Ca efflux		Ca-activated ^{45}Ca efflux	
	Ca (10^{-4} M)	Ca-free	Ca (10^{-4} M)	Ca-free	Ca (10^{-4} M)	Ca-free	Na	Tris
	(1)	(2)	(3)	(4)	(1)–(3)	(2)–(4)	(1)–(2)	(3)–(4)
A								
1	1·68	1·60	1·55	1·38	0·13	0·22	0·08	0·17
2	1·99	1·89	1·77	1·61	0·22	0·28	0·10	0·16
3	1·20	1·16	1·04	0·92	0·16	0·24	0·04	0·12
4	1·57	1·45	1·33	1·07	0·24	0·38	0·12	0·26
5	1·32	1·30	1·02	0·76	0·30	0·54	0·02	0·26
	1·55±0·14	1·48±0·13	1·34±0·15	1·15±0·15	0·21±0·03	0·33±0·06	0·07±0·02	0·19±0·0
B								
1	—	1·87	—	1·33	—	0·54	—	—
2	—	1·92	—	1·26	—	0·66	—	—
3	—	2·58	—	2·12	—	0·46	—	—
4	—	1·92	—	1·20	—	0·70	—	—
5	—	2·25	—	1·96	—	0·29	—	—
6	—	2·74	—	2·06	—	0·68	—	—
7	—	3·13	—	1·95	—	1·18	—	—
8	—	2·72	—	1·82	—	0·90	—	—
9	3·67	2·96	2·83	2·21	0·84	0·75	0·71	0·62
10	3·06	2·83	2·34	1·89	0·72	0·94	0·23	0·45
11	2·96	2·56	2·08	1·74	0·88	0·82	0·40	0·34
12	3·03	2·50	1·79	1·23	1·24	1·27	0·53	0·56
13	2·80	2·53	1·76	1·00	1·04	1·53	0·27	0·76
14	2·06	1·84	1·49	1·29	0·57	0·55	0·22	0·20
15	2·02	1·79	1·13	0·92	0·89	0·87	0·23	0·21
	2·80±0·22	2·41±0·12	1·92±0·21	1·60±0·11	0·88±0·08	0·81±0·08	0·37±0·07	0·45±0·0

This means that roughly 100 μM-Ca^{2+} extra was present in each tube. We prepared the various concentrations of $[Ca]_o$ buffering with 2 mM-EGTA as indicated in the abscissa of Fig. 6. The $[Ca]_o$ required for half maximal activation of $[Ca]_o$-activated ^{45}Ca efflux was approximately 50 μM in the presence of $[Na]_o$, while in the absence of $[Na]_o$ this value was slightly less. At high concentrations of $[Ca]_o$ (around 10^{-3} M-$[Ca]_o$), an extensive Ca influx would occur in the absence of $[Na]_o$, thus the amount of ^{45}Ca efflux may be underestimated, due to the reduction in the specific activity of the intracellular ^{45}Ca.

Residual ^{45}Ca efflux

As shown in Table 2, even in nominally Na- and Ca-free solution, there was evidence of ^{45}Ca efflux from cells of the taenia coli. This residual ^{45}Ca efflux contributed significantly to the total efflux. When tissues were loaded in Na-containing solution,

the residual efflux was 74% of the efflux into Na-containing, 10^{-4} M-$[Ca]_o$ solution, i.e. 1·15 as opposed to 1·55 n-mole/mg protein . 10 min. In tissues loaded in Na-free (Tris) solution, the residual efflux was 54% of the efflux into Na-containing, 10^{-4}-$[Ca]_o$ solution, i.e. 1·6 as opposed to 2·8 n-mole/mg protein . 10 min.

When levels of $[Ca]_i$ were increased by treatment with Na-free (Tris) solution, the value of the residual ^{45}Ca efflux was significantly increased from 1·15 to 1·60 n-mole/mg protein . 10 min ($P < 0·05$, Table 2), however the residual ^{45}Ca efflux was not affected by an increase in the $[Na]_i$ (Table 1).

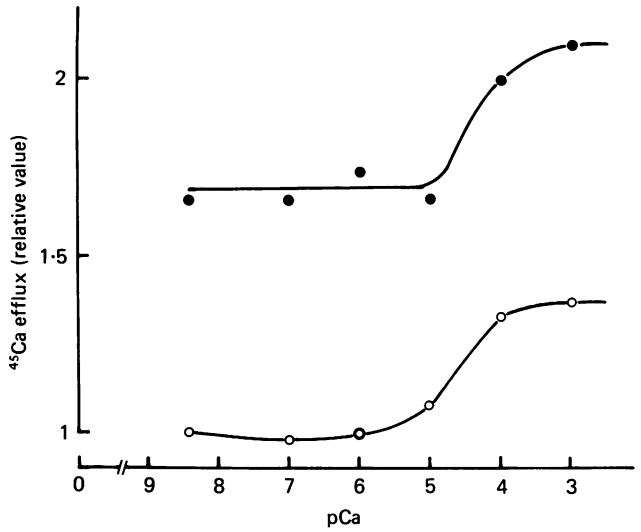

Fig. 6. Effect of $[Ca]_o$ on ^{45}Ca efflux in the single cell suspension prepared from the guinea-pig taenia coli. Cells were incubated at 36 °C for 10 min in Na-free (Tris), ^{45}Ca containing solution. After centrifugation of the suspension at 1,000 rev/min for 1 min, 1 ml. of an appropriate solution with various concentrations of $[Ca]_o$ was added to the cell pellet. Then ^{45}Ca efflux was measured at 10 min. The value was expressed relative to the ^{45}Ca efflux into Na-free (Tris), 10^{-6} M-$[Ca]_o$ solution. Each symbol is the mean value of three experiments. ○, ^{45}Ca efflux into Na-free (Tris) solution; ●, ^{45}Ca efflux into Na-containing solution.

DISCUSSION

The most convincing evidence that $[Na]_o$ is involved in Ca extrusion was obtained with squid axons (Baker & McNaughton, 1976; Blaustein & Russell, 1975; Blaustein, 1977). This particular tissue is probably the most suitable for Ca flux experiments because of its large size and the fact that both internal and external ionic compositions can be freely controlled.

In the smooth muscle tissue, it is extremely difficult to measure ^{45}Ca efflux as a transmembrane phenomenon. Therefore, to reduce the bulk of the extracellular space, single cells from the guinea-pig taenia colic and porcine coronary artery were prepared for measurement of the Ca efflux. The guinea-pig taenia coli showed a typical contracture in Na-free solution, while the porcine coronary artery did not contract in Na-free solution. In fact single cells prepared from the porcine coronary artery did

not show any change in Ca flux, whereas the single cells of the guinea-pig taenia coli showed a decrease in the Ca efflux, when suspended in Na-free (Tris) solution. When the single cells of the taenia coli were disrupted by water (osmotic shock), the typical Ca efflux disappeared. This means that the Ca efflux measured from the single cells is due to the properties of the intact cell.

With our experimental procedures, ^{45}Ca counts on the filter would represent the ^{45}Ca counts within the cell, but not the ^{45}Ca counts on the surface of the cells, because the cells were treated with a relatively high concentration of Ca chelating agent (EGTA). Thus, the Ca flux observed in the taenia coli is attributed to a particular phenomena involving the plasma membrane, and not to a non-specific phenomena on the surfaces of the cells.

The transmembrane Ca flux data obtained with single cells indicate that the Ca efflux in smooth muscle cells of the guinea-pig taenia coli consists of three major components: $[Na]_o$-activated, $[Ca]_o$-activated and a residual component persisting in the absence of both $[Na]_o$ and $[Ca]_o$. These three components have been postulated to be present in squid axon (Baker & McNaughton, 1976; Blaustein & Russell, 1975; Blaustein, 1977), and cardiac muscle (Reuter & Seitz, 1968; Busselen & Kerkhove, 1978). In this respect, the Ca efflux from the cell might be by the same processes as that in the squid axon and cardiac muscle.

The residual Ca efflux which persists in the absence of $[Na]_o$ and $[Ca]_o$ is a considerable proportion of the total Ca efflux. However, in the squid axon, DiPolo (1974) and Baker & McNaughton (1976) found that the residual Ca efflux was almost zero with the addition of metabolic inhibitors, namely cyanide or internal dialysis with ATP-free solution. Our preliminary experiments showed that plasma membrane of the single cells of the guinea-pig taenia coli seemed to become leaky with the metabolic inhibition produced by treatment with 2,4-dinitrophenol or iodoacetic acid (see also Casteels, Van Breemen & Wuytack, 1972; Brading, 1978). Therefore, we do not have sufficient data to assess whether the residual efflux is due to an ATP–dependent phenomena, as is the case in squid axon.

Regarding the Ca efflux, the interrelationship between Na–Ca exchange and Ca–Ca exchange should be considered. One possibility is that these two types of exchange are manifested by two completely independent transport mechanisms (for squid axon, Baker & McNaughton, 1976; for cardiac muscle, Busselen & Kerkhove, 1978). An alternative hypothesis is that Na–Ca and Ca–Ca exchanges are simply different modes of operation of a single carrier mechanism (for squid axon, Blaustein, 1977; Baker & McNaughton, 1978, for cardiac muscle, Jundt *et al.* 1975). In the guinea-pig taenia coli (1) the $[Ca]_o$-activated or $[Na]_o$-activated fraction of ^{45}Ca efflux is inhibited in the presence of $[Na]_o$ or $[Ca]_o$, respectively. This would be due to the displacement of Ca or Na from the external sites of the carrier by $[Na]_o$ or $[Ca]_o$, respectively. (2) The increase in $[Na]_i$ inhibits the $[Na]_o$-activated ^{45}Ca efflux, probably due to the displacement of Ca from the internal sites of the carrier by the increased $[Na]_i$. (3) The relationship between $[Ca]_o$ and the $[Ca]_o$-activated ^{45}Ca efflux is slightly shifted to the left in the absence of $[Na]_o$. Thus both Na–Ca and Ca–Ca exchange may involve a single carrier mechanism. It has also been suggested that the same mechanism could mediate Na:Na exchange (Brading, 1979).

However, the concentration of $[Ca]_o$ required for half maximal activation of

$[Ca]_o$-activated ^{45}Ca efflux was low. The affinity for $[Na]_o$ of the site responsible for the activation of ^{45}Ca efflux was much lower, more than 40 mM-$[Na]_o$ being required for its half maximal activation. Therefore, if all sites responsible for Ca efflux in physiological conditions (2–3 mM-$CaCl_2$) are saturated with $[Ca]_o$, the $[Na]_o$-activated fraction of ^{45}Ca efflux may not normally take place. In this respect, it cannot be concluded that Na–Ca, and Ca–Ca exchange operate on a single carrier mechanism.

In addition to the molecular nature of the carrier, the most interesting point is whether or not the Na–Ca exchange mechanism could be fully responsible for the low concentration of $[Ca]_i$. One could assume that the $[Ca]_i$ is determined by the equation

$$\frac{[Ca]_o}{[Ca]_i} = \frac{[Na]_o^2}{[Na]_i^2},$$

(Russell & Blaustein, 1974; Jundt *et al.* 1975) because the $[Na]_o$-activated Ca efflux was electro-neutral, but not electrogenic. Taking the approximate value $[Na]_o/[Na]_i$ and $[Ca]_o$ of 10–20 and 2·2 mM in the taenia (Brading, 1978), one can obtain the value of 5×10^{-6}–2×10^{-5} M-$[Ca]_i$ from the above equation. This value corresponds to a tension of about 70–80% of the maximum tension (Endo, Kitazawa, Yagi, Iino & Kakuta, 1977; Saida & Nonomura, 1979; T. Itoh, unpublished observations). Thus, it seems that the Na–Ca exchange mechanism alone could not fully explain the maintenance of a low concentration of $[Ca]_i$ in the guinea-pig taenia coli. Therefore, in addition to this exchange mechanism, other Ca efflux mechanisms, for example the residual Ca efflux and Ca sequestration in the cell might also contribute to maintain the low level of $[Ca]_i$. The ability of $[Na]_o$ to relax the contracture induced by Na-free solution was too rapid to be accounted for on the basis of the observed decrease in the ^{45}Ca content in the cells of the guinea-pig taenia coli on the readmission of $[Na]_o$ which was relatively slow. Furthermore, Katase & Tomita (1972) have shown that a dose of 7 mM-$[Na]_o$ is sufficient to relax the contracture maintained in Na-free solution. Although a direct comparison was impossible because of the different experimental conditions, the addition of 7 mM-$[Na]_o$ slightly activated the ^{45}Ca efflux. To clarify this point comparisons between the mechanical recording and ^{45}Ca efflux should be made using specific inhibitors of the internal Ca storage sites.

In conclusion, we have obtained evidence for three Ca efflux processes in the guinea-pig taenia coli, namely the $[Na]_o$-activated, $[Ca]_o$-activated fraction of ^{45}Ca efflux and the residual ^{45}Ca efflux. To elucidate the mechanisms involved in maintenance of the low concentration of $[Ca]_i$ in the taenia coli, the mechanism of Ca sequestration in the store sites is now being investigated.

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