Arch. Pharm. Res. 10(2), 80-87 (1987)

Effect of Sodium on Transmembrane Calcium Movement in the Cat Ileal Longitudinal Muscle

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(Received January 21, 1987)

Abstract□To get a better insight into the existence and the role of a Na-Ca exchange mechanism in smooth muscle, the effect of Na substitution with sucrose on tension development, cellular Ca uptake and ^{45}Ca efflux was investigated using isolated cat ileal longitudinal muscle strips. Experimental results were summarized as follows; 1) Exposure of the cat ileal longitudinal muscle to Na-free solution induced a contraction, and the magnitude of the contraction increased after incubation of the muscle strips with ouabain (2×10^{-5}M) for 1 hr. 2) Cellular Ca uptake in Na-free solution increased with an increase in Na content of the Na-loading media, and a linear relationship existed between tissue Na content and cellular Ca uptake for 10 min. 3) After tissues were equilibrated in PSS containing ^{45}Ca for 2 hr, cellular Ca uptake decreased with raising the external Na concentration. 4) Removal of medium Na or inhibition of the Na-K pump decreased the rate of ^{45}Ca efflux. These results strongly suggested that Na substitution increases cellular Ca uptake and decreases the rate of ^{45}Ca efflux via a Na-Ca exchange mechanism.

Key words□ Na-Ca exchange mechanism in smooth muscle, cat ileal longitudinal muscle, cellular Ca uptake, ^{45}Ca efflux.

Force development by smooth muscle cells is directly regulated by the concentration of free Ca ions in the myoplasm[1]. The level of ionized Ca in the myoplasm of the smooth muscle cell is kept very low ($\leq 10^{-7}$ M) at rest and regulated by the plasma membrane and subcellular organelles, i.e., sarcoplasmic reticulum and mitochondria[2].

It has been well known that variations in intra- and extracellular Na concentrations affect smooth muscle function by changing the levels of myoplasmic free Ca ion. When the external medium is depleted of Na, tonic and/or phasic contractions occur in various visceral and vascular smooth muscles[3-9]. And the high-K contracture of guinea-pig taenia coli induced no relaxation in Na-free solution[10,11]. Na substitution or inhibition of the Na-K pump has been reported to cause contraction and increase the cellular Ca content[11,12] and to decrease the rate of ^{45}Ca efflux[13,14].

These observations have been interpreted in terms of a model for Na-Ca exchange originally developed to explain the potentiation of ^{45}Ca efflux from rabbit atria by medium Na[15]. Na-Ca exchange is a transport process mediated by a carrier which couples oppositely directed transmembrane Na and Ca fluxes such that the electrochemical Na gradient participates in the maintenance of a large electrochemical Ca gradient.

In cardiac muscle, there is strong evidence for the existence of a Na-Ca exchange and its role in the control of intracellular free Ca. The exchange is thought to be electrogenic and voltage sensitive, and thus to affect not only tension development[16-18] but also the membrane potential itself[19,20].

In contrast, more recently several workers, as a result of more detailed studies, have become increasingly reluctant to assign much importance to Na-Ca exchange in the regulation of intracellular Ca of intestinal and vascular smooth muscles[21-25]. It has been considered that these conflicting views are due to the diversity of smooth muscle types, the complexity of Ca metabolism in these tissues, and the technical difficulties involved in measuring ^{45}Ca fluxes, cellular Ca and the Na gradient[21].

In the present study, we investigated the effect of Na substitution with sucrose on tension development, cellular ^{45}Ca uptake and ^{45}Ca efflux in cat ileal longitudinal muscle strips in order to get a better insight into the existence and the role of a Na-Ca exchange in smooth muscle.

EXPERIMENTAL METHODS

Dissection

Cats of either sex (2-3 kg) were stunned and bled. A length of about 10 cm of the terminal ileum was removed and the longitudinal muscle was separated from the underlying circular muscle. For tension recording, the longitudinal muscle was prepared in strips, 0.5 cm in width and 1.5-2 cm in length. For determination of tissue Na and ^{45}Ca content, the longitudinal muscle was cut into segments (20-30 mg).

Solutions

The physiological salt solution (PSS) contained (mM): NaCl, 130; KCl, 5; $CaCl_2$, 2.4; $MgCl_2$, 1.0; Glucose, 10; Tris, 2.4; equilibrated with 100% O_2, pH 7.4. The washout solution (EGTA-PSS) was made by adding 2mM-ethyleneglycol-bis-(β-aminoethylether) N, N'-tetraacetic acid (EGTA) to Ca-free solution where $CaCl_2$ was omitted from the PSS without any substitution. Sucrose-PSS was identical to PSS, except that it contained 260 mM-sucrose instead of 130 mM-Na. Similarly Tris-PSS contained additional 130 mM-Tris instead of Na. Variations in the Na concentration were made by replacing NaCl isosmotically with sucrose. Except where specified, all solutions were maintained at 36-37 °C.

Tension recording

The muscle strips were suspended in a 20 ml organ bath containing PSS at 37 °C. Initial tension on the muscle was set at 1 g and at least 30 min was allowed for equilibration before initiation of the experiment. Contractile responses were isometrically measured by Narco F60 microdisplacement myograph transducer and recorded on a Narco Biosystem.

Measurement of cellular ^{45}Ca *Uptake*

Paired muscle segments were pretreated with various Na-loading solutions containing ouabain (2×10^{-5} M). After the Na-loading, tissues were incubated with 3 ml-Sucrose-PSS containing ^{45}Ca(1.3 μCi/ml). Under equilibrium exchange conditions, tissues were equilibrated in PSS containing ^{45}Ca for 2 hr and then incubated in various experimental media, each containing the same specific activity of ^{45}Ca as PSS. At appropriate times, tissues were removed from the incubation media and immediately placed in an ice-cold EGTA-PSS for 30 min in order to remove extracellular bound ^{45}Ca (Fig. 1). Tissues were then blotted briefly, weighed and digested with 3ml of N-NaOH. After neutralization with 3 N-HCl, a scintillation cocktail was added to each sample and the ^{45}Ca content of each tissue measured in a liquid scintillation counter (Packard, Tricarb 300C). Samples of the incubation media were also counted.

Measurement of cellular Na *content*

Tissues were incubated for 2 hr in the Na-loading solution containing different $[Na]_o$ and ouabain (2×10^{-5} M). After the Na-loading, tissues were blotted to obtain the wet weight before analysis. Tissues were then exposed for 40 min to an ice-cold solution containing 250 mM-sucrose and 10 mM-$LaCl_3$ in order to remove extracellular Na. The pH was adjusted to 7.2 with 15 mM LiOH. Subsequently, tissues were leached in N-HNO_3 for 24 hr. Na was then measured using a flame photometer (Beckman, Klina).

^{45}Ca *efflux analysis*

Tissues were equilibrated in PSS containing ^{45}Ca (1.3 μCi/ml) for 3 hr. Tissues were then placed in an ice-cold EGTA-PSS for 30 min in order to

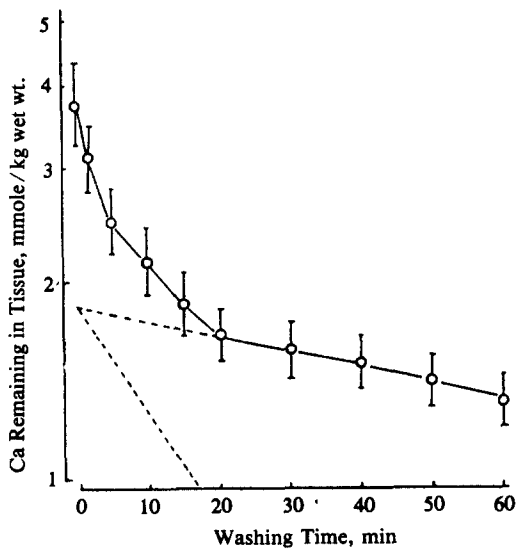

Fig. 1. ^{45}Ca washout into washing solution (ice-cold EGTA-PSS).

Tissues were equilibrated in PSS containing ^{45}Ca (1.3 μCi/ml) for 2 hr before initiation of ^{45}Ca wash out into ice-cold EGTA-PSS. The efflux curve was dissected into two exponential components. The rate constants of fast and slow components were 0.038 min^{-1} and 0.0052 min^{-1}, respectively. Each point represents mean ± S.E. of three experiments.

remove extracellular bound ^{45}Ca (Fig. 1). Subsequently, ^{45}Ca washout was monitored in the appropriate non-labelled media. Tissues were transferred at 5 min intervals into sequential 3 ml aliquots of medium. After efflux, tissues were blotted, weighed and residual tissue label was measured. The samples were counted, as were blanks and samples of the loading media. The radioactivity in each tissue at each time point was calculated by adding the counts in each sample in reverse order to the residual tissue ^{45}Ca.

Drugs used

^{45}Ca(Amersham/Searle Corp.), Tris (Sigma), acetylcholine (Sigma), ouabain (Sigma), EGTA (Sigma), nifedipine (Sigma), verapamil (Sigma), $LaCl_3$ (Sigma), $MnCl_2$ (Sigma), sodium vanadate (Aldrich)

Statistics

Statistical analysis of the data was performed in each case according to Student's t-test. Significance was taken as $p < 0.05$.

RESULTS

The measurement of ^{45}Ca fluxes in smooth muscle has been hampered by the existence of large, extracellularly bound pools. Extracellular exchange of ^{45}Ca has been difficult to distinguish from the true plasmalemmal Ca flux. This extracellular label is especially problematic in Na substitution experiments since it exchanges differentially with various Na substitutes [24].

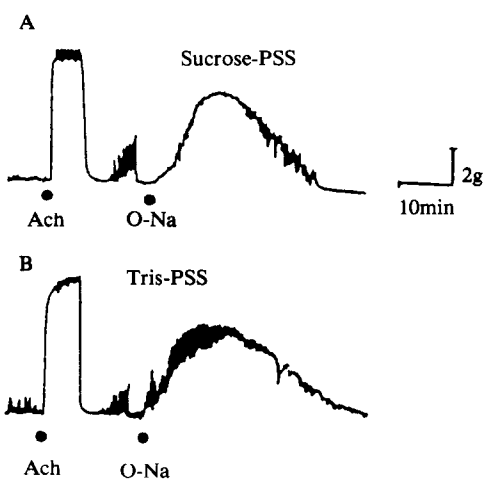

Fig. 2. Effect of Na-free solutions (A, Sucrose-PSS; B, Tris-PSS) on tension development in cat ileal longitudinal muscle strips.

The contractile responses induced by Na-free solutions were compared with those induced by 5×10^{-6} M-Ach.

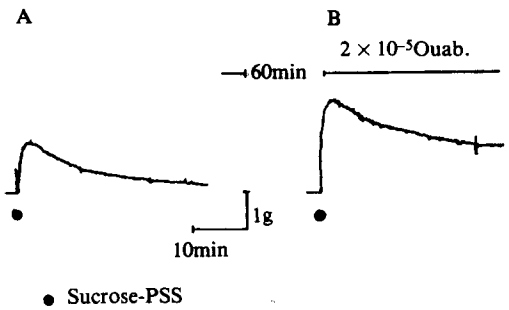

Fig. 3. Tension development in normal tissue (A) and Na-loaded tissue (B) on exposure to Na-free solution (Sucrose-PSS) in cat ileal longitudinal muscle strips.

Na-loaded tissue was prepared by treating the muscle with ouabain (2×10^{-5} M) for 60 min before the application of Sucrose-PSS.

A number of 'quenching' methods have been developed to remove extracellular label while maintaining cellular ^{45}Ca pools [21,26]. The use of these techniques generally leads to the resolution of a slow, monoexponential component of ^{45}Ca efflux, which is then considered to be of intracellular origin. The efflux of ^{45}Ca from prelabelled tissues was measured in ice-cold EGTA-PSS in order to determine the cellular Ca fraction. As shown in Fig. 1 the efflux curve was dissected into two exponential components and the rate constants of the fast and the slow components were 0.038 min^{-1} and 0.0052 min^{-1}, respectively.

Since the fast component was essentially removed after 20 min washout in ice-cold EGTA-PSS, tissues were washed for 30 min in this medium in the following ^{45}Ca uptake experiments, and the remaining ^{45}Ca was considered to be of an exclusively cellular origin. The cellular Ca content at this time was about 1.55 ± 0.15 mmole/kg wet wt., and this value is similar to that obtained for guinea-pig taenia coli [27].

Effect of Na-*free solution on tension development*

Fig. 2 shows the effect of complete substitution of NaCl with sucrose or Tris on the mechanical response. Exposure of the ileal longitudinal muscle strips to Na-free solutions induced contractions. The magnitude of the contractions was about 50-70% of that induced by 5×10^{-6} M-acetylcholine (Ach).

Fig. 3 illustrates that, after incubation of the

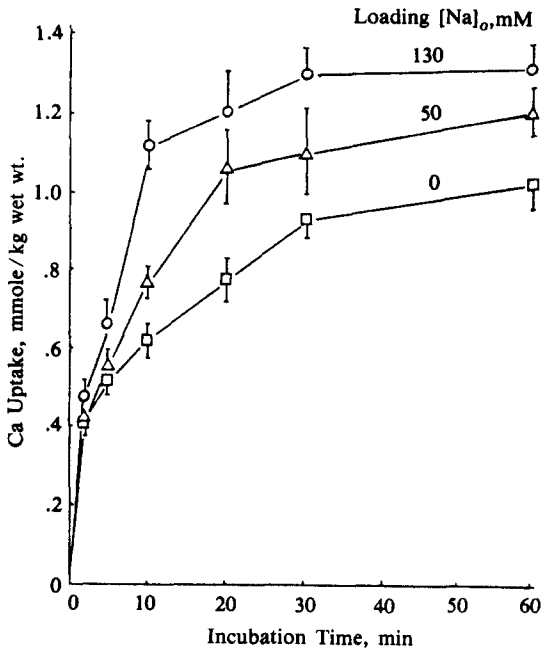

Fig. 4. Effect of the Na concentration of Na-loading solution on cellular Ca uptake in Na-free solution (Sucrose-PSS).

Tissues were pretreated with Na-loading solution containing different $[Na]_o$ (sucrose-substituted) and ouabain (2×10^{-5} M) for 2 hr and then incubated in Sucrose-PSS containing ^{45}Ca. At appropriate times, tissues were removed from the labelled media and immediately placed into washing solution (ice-cold EGTA-PSS) for 30 min. Each point represents mean ± S.E. of four experiments.

muscle strips with ouabain (2×10^{-5} M) for 60 min, Sucrose-PSS caused larger contraction than that in normal tissue. This implies that the contraction induced by Sucrose-PSS was not due to the specific effect of sucrose itself, but related to cellular Na content.

Effect of Na *on cellular* Ca *uptake in Na-free solution (Sucrose-Pss)*

In order to test whether cellular Na content affects cellular Ca uptake, after tissues were pretreated with Na-loading solution containing different $[Na]_o$ (sucrose-substituted) and ouabain (2×10^{-5} M) for 2 hr, cellular Ca uptake was measured in Na-free solution. As shown in Fig. 4, cellular Ca uptake in Na-free solution increased with an increase in the Na concentration of the Na-loading media.

In fact, a linear relationship existed between cellular Na content and the Ca uptake for 10 min (Fig. 5). This indicates that, when tissues were exposed to Na-free solution, cellular Ca uptake was dependent on cellular Na content. The intercept of ordinate reveals the Ca uptake when cellular Na content is theoretically zero, indicating Na-independent Ca uptake. This value was 0.58 mmole/kg wet wt..

Net Ca *uptake under equilibrium exchange conditions*

In this experiment, the effect of Na removal on cellular Ca uptake was determined under equilibrium exchange conditions. Tissues were prelabelled in PSS for 2 hr, and then incubated in various experimental media, each containing the same specific activity of ^{45}Ca as PSS, so that any changes in tissue ^{45}Ca were due to a net Ca uptake or loss by the tissue.

As shown in Fig. 6, after equilibration in PSS for 2 hr, cellular Ca was 1.30±0.127 mmole/kg wet wt.. In PSS, an increase in cellular Ca for 60 min incubation was only about 0.13 mmole/kg wet

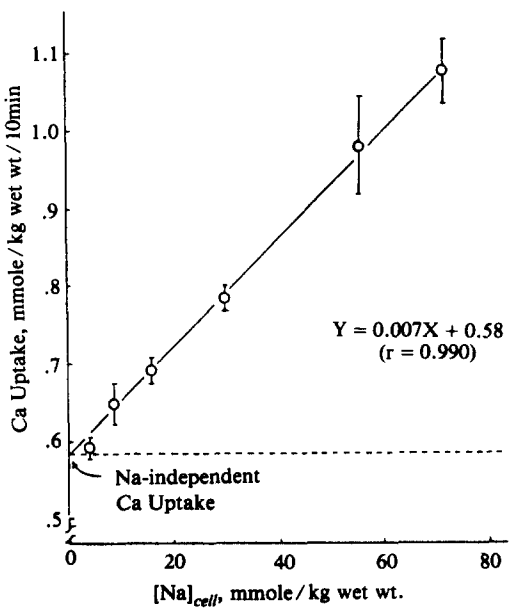

Fig. 5. Relationship between cellular Na content and Ca uptake for 10 min in Sucrose-PSS.

Tissues were incubated for 2 hr in the Na-loading solution containing different $[Na]_o$, as shown in Fig. 4. After the Na-loading, tissues were washed for 40 min with ice-cold 10 mM-La-sucrose solution for the measurement of cellular fraction of Na. Other legends are the same as in Fig. 4.

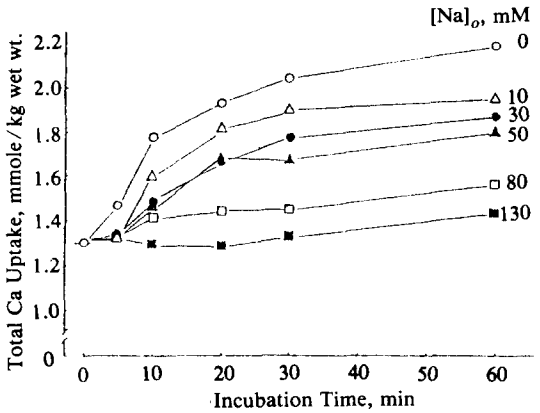

Fig. 6. Net Ca uptake under equilibrium exchange conditions.

Tissues were equilibrated in PSS containing ^{45}Ca for 2 hr and then incubated in media containing different $[Na]_o$ (sucrose-substituted) and ^{45}Ca. At appropriate times, tissues were removed from the labelled media and immediately placed into washing solution (ice-cold EGTA-PSS) for 30 min. Each point represents mean of four experiments.

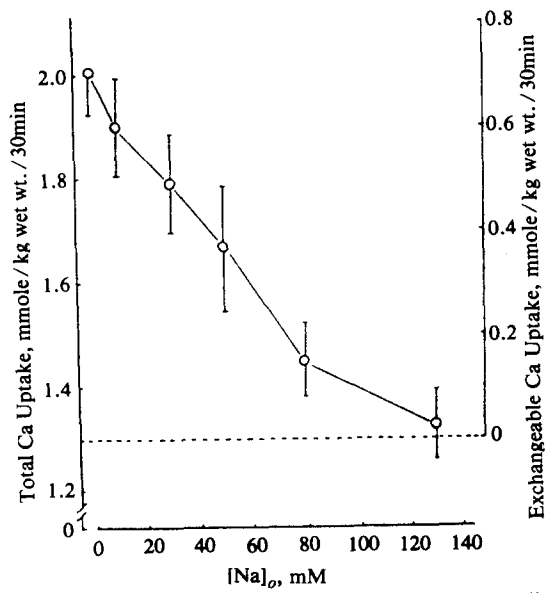

Fig. 7. Relationship between $[Na]_o$ in incubation media and the Ca uptake observed after a 30 min incubation of tissues in these media.

The exchangeable Ca uptake was calculated by subtracting the total cellular Ca content measured at time zero of incubation from that measured after 30 min incubation. The data shown in Fig. 6 were used. Each point represents mean ± S.E. of four experiments.

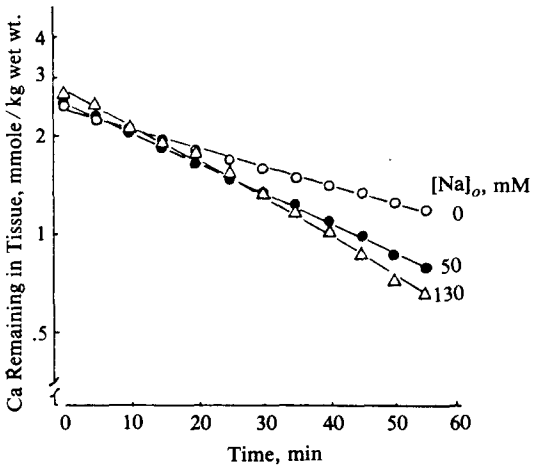

Fig. 8. Effect of the external Na concentration on transmembrane ^{45}Ca efflux.

Tissues were equilibrated in PSS containing ^{45}Ca for 3 hr and then washed for 30 min in ice-cold EGTA-PSS. Tissues were then placed in the appropriate unlabelled media at 37°C and the washout of tissue ^{45}Ca monitored. The rate constant of each line was as follows (mean ± S.E. of three experiments): 0-Na, 0.0117 ± 0.0014 min^{-1}; 50 mM-Na, 0.0207 ± 0.0025 min^{-1}; 130 mM-Na, 0.0287 ± 0.0027 min^{-1}

wt. However, when tissues were incubated in Na-depleted media, cellular Ca began to increase significantly at 10 min. The relative magnitude of the net Ca uptake for 30 min is plotted against $[Na]_o$ in Fig. 7. It can be seen that cellular exchangeable Ca uptake, which amounted to about 0.7 mmole/kg wet wt. upon total Na replacement, decreased with raising $[Na]_o$.

Effect of Na *substitution on transmembrane* ^{45}Ca *efflux*

The net Ca uptake observed upon Na removal might also be partially mediated by a decrease in the Ca extrusion rate. In the present study, ^{45}Ca efflux into PSS was compared with that into Na-depleted media. Fig. 8 shows that Na substitution by sucrose caused a decrease in the rate of ^{45}Ca efflux.

Effects of Ca *antagonists and* Na*-pump inhibitors on transmembrane* Ca *fluxes*

Table I shows the effects of Ca antagonists on cellular Ca uptake in Na-free solution (Sucrose-PSS). The Na-gradient Ca uptake was calculated by subtracting the Ca uptake of Na-unloaded tissue (preincubation in O-Na) from that of Na-loaded tissue (preincubation in 130 mM-Na). All Ca an-

Table I. Effects of calcium antagonists on Ca uptake in Sucrose-PSS

Drugs	Preincubation Conditions		Na-gradient Ca uptake
	130 mM-Na (mmole/kg wet wt.)	0-Na(Sucrose) (mmole/kg wet wt.)	(mmole/kg wet wt.)
Control	1.405 ± 0.032	0.874 ± 0.028	0.532 ± 0.029
Nifedipine, 0.01 mM	0.772 ± 0.025	0.449 ± 0.029	0.323 ± 0.029*
Verapamil, 0.05 mM	0.830 ± 0.018	0.426 ± 0.059	0.404 ± 0.039
La, 1 mM	0.461 ± 0.029	0.245 ± 0.015	0.216 ± 0.022**
Mn, 1 mM	0.516 ± 0.032	0.242 ± 0.040	0.274 ± 0.038*

Muscle strips were preincubated in PSS (130 mM-Na) or Sucrose-PSS (O-Na) containing ouabain (2×10^{-5} M) for 2 hr and then incubated in ^{45}Ca-containing Sucrose-PSS for 1 hr. After incubation, tissues were washed in ice-cold EGTA-PSS for 30 min to remove extracellular bound ^{45}Ca. All Ca antagonists were added in incubation media. All values indicate mean ± S.E. of three experiments.

* $p<0.05$ (Significantly different from the control)

** $p<0.01$ (Significantly different from the control)

Table II. Effects of Na-pump inhibitors and Ca antagonists on transmembrane ^{45}Ca efflux

Drugs	Efflux rate constants (k), min^{-1}
Control	0.02767 ± 0.0029
Ouabain, 0.1 mM	0.01375 ± 0.0021*
Vanadate, 0.1 mM	0.01663 ± 0.0011*
Control	0.02417 ± 0.0021
La, 2 mM	0.01451 ± 0.0010*
Mn, 5 mM	0.01344 ± 0.0020*
Nifedipine, 0.01 mM	0.02614 ± 0.0030
Verapamil, 0.1 mM	0.02451 ± 0.0028

Muscle strips were equilibrated in ^{45}Ca-containing PSS for 3 hr and then placed in ice-cold EGTA-PSS for 30 min to remove extracellular bound ^{45}Ca. Tissues were transferred at 5 min intervals into sequential 3 ml aliquots of PSS. All values indicate mean ± S.E. of three experiments.

* $p<0.05$ (Significantly different from the control)

tagonists except verapamil decreased the Na-gradient Ca uptake significantly. Nifedipine, $LaCl_3$ and $MnCl_2$ decreased the Na-gradient Ca uptake by 39%, 59% and 48%, respectively.

Table II shows the effects of Na-pump inhibitors and Ca antagonists on transmembrane ^{45}Ca efflux. All Na-pump inhibitors and inorganic Ca antagonists decreased the rate of ^{45}Ca efflux significantly. Organic Ca antagonists, defined as Ca influx inhibitors, showed little effect at the given concentrations.

DISCUSSION

The existence of a Na-Ca exchange mechanism has been demonstrated in a range of membranes including the squid giant axon[28], cardiac muscle[15] and mitochondria[29]. There is also strong evidence for the existence of such a mechanism in various types of smooth muscle including the guinea-pig taenia coli[27], ureter[3] and vascular smooth muscle[8].

Since direct evidence of Na-Ca linked movements across the plasmalemma of smooth muscle cells is difficult to obtain, no consensus has been reached regarding the role of Na-Ca exchange in maintenance of the transmembrane Ca gradient in smooth muscle.

Most studies of Na-Ca exchange have used contracture tension as an indicator of $[Ca]_i$. However, there have been reports that two other factors may influence tension during the low Na contracture. (i) Changes of intracellular pH can affect tension: alkalinization increases and acidification decreases tension at a given level of $[Ca]_i$[30]. Coray and McGuigan[31] have reported that Na removal produces an intracellular alkalinization in ferret papillary muscles (22-25 °C). (ii) Changes in the intracellular ATP concentration ($[ATP]_i$) can affect tension: decreasing $[ATP]_i$ increases the sensitivity of contractile proteins to Ca and eventually leads to the development of rigor tension[32]. Ventura-Clapier and Vassort[33] have found a decrease of $[ATP]_i$ during the low Na contracture and have suggested that the consequent development of rigor tension can account for much of this contracture. Thus, we

measured ^{45}Ca fluxes rather than contracture tension during the low Na contracture.

Although participation of a Na-Ca exchange mechanism in the control of cytoplasmic free Ca concentration in the smooth muscle cell is still a matter of controversy, the following results obtained from this study suggest the involvement of a Na-Ca exchange mechanism in the cat ileal longitudinal muscle. (i) Exposure of the muscle strips to Na-free solutions induced a contraction, and the magnitude of the contraction increased after incubation of tissues with ouabain (2×10^{-5} M) for 1 hr. (ii) Cellular Ca uptake in Na-free solution (Sucrose-PSS) increased with an increase in Na content of the Na-loading media, and a linear relationship existed between tissue Na content and cellular Ca uptake for 10 min. (iii) After tissues were equilibrated in PSS containing ^{45}Ca for 2 hr, cellular Ca uptake decreased with raising the external Na concentration. (iv) Removal of medium Na or inhibition of the Na-K pump decreased the rate of ^{45}Ca efflux.

In the present study, sucrose was chosen as the primary Na substitute. Casteels and Raeymaekers[34] have pointed out that sucrose solution exerts osmotic effects because of the low membrane permeability. It must also be taken into account that sucrose-solution is deficient in $[Cl]_o$ and that the ionic strength of the solution is low. However, there is no consensus of opinion on the most inert Na substitute or the one which clearly represents the effect of Na depletion *per se*.

Aaronson and van Breemen[21] have shown that sucrose appears not to cause the kind of gross damage to the cell membrane. It has also been shown that the changes in tension, cellular Ca uptake and ^{45}Ca fluxes are not due to the specific effect of sucrose itself[9,11,21]. In addition, sucrose has been widely employed as a Na substitute in previous studies of various smooth muscles[11,14,21,25].

Na removal itself might increase the Ca permeability of the membrane[10]. However, cellular Ca uptake when tissues were preincubated in PSS was greater than that when tissues were preincubated in Sucrose-PSS (Table I). Brading[27] also reported a nonspecific increase in membrane permeability in guinea-ping taenia coli with a reversed Na gradient. She proposed that the increase in $[Ca]_i$ due to Na-Ca exchange precedes and causes the general membrane alteration, which permits the secondary increase in Na efflux, and a simultaneous uptake of Ca.

It has been reported that Na removal caused depolarization in the cirmular muscle of the guinea-pig stomach[7,9], which might itself influence transmembrane Ca fluxes. Cellular Ca uptake decreased when tissues were preincubated in Sucrose-PSS (control in Table I). This shows that a membrane depolarization did not occur in Sucrose-PSS.

Although the data are incomplete, the results obtained from isolated cat ileal longitudinal muscle strips suggested the involvement of a Na-Ca exchange mechanism in this tissue. Van Breemen *et al.*[25] suggested that Na may compete for binding with Ca at a variety of sites throughout the cell membrane, the cell interior[35], and the extracellular matrix, thereby influencing Ca homeostasis in complex and multifocal ways. Clear dissection of the processes of the transmembranal ion transport will be expedited by the isolation of biochemically pure plasmalemma as pointed out by Aaronson and van Breemen[21].

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