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# EFFECTS OF SODIUM GRADIENT MANIPULATION UPON CELLULAR CALCIUM, $^{45}Ca$ FLUXES AND CELLULAR SODIUM IN THE GUINEA-PIG TAENIA COLI

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SUMMARY

1. Sucrose and choline were utilized as NaCl substitutes in order to investigate Na–Ca interactions in the smooth muscle of the guinea-pig taenia coli.

2. Progressive substitution of NaCl by sucrose caused a progressive increase in cellular exchangeable Ca. This uptake, which amounted to about 300 $\mu$mole Ca/kg tissue upon total Na replacement, reached a plateau within 20 min. Complete substitution of NaCl by choline chloride caused cellular Ca to increase rapidly to an initial peak, and then decrease to a stable plateau which was also about 300 $\mu$mole/kg above control.

3. Replacement of NaCl by either sucrose or choline chloride caused a transient increase in the Ca influx rate, which was measured using a 3 min pulse labelling with $^{45}Ca$. This increase was more pronounced in choline chloride.

4. NaCl substitution by either sucrose or choline chloride caused a decrease in the $^{45}Ca$ efflux rate. Two exponential components of transmembrane $^{45}Ca$ efflux were found in control and Na-free media.

5. Treatment of tissues with $3 \times 10^{-5}$ M-ouabain did not significantly affect the cellular Ca content after 80 min, at which time the Na and K gradients were largely dissipated.

6. Removal of medium K caused a slower dissipation of the Na and K gradients. This treatment decreased cellular Ca, did not affect the Ca influx rate, and increased the $^{45}Ca$ efflux rate.

7. Tissues were incubated in depolarizing media containing $10^{-4}$ M-ouabain in order to remove the Na gradient. Subsequent measurement of cellular Na indicated the absence of a significant fraction of bound Na.

8. The ratio $[Na]_o/[Na]_i$ had a value of 6·3 in control medium, and decreased as $[Na]_o$ was progressively lowered by sucrose substitution, reaching a value of $< 1$ in a medium containing 5 mM-Na.

9. These experiments provide evidence that a Na–Ca exchange carrier does not play an important role in regulation of tension in this muscle, and also indicate that the Ca gradient is not solely dependent on the Na gradient in guinea-pig taenia coli.

## INTRODUCTION

Research on the interaction of Na and Ca ions with biological enzyme and transport systems has a long and complex history.

In smooth muscle, Na substitution or inhibition of the Na–K pump has been reported to cause contraction and increase the cellular Ca content (Friedman, Jamieson & Friedman, 1959; Ma & Bose, 1977). In addition, in some cases Na substitution has been shown to decrease the rate of $^{45}Ca$ efflux (Reuter, Blaustein & Haeusler, 1973; Brading & Widdicombe, 1975).

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 (Reuter & Seitz, 1968). Briefly, this hypothesis states that a membrane carrier couples oppositely directed transmembrane Na and Ca fluxes such that the electrochemical Na gradient participates in the maintenance of a large electrochemical Ca gradient.

Unfortunately, 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, have prevented the development of a consensus regarding the role of an exchange process in maintenance of the transmembrane Ca gradient. For example, it has been proposed that Na–Ca exchange is the major determinant of this gradient in arterial smooth muscle (Blaustein, 1977). However, a great deal of evidence in arterial, intestinal, and uterine smooth muscle suggests that the Na gradient may regulate transmembrane Ca movement only to a limited degree or only during extreme experimental perturbation (van Breemen, Aaronson & Loutzenhiser, 1979). If this is the case, physiological Ca homeostasis would be determined predominantly by another Ca extruding process, probably involving a Ca ATPase. Several workers have proposed fundamentally similar models which incorporate a series arrangement of a Ca pump and an exchange process, in order to explain apparent contradictions in the available data (Reuter *et al.* 1973; Brading & Widdicombe, 1975; Brading, 1979; van Breemen *et al.* 1979).

The experiments described in this report were designed to provide basic information concerning the role of extracellular Na and the Na gradient in the modulation of Ca fluxes, and the maintenance of cellular Ca in the guinea-pig taenia coli. To this end, measurements were made of exchangeable cellular Ca, unidirectional $^{45}Ca$ influx, unidirectional $^{45}Ca$ efflux and cellular Na, during manipulation of the Na gradient. The data indicate that Ca extrusion is not singly dependent upon the Na gradient, although an exchange process may be involved in regulating transmembrane Ca fluxes in a specialized region of the cell. Alternatively, Na and Ca may interact in a complex fashion at multiple cell loci. Preliminary reports of this data have been presented previously (van Breemen, Aaronson & Loutzenhiser, 1980).

## METHODS

### *Dissection procedure*

Female English Shorthair guinea-pigs weighing 300–500 g were stunned and bled, and the taenia coli was dissected from the caecum while being continually superfused with a control physiological salt solution (PSS) containing (mM): NaCl, 142·0; $CaCl_2$, 1·5; $MgCl_2$, 1·0; KCl, 5·0; glucose 10·0;

HEPES, 5·0; and bubbled with 100 % $O_2$, pH 7·2. Tissues were subsequently cleaned in this medium, and cut into sections (3–25 mg) which were allowed to equilibrate for at least 1 hr in PSS. Except where specified, all media were maintained at 36–37 °C.

*Measurement of cellular Ca*

Following equilibration, tissues were placed into PSS containing $^{45}Ca$ ($1–5 \times 10^6$ c.p.m./ml.) for at least 100 min to allow maximal labelling of cellular Ca pools. Tissues were then removed to 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. At appropriate times, tissues were removed from the labelled media and immediately placed into an ice-cold, vigorously bubbled solution identical to PSS except that Ca was omitted, and 2 mM-ethyleneglycol-bis-(β-aminoethyl ether)*N,N'*-tetraacetic acid (EGTA) was added (EGTA-PSS) for 45 min. The purpose of this wash was to remove all extracellular bound label while leaving intracellular $^{45}Ca$ stores intact (van Breemen *et al.* 1980). Tissues were then blotted briefly, weighed and left overnight in scintillation vials containing 3 ml. 5·0 mM-ethylenediaminetetraacetic acid (EDTA). This treatment disperses $^{45}Ca$ into the vial solution as effectively as does overnight digestion with 30 % $H_2O_2$ at 90 °C (Aaronson, P., unpublished observation). Subsequently, 7·0 ml. of a scintillation cocktail containing Triton X-100 was added to each vial and the $^{45}Ca$ content of each tissue measured in a liquid scintillation counter (Tracor Analytic, Des Plaines, IL). Blanks and samples of the labelling media were also counted; the specific activities of the latter were used to convert tissue counts to tissue Ca content which was expressed as μmole Ca/kg tissue.

*Unidirectional $^{45}Ca$ influx mesurement*

Following a 2 hr equilibration in PSS, tissues were placed into non-labelled experimental media. At appropriate times, tissues were removed from either PSS or experimental media and placed in identical media containing $^{45}Ca$ for 3 min. Tissues were then washed for 45 min in ice-cold EGTA-PSS and processed to quantitate cellular $^{45}Ca$. During the 3 min 'pulse labelling' period $^{45}Ca$ uptake is almost entirely a function of Ca influx; $^{45}Ca$ efflux has a negligible effect on cellular label due to the initial low intracellular specific activity. By placing a large number of tissues in a particular medium and removing small groups for pulse labelling at various times, a temporal profile of the effect of the medium on Ca influx could be obtained.

*$^{45}Ca$ efflux analysis*

Tissues were equilibrated with the appropriate $^{45}Ca$-containing medium for 3–4 hr. They were then placed in an ice-cold medium identical to PSS except that it contained 6·5 mM-$CaCl_2$ and 5·0 mM-EGTA instead of 1·5 mM-$CaCl_2$. A 40 min wash in this medium (Ca-EGTA-PSS) removes extracellular $^{45}Ca$ leaving intracellular label largely intact (Aaronson, van Breemen, Loutzenhiser & Kolber, 1979). Ionic Ca is left in the medium to prevent alterations in membrane function which might occur in its absence. 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 $^{45}Ca$. Results were expressed as fraction of tissue $^{45}Ca$ present *vs.* time in order to facilitate curve peeling.

*Measurement of cellular Na and K*

Tissues were removed from PSS or from experimental media at appropriate times and placed for 40 min into an ice-cold solution containing 250 mM-sucrose and 10 mM-$LaCl_3$. The pH was adjusted to 7·2 with LiOH. Fig. 1 illustrates the effect of the La wash on tissue Na and K. After about 35 min, the desaturation of tissue Na approximated a monoexponential function. It was found that during the La wash, the dry/wet wt. ratio of the tissues progressively increased, and that this effect could be described by the expression:

$$(\text{dry/wet})_t = (\text{dry/wet})_{t=0}\, e^{kt}, \quad k = 0{\cdot}0046\ \text{min}^{-1}$$

where the subscript $t$ indicates the time of observation and $k$ is a rate constant. The mechanism of this water loss was not investigated thoroughly, but appeared to be an osmotic effect due both to the replacement of medium Na by sucrose and to the presence of La in the medium.

Since tissues were weighed following the washing period, this expression was used to correct the raw data shown in Fig. 1, resulting in the lower curves in this Figure. The rate constant of the monoexponential tail of the corrected Na washout curve was determined to be $-0{\cdot}0072$ min$^{-1}$, such that 25 % of this component was lost after 40 min. This component was assumed to represent cellular Na. The K desaturation curve was approximately monoexponential, as expected from the tissue distribution of this cation, and its rate constant of 0·0037 indicated a 14 % cellular K loss during the 40 min wash.

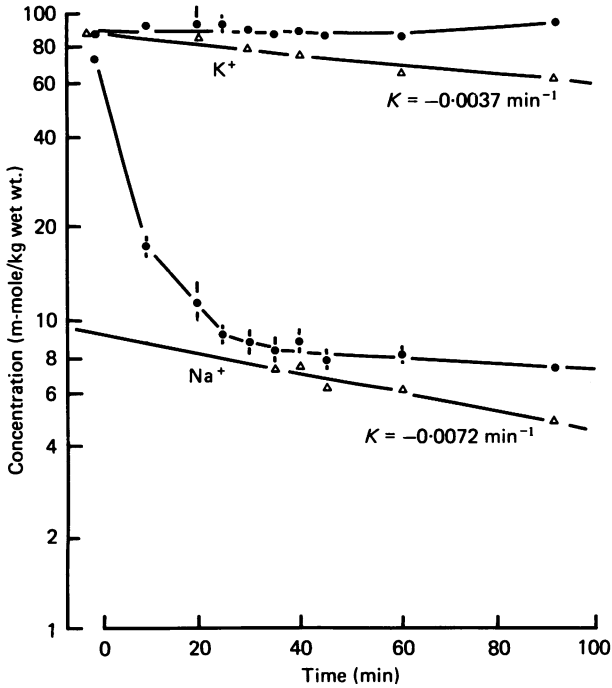


Fig. 1. The washout of tissue K (top) and Na (bottom) in an ice-cold solution of 250 mM-sucrose and 10 mM-$LaCl_3$. ●, raw data; △, represent the values for tissue Na and K which have been corrected for progressive tissue shrinkage during the La wash.

Following a 40 min wash, tissues were left overnight in washed scintillation vials containing 2–4 ml. 15 mM-LiCl. Na and K were subsequently measured using a flame photometer (Instrumentation Laboratories, Lexington, Mass.). After a correction for loss during the La wash, cellular Na and K were expressed as m-mole/kg tissue, or m-mole/l. cell water.

*Experimental media*

Sucrose-PSS was identical to PSS, except that it contained 260 mM-sucrose (Sigma) instead of 142 mM-Na. Choline-PSS similarly contained 142 mM-choline chloride (Sigma) instead of Na. $5{\cdot}0 \times 10^{-7}$ M-atropine was added to choline-PSS to prevent activation of muscarinic receptors. In both media, the pH was adjusted to 7·2 with KOH; an equimolar quantity of KCl was left out of the medium.

*Statistics*

Student's $t$ test for unpaired data was used in statistical analyses. A probability of $P < 0{\cdot}05$ was considered to indicate significantly different populations.

RESULTS

A major obstacle to the measurement of $^{45}Ca$ fluxes in smooth muscle has been the presence of a relatively large amount of extracellularly bound label, the presence of which also complicates estimations of cellular exchangeable Ca. This extracellular label is particularly problematic in Na substitution experiments since it exchanges differentially with various Na substitutes (Raeymaekers, Wuytack & Casteels, 1974).

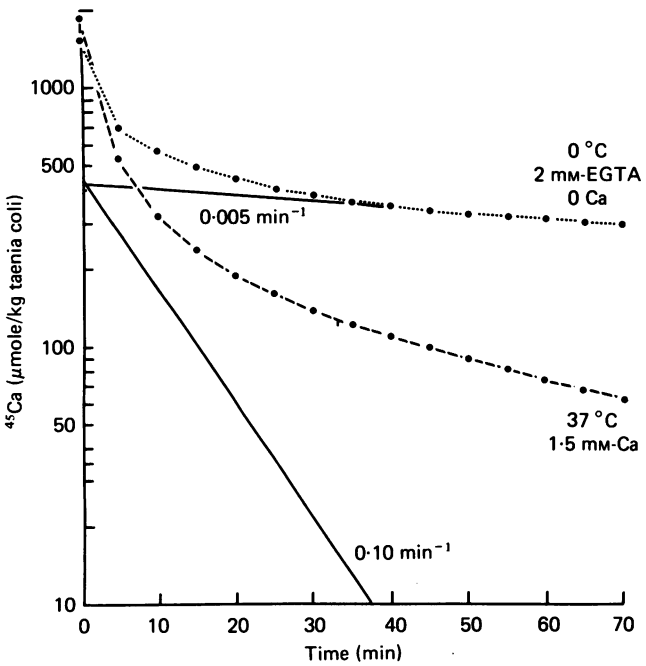


Fig. 2. $^{45}Ca$ washout into either PSS at 37 °C or EGTA-PSS at 0 °C. Tissues were labelled in PSS for at least 2 hr before washout into non-radioactive media was commenced. The efflux curve in EGTA was dissected into two exponential components by curve peeling. The rate constant of each process is shown. The sum of these two components was equal to the curve at each point except at $t = 0$; this discrepancy was due to the presence of a very fast component of efflux which washed out completely during the first 5 min efflux period.

A variety of 'quenching' methods have been developed to remove extracellular label while maintaining cellular $^{45}Ca$ pools (van Breemen, Hwang & Siegel, 1979; Droogmans, Raeymaekers & Casteels, 1977). 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 taenia coli sections was followed in ice-cold EGTA-PSS in order to resolve and measure the cellular Ca fraction. Fig. 2 shows that in this medium the slow efflux component had a rate constant of 0·0053 min$^{-1}$. Two other washout components also occurred. The faster, with a rate constant too large to measure with 5 min collection periods, represented 0·6 m-mole Ca/kg tissue. The size of this component and its rapid removal suggest that it is due to the washout of free extracellular label, since a $[Ca]_o$

of 1·5 mM in an extracellular space of 0·35 l./kg tissue (Jones, Somlyo & Somlyo, 1973), would give 0·53 m-mole/kg free extracellular Ca. The intermediate washout component of 0·45 m-mole Ca/kg tissue may have been due to the efflux of bound extracellular label.

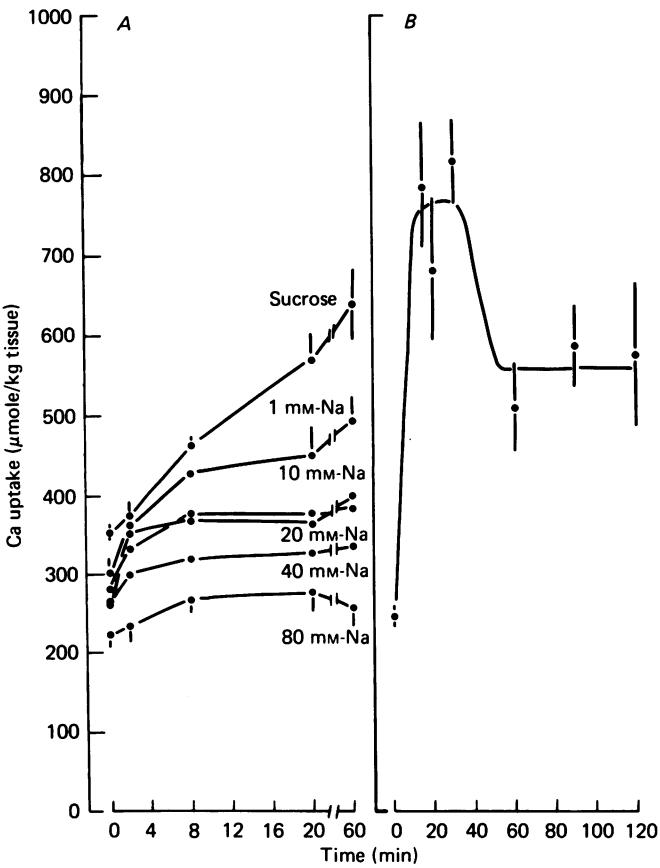


Fig. 3. The net uptake of exchangeable cellular Ca by taenia coli in Na-depleted media. Tissues were pre-labelled in PSS for 2 hr and then placed in a Na-depleted medium at time zero. *A*, effect of media in which NaCl was partially or completely replaced with sucrose. *B*, effect of complete substitution of NaCl with choline chloride. Each point in both panels represents the mean and S.E.M. of mean for at least four tissues.

Since the faster components were essentially removed after 40 min in cold EGTA-PSS, tissues were washed for 45 min in this medium in all $^{45}Ca$ uptake experiments, and the remaining $^{45}Ca$ was considered to be of an exclusively cellular origin. The mean exchangeable Ca content at this time was $304{\cdot}1 \pm 5{\cdot}3$ μmole/kg tissue ($n = 290$); although this value underestimates the content of the slow component by 27%, it will be used hereafter as an approximation of cellular Ca content. This value is much higher than those which have been previously obtained for taenia coli (Ma & Bose, 1977; van Breemen, Farinas, Casteels, Gerba, Wuytack & Deth, 1973) using the 'La method', although it is similar to values which have been obtained for vascular smooth muscle using media with lowered temperatures (van

Breeman *et al.* 1979; Deth, 1978; Ozaki & Urakawa, 1979; Dutta, Mustafa & Jones, 1980). It is likely that the reduced temperature of EGTA and La media retards the loss of Ca by cells.

*Na substitution experiments*

Sucrose was used as the primary Na substitute in this study. Choline chloride was also used as a Na substitute in order to establish that the changes seen in sucrose were not due to changes in ionic strength, or in medium [Cl]. Replacement of PSS by either choline-PSS or sucrose-PSS induced an uptake of cellular Ca, as shown in

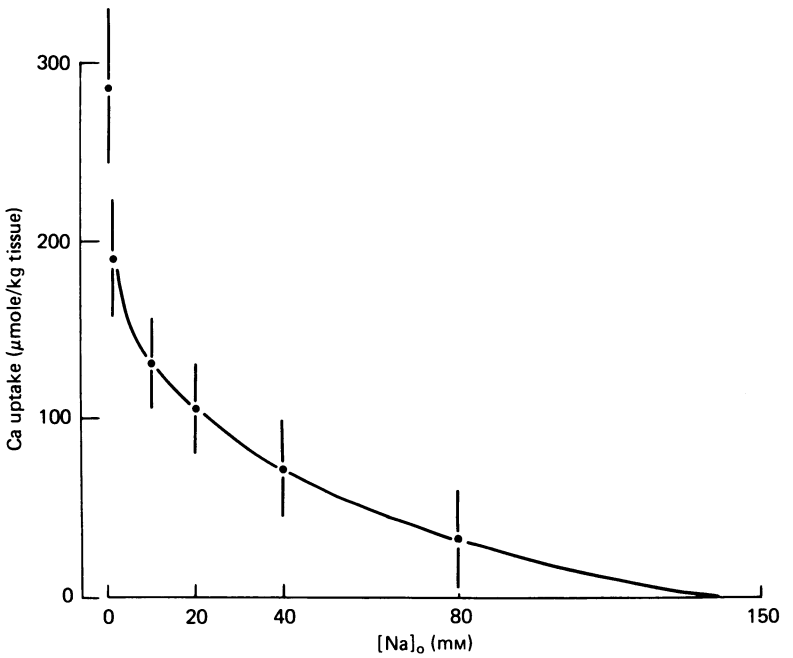


Fig. 4. The relationship between the Na concentration in Na-depleted media and the cellular uptake of exchangeable Ca observed after a 60 min incubation of tissues in these media. The points were calculated by subtracting the total cellular Ca measured in PSS from that measured after 60 min in the low-Na media; the data shown in Fig. 3 were used for these calculations.

Fig. 3. In sucrose the uptake was asymptotic, while in choline there was an initial large Ca gain, followed by a derease which resulted in a plateau Ca level similar to that seen in sucrose. Fig. 3 also shows that partial replacement of Na with sucrose caused a graded Ca uptake. In 80 mM-Na cellular Ca was significantly elevated only at 20 min. The relative magnitude of the net uptake is plotted against $[Na]_o$ in Fig. 4. It can be seen that half-maximal uptake occurred at $[Na]_o$ about 7 mM. The large difference between the uptake observed in sucrose-PSS and 1 mM-Na-PSS, and the small gain seen upon replacement of 72 mM-Na again suggest that changes in Cl or ionic strength were not important in mediating the observed effects.

The asymptotic nature of the net Ca uptake in the sucrose media suggested that a progressive, non-specific increase in membrane permeability to Ca was not occurring. In order to test for such an effect, the extracellular space was measured, using a 30 min tissue incubation in [$^{14}$C]sorbitol. Fig. 5 shows that the sorbitol space

did not increase significantly within the first 60 min of tissue incubation in sucrose-PSS. Na removal did, however, cause a loss of tissue water, which may have been due to an osmotic loss of water from cells and a shrinking of the extracellular glycoprotein matrix caused by increased Ca binding in the absence of Na (Friedman, 1977).

Fig. 5 also shows that complete Na substitution with sucrose caused a transient contracture with a subsequent complete and sustained relaxation accompanied by a loss of all spontaneous contractile activity. It should be stressed that tissues were relaxed during the period in which cellular Ca was elevated by more than 60%. The relaxation was not due to a direct effect of sucrose on the myofilaments, since elevation of $[K]_o$ to 20 mM in sucrose-PSS caused an instantaneous marked contracture, probably due to membrane depolarization.

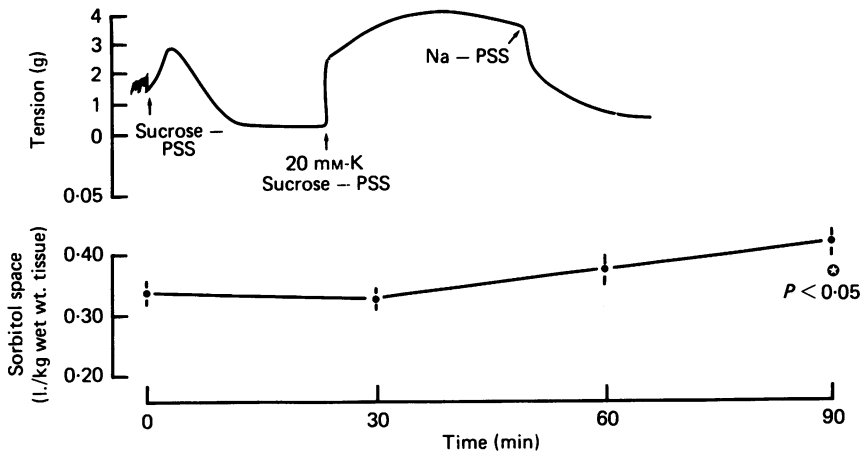


Fig. 5. The effect of Na removal on tension development and the sorbitol space of taenia coli. For tension measurements tissues were equilibrated in PSS for 1 hr; the control medium was replaced by sucrose-PSS at time 0 on the graph. After tissues had relaxed maximally, 20 mM-K, sucrose-PSS was substituted for sucrose-PSS. For measurement of the sorbitol space, tissues were incubated in PSS for 2 hr and then placed in sucrose-PSS. At appropriate times, groups of four to five tissues were removed either from PSS or from sucrose-PSS and placed for 30 min into an identical medium containing [$^{14}$C]sorbitol.

### *Effects of Na substitution on cellular Na*

Calculation of the transmembrane Na gradient in taenia coli involved the measurement of total cellular Na, and also the estimation of the fraction of cellular Na which is free. Friedman (1977) has provided evidence that about 60% of cellular Na is free in rat tail artery cells, while the use of Na-sensitive micro-electrodes has indicated that less than one third of the cellular Na in skeletal and cardiac muscle cells may be free (Lee & Armstrong, 1978; Ellis, 1977).

The Na gradient in taenia coli is maintained by the Na–K pump, and therefore inhibition of the pump should result in an increase in free $[Na]_i$, such that after equilibration free $[Na]_i = [Na]_o$. In this case, since $[Na]_i$ bound = total $[Na]_i$ − free $[Na]_i$, it is possible to estimate bound cellular Na by measuring total cellular Na in the presence of a known concentration of medium Na.

Therefore, tissues were incubated in depolarizing media in which [Na] was reduced

to a nominal concentration of 0, 10 or 25 mM by isotonic substitution with K. These media also contained $10^{-4}$ M-ouabain. After 5 hr exposure to these conditions, tissues were used to measure either the extracellular space (sorbitol space) and the dry/wet weight ratio or total cellular Na. The medium Na concentration was also measured to account for any changes due to evaporation or leaching of Na from the glassware.

TABLE 1. Data and calculations for estimation of a possible bound component of intracellular Na. All values shown as mean ± S.E. of mean of ten individual measurements. Total cellular Na, the sorbitol space and the dry/wet wt. ratio were measured in tissues which had been incubated for 5 hr in media containing a nominal [Na] of 0, 10 or 25 mM. These media were made isotonic with PSS by addition of K and also contained $10^{-4}$ M-ouabain. Cellular Na was quantitated following a 40 min wash of tissues in the ice-cold 10 mM-La-sucrose solution. The values shown are corrected for the loss of cell Na during the washing procedure. Cell space was considered to occupy that portion of the tissue not occupied by the dry wt. or the sorbitol space (extracellular space). Note that the measured Na concentration of the depolarizing media was higher than the nominal concentration; this was probably due to evaporation occurring during the experiment as well as Na contamination occurring during transfer of tissues from PSS to the experimental media

| *A* | *B* | *C* | *D* |
|---|---|---|---|
| Nominal $[Na]_o$ (mM) | Cellular Na (m-mole/kg dry wt.) | Sorbitol space (l./kg wet wt.) | Dry/wet wt. ratio |
| 0 | 4·42 ± 0·271 | 0·468 ± 0·012 | 0·141 ± 0·003 |
| 10 | 31·51 ± 2·42 | 0·414 ± 0·010 | 0·141 ± 0·002 |
| 25 | 79·10 ± 4·42 | 0·373 ± 0·011 | 0·152 ± 0·0014 |

| *E* | *F* | *G* |
|---|---|---|
| Cell space (l./kg wet wt.) | Total $[Na]_i$ (m-mole/l. cell space) | Measured $[Na]_o$ (= free $[Na]_i$) (mM) |
| 0·391 ± 0·012 | 1·60 ± 0·11 | 0·63 |
| 0·445 ± 0·010 | 9·99 ± 0·81 | 11·66 |
| 0·475 ± 0·011 | 25·31 ± 1·55 | 26·16 |

Total Na was expressed as m-mole/l. cell space and compared to the measured $[Na]_o$, as shown in Table 1. A comparison of columns *F* and *G* shows that in tissues incubated in the Na-containing depolarizing media total intracellular Na was not significantly higher than free cellular Na, indicating the absence of a measurable fraction of bound cellular Na. In the nominally Na-free medium, total $[Na]_i$ was in slight excess of free $[Na]_i$, suggesting the presence of a small bound fraction of cellular Na. In general, however, these data were consistent with the hypothesis that taenia coli cells contained only a negligible amount of bound Na. These results were additionally supported by experiments in which total cellular Na was measured during incubation of tissues in sucrose-PSS. Removal of medium Na caused a rapid initial loss of cellular Na which was followed by a slow, monoexponential component of Na washout which by extrapolation to $t = 0$ appeared to comprise 5–10 % of the total cellular Na (data not shown). Recently, Brading, Burnett & Sneddon (1980) have similarly shown that upon removal of medium Na almost all cellular Na is lost within 30 min. It is interesting that under analogous conditions, rat tail artery cells retain a large cellular Na fraction (Friedman, 1977), suggesting that Na binding and/or sequestration differ profoundly in these two smooth muscles.

Unless treatment of tissues in the depolarizing media changed the binding of cellular Na and thus its activity coefficient, the data suggest that free $[Na]_i$ may be

estimated roughly by measuring total $[Na]_i$. Under the assumption that such changes were not occurring, the Na ratio $[Na]_o/[Na]_i$ was calculated at various $[Na]_o$ by placing tissues in PSS, or in several isotonic low Na (sucrose) media. Depletion of medium Na resulted in a rapid loss of cellular Na with the establishment of a new steady state within 30 min (Fig. 6). Table 2 shows the calculation of total cellular Na and the ratio $[Na]_o/[Na]_i$ in PSS and in several low Na media after 30 min incubation. In taenia coli, unlike rat tail artery, the Na gradient decreases with progressive depletion of medium Na. It is important to note that in PSS, the ratio $[Na]_o/[Na]_i$ has a value of approximately 6·3. At this ratio, a 3 Na/Ca exchange carrier would tend to set $[Ca]_i$ at about $9{\cdot}2 \times 10^{-7}$ M and would be expected to mediate a net influx if $[Ca]_i$ fell below this value.

TABLE 2. Data and calculations for estimation of the transmembrane Na concentration ratio in PSS and several Na-depleted media. All values are shown as mean ± S.D. (the number of measurements is shown in parentheses next to each column). Total cellular Na, the sorbitol space and the dry/wet wt. ratio were measured in tissues which had been equilibrated in PSS or in tissues which were incubated in low-Na media for 30 min following equilibration in PSS. Cellular Na was quantitated following a 40 min wash of tissues in the ice-cold 10 mM-La sucrose solution. The values shown are corrected for tissue shrinkage and loss of cell Na during the washing procedure. Cell space was considered to occupy that portion of the tissue not occupied by the dry wt. or the sorbitol space (extracellular space).

| | *A* | *B* | *C* | *D* | *E* | *F* | *G* |
|---|---|---|---|---|---|---|---|
| $[Na]_o$ (mM) | Cellular Na (m-mole/kg dry wt.) | Sorbitol space (l./kg tissue) | Dry/wet wt. ratio | Cell space (l./kg tissue) | Dry/wet wt. / cell space | $[Na]_i$ (m-mole/l. cell space) | $[Na]_o/[Na]_i$ |
| 0 | 6·46 ± 3·14 (10) | 0·328 ± 0·034 (4) | 0·254 ± 0·007 (4) | 0·418 ± 0·035 | 0·608 ± 0·053 | 3·01 ± 1·93 | — |
| 5 | 10·11 ± 0·882 (8) | 0·368 ± 0·032 (4) | 0·257 ± 0·004 (4) | 0·375 ± 0·032 | 0·685 ± 0·059 | 6·94 ± 0·85 | 0·72 |
| 15 | 13·88 ± 3·68 (6) | 0·338 ± 0·022 (4) | 0·265 ± 0·015 (4) | 0·397 ± 0·026 | 0·668 ± 0·057 | 9·27 ± 2·58 | 1·62 |
| 30 | 20·57 ± 3·88 (7) | 0·370 ± 0·024 (4) | 0·251 ± 0·005 (4) | 0·379 ± 0·024 | 0·662 ± 0·044 | 13·61 ± 2·72 | 2·20 |
| 75 | 28·13 ± 6·01 (5) | 0·384 ± 0·029 (6) | 0·217 ± 0·010 (5) | 0·399 ± 0·031 | 0·544 ± 0·050 | 15·47 ± 3·57 | 4·85 |
| 142 | 52·95 ± 14·54 (36) | 0·349 ± 0·057 (10) | 0·194 ± 0·013 (5) | 0·457 ± 0·059 | 0·425 ± 0·062 | 22·4 ± 6·97 | 6·34 |

The K content of taenia coli cells in PSS was found to be 75·7 ± 3·4 m-mole/kg wet wt. ($n = 22$). Correcting for cation loss during the La wash, and assuming a cell space of 0·457 l./kg wet wt. (Table 2), $[K]_i$ was found to be 166 m-mole/l. cell space, a value which is similar to previous estimates (Casteels, 1969).

### *Unidirectional Ca influx and efflux in Na-free medium*

As shown in Fig. 7, substitution of PSS by either sucrose-PSS or choline-PSS caused a dramatic, but transient, increase in the unidirectional Ca influx rate. The control influx rate was determined by pulse labelling one group of tissues in PSS for 3 min.

Several other groups of tissues were first equilibrated in PSS, placed in a Na-free medium, and individually pulsed at the indicated times. The period during which the Ca influx rate was elevated corresponds to that during which net Ca uptake occurred (Fig. 3).

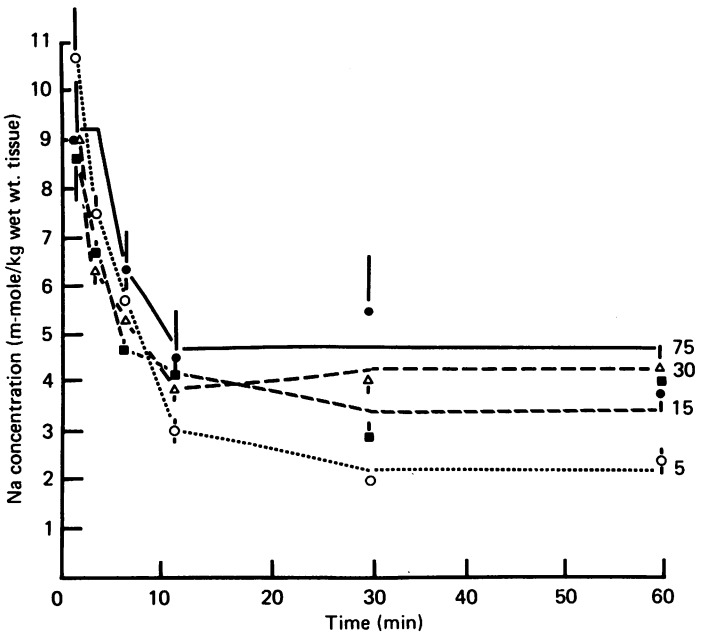


Fig. 6. The effect of several low-Na media upon cellular Na. Tissues were equilibrated in PSS for 2 hr and then placed into media containing 5, 15, 30 or 75 mM-Na. At the time shown, tissues were removed from the incubation media and washed for 40 min in ice-cold 10 mM-$LaCl_3$, 250 mM-sucrose solution in order to remove extracellular Na. The values for cellular Na shown are uncorrected for tissue shrinkage and Na loss during the La wash, and are expressed as m-mole/kg wet wt. tissue.

The net Ca uptake observed upon Na removal might also be partially mediated by a decrease in the Ca extrusion rate. Such an inhibitory effect of Na substitution upon Ca extrusion ($^{45}$Ca efflux) has been previously described for Na-loaded taenia coli in the presence of 5 mM-La medium (Brading & Widdicombe, 1975).

In order to quantitate a possible inhibition under less extreme conditions, tissues were labelled with $^{45}$Ca in PSS, and then washed in ice-cold PSS containing 5·0 mM-Ca EGTA, as described under Methods. We have previously shown that this washing procedure removes extracellular $^{45}$Ca, while leaving intracellular Ca stores largely intact. When tissues are rewarmed to 37 °C and $^{45}$Ca efflux in PSS monitored, two exponential transmembrane efflux components are observed (Aaronson *et al.* 1979). In the present study, $^{45}$Ca efflux into PSS was compared with that into sucrose-PSS, and choline-PSS followed the wash in Ca-EGTA-PSS. Both Na substitutes slowed $^{45}$Ca efflux from taenia coli cells. Figure 8*A* shows that sucrose substitution slowed efflux both by decreasing the rate constant of the slower efflux component, and by increasing the size of the slower component from 41 to 63 % of the total initial cellular label. Choline substitution decreased the rate of the faster efflux component, and also increased the size of the slower efflux component from 37 to 50 % of initial cellular label (Fig. 8*B*).

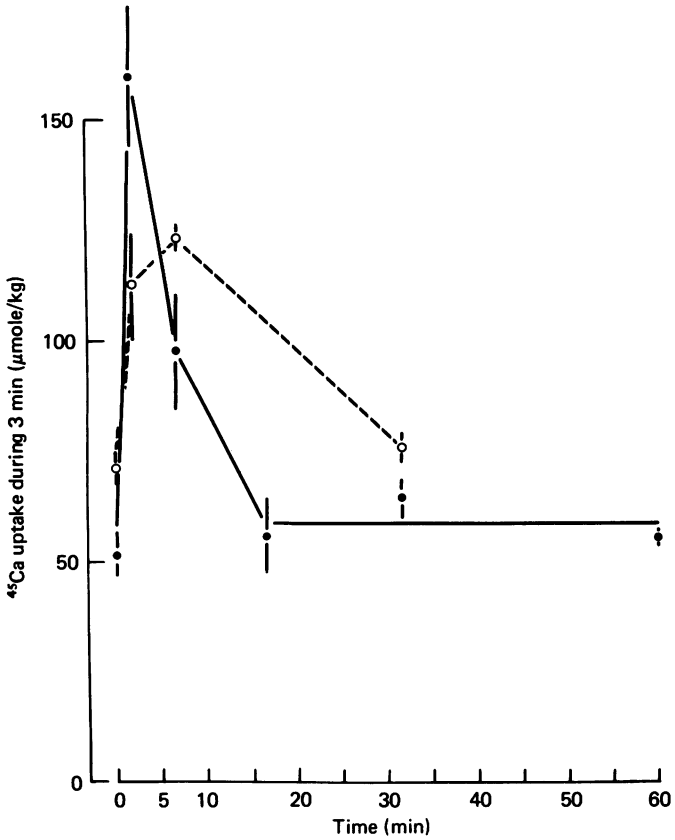


Fig. 7. The effect of complete substitution of NaCl with sucrose (○) or choline chloride (●) upon the Ca influx rate, measured during a 3 min $^{45}$Ca labelling period. Tissues were equilibrated with PSS for 2 hr and then placed into either sucrose-PSS or choline-PSS at time zero. At various times, groups of four to five tissues were removed from the medium and placed for 3 min in an identical medium containing $^{45}$Ca. After this labelling period, tissues were washed in ice-cold EGTA-PSS to remove extracellular label.

TABLE 3. The effect of $3 \times 10^{-5}$ M-ouabain upon cellular Ca, Na and K. Cellular cation content was measured immediately prior to, and 13 and 80 min after, addition of ouabain to the medium. Cellular Ca was quantitated following a 45 min wash of tissues in ice-cold EGTA-PSS, and cellular Na and K were measured following a 40 min wash of tissues in the ice-cold 10 mM-La sucrose medium. Each value shown is the mean ± S.E. of the mean of the number of individual measurements shown in parentheses. Note that the cellular Ca content did not change significantly during the 80 min ouabain incubation

| *A* | *B* | *C* | *D* |
|---|---|---|---|
| Min of incubation in $3 \times 10^{-5}$ M-ouabain | Cellular Na (m-mole/kg tissue) | Cellular K (m-mole/kg tissue) | Cellular Ca (μmole/kg tissue) |
| 0 | 13·2 ± 0·9 (4) | 74·2 ± 3·4 (4) | 190·1 ± 15·3 (4) |
| 13 | 28·9 ± 1·6 (5) | 50·5 ± 4·9 (5) | 194·8 ± 14·2 (4) |
| 80 | 74·3 ± 9·6 (4) | 9·1 ± 1·3 (4) | 214·7 ± 6·1 (4) |

*Effects of ouabain on cellular Ca*

Treatment of guinea-pig taenia coli with ouabain causes an inhibition of the Na–K pump and a subsequent dissipation of the electrochemical Na and K gradients (Casteels, 1966). Taenia coli were treated with $3 \times 10^{-5}$ M-ouabain, and cellular Ca, Na and K were subsequently measured. During Na gradient breakdown there was no significant increase in the cellular Ca content (Table 3). In addition, tissues remained completely relaxed after an initial, transient contracture, as first noted by van Esveld (1928). These results indicate that cellular maintenance of Ca homeostasis is not dependent upon the presence of an intact Na gradient, confirming earlier reports in this and other smooth muscles (van Breemen *et al.* 1973; Droogmans & Casteels, 1979).

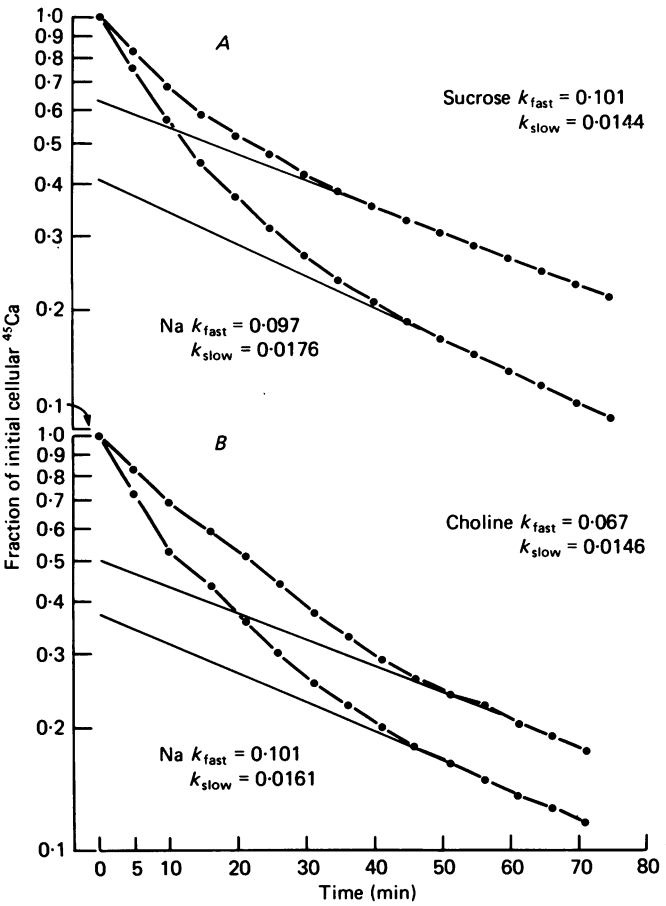


Fig. 8. The effect of complete substitution of NaCl with either sucrose or choline chloride, upon transmembrane $^{45}$Ca efflux. Tissues were labelled for 3 hr in PSS and then washed for 45 min in ice-cold Ca-EGTA-PSS. Tissues were then placed in the appropriate unlabelled medium at 37 °C and the washout of tissue $^{45}$Ca monitored. *A*, $^{45}$Ca efflux in PSS *vs.* sucrose-PSS; each line represents the average of five washout experiments. *B*, $^{45}$Ca efflux in PSS *vs.* choline-PSS; each line represents the average of four experiments. In both panels, only the slow efflux component is drawn, although the rate constants for both transmembrane components are shown.

*Effects of K removal on cellular Ca metabolism*

Removal of K from the medium also causes a dissipation of the Na and K concentration gradients by inhibiting the Na–K pump of taenia coli. K removal exerts a more complex effect upon the membrane potential, first hyperpolarizing the membrane and then causing a secondary, sustained, depolarization (Casteels, Droogmans & Hendrickx, 1971). Fig. 9 shows the effects of K removal upon tension, cellular Na and K, and the unidirectional Ca influx rate.

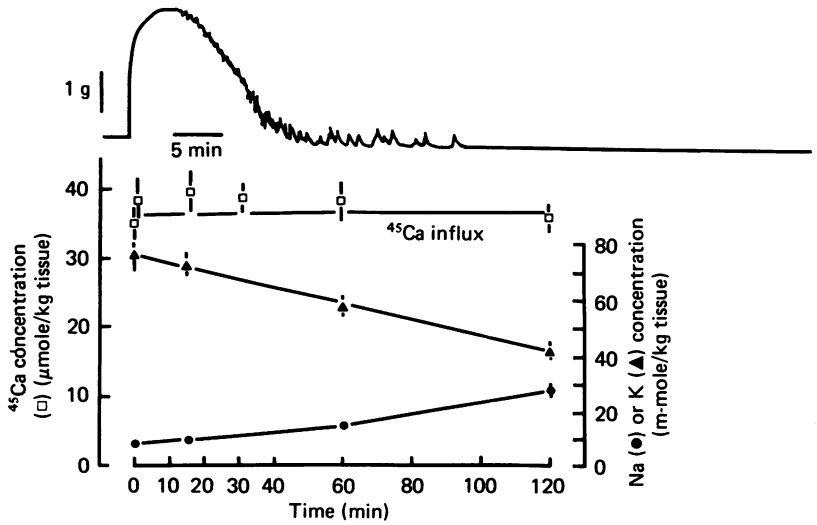


Fig. 9. The effect of K-free medium upon tension development, the Ca influx rate and total cellular Na and K. Tissues were equilibrated in PSS for 1 hr (tension measurements) or 2 hr (Ca influx and Na and K quantitation) and then placed into K-free PSS at time 0 on the graph. In order to measure the Ca influx rate, tissues in PSS or K-free PSS were removed at appropriate times, pulse-labelled for 3 min in identical media containing $^{45}$Ca and then washed in ice-cold EGTA-PSS to remove extracellular label. For measurement of cellular Na and K, tissues were removed from PSS or K-free PSS at appropriate times and washed for 40 min in the ice-cold 10 mM-La sucrose medium in order to remove extracellularly bound cation. The Na and K data shown are not corrected for cation loss and tissue shrinkage during the La wash.

K removal caused a transient contracture followed by complete tissue relaxation and a loss of spontaneous tension oscillations, as previously observed (Holman, 1958). K removal caused a more gradual cellular gain of Na than did ouabain treatment, which is consistent with the proposal (Casteels, Droogmans & Hendrickx, 1973) that the loss of K from cells in this medium may raise $[K]_o$ in the vicinity of the membrane to levels which partially activate the Na–K pump. The upper part of Fig. 9 shows that the Ca influx rate, estimated using the 3 min pulse-labelling protocol, did not change significantly during 2 hr of K depletion.

The effect of K-free medium on $^{45}$Ca efflux was measured as follows. Tissues were labelled with $^{45}$Ca in either PSS or K-free PSS, washed for 47 min in cold Ca-EGTA-PSS, and then allowed to washout into the same (unlabelled) medium in which they had been labelled. Fig. 10 shows that tissues which were labelled in K-free

PSS and washed out in this medium showed an apparent rate constant of $^{45}Ca$ efflux which was slightly higher than that seen in control tissues. In this case, partial breakdown of the Na gradient potentiated $^{45}Ca$ efflux, rather than causing the inhibition predicted by the Na–Ca exchange hypothesis.

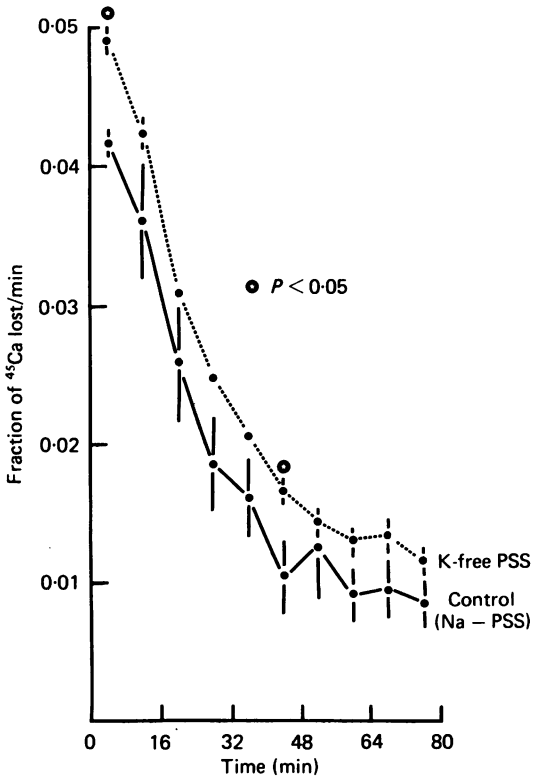


Fig. 10. The effect of K-free medium on the apparent rate constant of transmembrane $^{45}Ca$ efflux. Control tissues (——) were labelled in PSS for 3 hr, washed in ice-cold Ca-EGTA-PSS for 47 min and allowed to wash out into PSS at 37 °C. Experimental tissues (– – –) were labelled in PSS for 1 hr and then further labelled in K-free PSS for 2 hr. They were then washed in ice-cold Ca-EGTA-PSS for 47 min and allowed to wash out into K-free PSS at 37 °C. Each set of data points represents the mean ± S.E. of mean for eight separate washout experiments.

By measuring the absolute amounts of $^{45}Ca$ lost during these efflux experiments, it was possible to calculate the cellular Ca content prior to washout. Tissues labelled in K-free PSS had a total Ca content of $178 \pm 8$ $\mu$mole Ca/kg tissue ($n = 8$), compared to $272 \pm 13$ $\mu$mole/kg tissue ($n = 8$) for tissues labelled in PSS. These values refer to the residual tissue after the cold Ca EGTA wash, a procedure which gives values for cellular Ca similar to those obtained using the cold EGTA wash (Aaronson, P., unpublished results). It therefore appears that incubation of tissues in K-free medium resulted in a significant and substantial loss of cellular Ca after 2 hr, an effect which is consistent with the observations that the influx rate was not altered in this medium, and that $^{45}Ca$ efflux was slightly stimulated.

## DISCUSSION

Sucrose was chosen as the primary Na substitute in this study for several reasons. This compound does not penetrate the cell membrane (Casteels, 1970), and its substitution for Na does not cause a membrane depolarization, which might itself influence transmembrane Ca fluxes (Katase & Tomita, 1972). Sucrose appears not to cause the kind of gross damage to the cell membrane which would be evidenced by an increase in the sorbitol space (Fig. 5). In addition, this compound has been widely employed as a Na substitute in previous studies of taenia coli and other types of smooth muscle (van Breemen *et al.* 1979; Reuter *et al.* 1973; Ma & Bose, 1977). It should be pointed out that the low $[Cl]_o$ obtained in sucrose-PSS may decrease the K permeability of the membrane (Casteels, 1970), and that spontaneous action potentials are also abolished upon replacement of NaCl with sucrose (Brading, Bülbring & Tomita, 1969). However, the finding that substitution of Na with choline chloride causes changes in total cellular Ca, unidirectional $^{45}Ca$ influx and $^{45}Ca$ efflux which are qualitatively similar to those observed in sucrose is inconsistent with the possibility that these effects are predominantly due to the low $[Cl]_o$, or ionic strength of sucrose-PSS. We have also observed similar changes in these three parameters of Ca metabolism upon complete substitution of Na with K (Aaronson, van Breemen & Zera, 1980).

The data show that cells progressively gain Ca as the Na gradient is reduced by removal of medium Na. Intriguingly, cells do not take up Ca when the Na gradient is decreased during Na pump inhibition with ouabain, and indeed lose Ca if the Na gradient is partially dissipated following removal of medium K. These results imply strongly that it is not the Na gradient *per se* which is responsible for controlling the cellular Ca content. The relaxation of tissues observed in both Na-free media, and in the K-free and ouabain-containing media, as well as the lack of a Ca uptake in the latter two solutions indicate that, in this tissue, Ca homeostasis is not solely dependent upon a Na–Ca exchange process utilizing the inward Na gradient as the energy source for Ca extrusion. We conclude therefore that in this tissue, Ca homeostasis in the cellular compartment which is in direct contact with the myofilaments is mainly dependent upon another Ca extruding mechanism, probably an ATP-driven Ca pump (van Breemen *et al.* 1973; Casteels & van Breemen, 1975).

Replacement of NaCl with either sucrose or choline chloride results in an increase in exchangeable cellular Ca, which eventually plateaus at a level which is roughly 300 $\mu$mole/kg higher than control. The large Ca uptake in the absence of a tissue contracture indicates the existence of a cellular Ca pool which is Na-sensitive, but not directly involved with myofilament activation. The return of extracellular Na caused a rapid return of cellular Ca to control levels while the muscle remained relaxed, although the quantity of Ca released was much larger than that required for maximal activation of the contractile proteins (van Breemen, 1976). It therefore appears that this Na-sensitive Ca pool is superficially located, perhaps within the peripheral sarcoplasmic reticulum (s.r.) or on the inner plasmalemmal surface. This possibility is consistent with the concept that one Ca extrusion pathway in taenia coli may involve two separate steps. In the first, Ca would be actively concentrated in the s.r. by a Ca ATPase similar to that present in skeletal and cardiac muscle s.r.

Ca might then be extruded from the cell via a Na–Ca exchange process located in the areas of close apposition between the plasmalemma and the superficial s.r. This model has been previously discussed (Brading, 1979; van Breemen *et al.* 1979).

It should be stressed that Na might be able to regulate the Ca content of such a pool through mechanism other than Na–Ca exchange. For example, the increased unidirectional Ca influx observed upon Na removal is consistent with a competition between these cations for a plasmalemmal influx pathway. However, the lack of a potentiation of unidirectional Ca influx during partial Na gradient dissipation in K-free PSS suggests that Ca influx is not related to the Na gradient, or to an exchange process. The effect of Na removal on the Ca influx rate may thus be due to competition for another type of influx pathway; one possibility is that this pathway involves a membrane channel (van Breemen *et al.* 1979; Droogmans & Casteels, 1979).

The asymptotic cellular uptake of Ca in Na-free media and the loss of Ca following Na-pump inhibition may be explained by the existence of a number of intracellular binding sites at which Na and Ca compete. The decrease of cellular Na in sucrose-PSS would be expected to deplete these sites of Na and cause them to bind Ca. This bound Ca would not be available for myofilament activation, especially if it were located within intracellular membrane-bound structures such as the s.r. The decrease in the rate of $^{45}$Ca efflux observed when medium Na is removed has been generally explained as being a result of the inhibition of Na–Ca exchange (Reuter *et al.* 1973; Brading & Widdicombe, 1975). Our data confirm that this effect is due to an inhibition of transplasmalemmal Ca extrusion. However, when the Na gradient is decreased following Na-pump inhibition in K-free PSS, $^{45}$Ca efflux is potentiated. A similar effect has been demonstrated in the arteria carotis communis of cattle (Preiss & Banaschak, 1979). This phenomenon is not explained by the Na–Ca exchange model.

Although the data are incomplete, it may be fruitful with respect to future research to speculate that Na may modulate $^{45}$Ca efflux through interactions that do not necessarily involve Na–Ca exchange. For example, if Na and Ca do compete for intracellular binding, a change in $[Na]_i$ should affect intracellular Ca binding and thus $[Ca]_i$. In this case, a change in medium Na would be expected to influence the activity of a plasmalemmal Ca-extruding pump, even if it were not directly Na-controlled. Alternatively, if a plasmalemmal Ca ATPase in smooth muscle is similar to those found in skeletal and cardiac muscle s.r., the pump may be stimulated by the presence of a suitable monovalent cation on the side of the membrane to which Ca is being actively transported (Duggan, 1977; Chiu & Haynes, 1980). This hypothesis would explain the observation that the inhibition of $^{45}$Ca efflux in Li-PSS is much smaller than that seen in sucrose or choline media. Clear dissection of these processes will be expedited by the isolation of biochemically pure plasmalemmal and s.r. membrane fractions from smooth muscle.

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