Na^+/Ca^{2+} exchange in isolated smooth muscle cells demonstrated by the fluorescent calcium indicator fura-2

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Fura-2, a novel fluorescent indicator of cytoplasmic calcium concentrations ($[Ca_i^{2+}]$), was 'loaded' into smooth muscle cells isolated from guinea pig taenia coli. Resting cells maintained a stable $[Ca_i^{2+}]$ of 107 ± 26 nM ($n = 13$), which could be perturbed with ionomycin. $[Ca_i^{2+}]$ was elevated by stimulation of the cells with carbachol or 50 mM KCl. Reduction of the plasmalemmal Na^+ concentration gradient by inhibition of the Na^+/K^+-ATPase with ouabain markedly elevated $[Ca_i^{2+}]$; this elevation was dependent on extracellular Ca^{2+}. $[Ca_i^{2+}]$ was also increased by replacement of the extracellular Na^+ with an organic cation.

(Smooth muscle) Fura-2 Na^+/Ca^{2+} exchange

1. INTRODUCTION

Little direct information has been available about the extent and nature of processes of ion transport across the plasmalemma of smooth muscle cells. The numerous studies using isotopes [1,2] have been hampered by the existence of large, extracellularly bound pools, particularly of Ca^{2+}. Extracellular exchange of isotope has been difficult to distinguish from the true plasmalemmal Ca^{2+} flux. Other studies have employed less direct methods of estimating Ca^{2+} fluxes or $[Ca_i^{2+}]$, such as tension and membrane potential [3]. Here, we report upon the first direct demonstration of Na^+/Ca^{2+} exchange in isolated smooth muscle cells from the guinea pig taenia coli, using the recently synthesized intracellular calcium indicator, fura-2 [4]. This technique has enabled agonist-induced changes in $[Ca_i^{2+}]$ to be recorded directly and compared to the time course of the $[Ca_i^{2+}]$ change detected with more classical indicators such as the photoprotein, aequorin [5]. This is of particular importance since photoproteins have a non-linear stoichiometry with respect to Ca^{2+} and hence would be strongly influenced by fluxes occurring across the surface membrane of the smooth muscle cell, which has a high surface area to volume ratio compared to skeletal muscle fibres.

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Abbreviations: $[Ca_i^{2+}]$, cytoplasmic free calcium concentration; $[Ca_o^{2+}]$, extracellular free calcium concentration; $[Na_i^+]$, cytoplasmic sodium concentration; $[Na_o^+]$, extracellular sodium concentration; $[K_o^+]$, extracellular potassium concentration; fura-2/AM, fura-2 acetomethoxy pentaester; DMSO, dimethyl sulphoxide; BSA, bovine serum albumin

2. MATERIALS AND METHODS

2.1. *Preparation of fura-2-loaded smooth muscle cells*

Taenia coli smooth muscle strips from adult male guinea pigs were chopped transversely into 1 mm segments, washed in 1% BSA saline and incubated at 37°C for 2×30 min periods in 10 ml of 1% BSA saline containing 1 mg/ml collagenase, 0.25 mg/ml trypsin inhibitor. After washing twice by gentle centrifugation (1 min at $100 \times g$) to remove the enzyme, the tissue was dispersed into

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3 ml of 1% BSA saline by 30 s gentle pipetting, and filtered through nylon gauze to obtain a suspension of individual smooth muscle cells (smc). smc suspension was incubated with 4 μM fura-2/AM at 37°C for 1 h and then diluted to 20 ml before centrifugation at 250 × g for 12 min, onto a silicone fluid cushion (Dow Corning FS-1265/300). The smc pellet was resuspended in 5 ml of 1% BSA saline and stored at room temperature (~20°C).

2.2. *Fluorimetry*

Fluorescence signals were recorded from smc suspensions at 37°C in 1 cm, UV-compatible plastic cuvettes using a Shimadzu RF 540 fluorimeter. For details of spectrum recording see figure legends. smc suspensions were periodically mixed by hand. This produced a negligible mixing artefact. The smc were subsequently lysed by adding 50 μM digitonin to obtain the signal of the calcium-saturated dye (F_{max}); the signal (F_{min}) from the Ca-free form of the dye was recorded by adding 10 mM Na-EGTA at pH 8.3. The $[Ca_i^{2+}]$ corresponding to an intracellular fura-2 fluorescence F was calculated by application of the equation $[Ca_i^{2+}] = K_d(F - F_{min}/F_{max} - F)$ to the 510 nm emission values excited at 340 nm (F), using K_d = 224 nM [4]. This method utilizes the largest fura-2 fluorescence signal relative to smc autofluorescence [4]. A component (15–30%) of the total fura-2 fluorescence originates from extracellular fura-2 free acid, mainly residual from the loading procedure. This component was subtracted by quenching the extracellular fura-2 signal by addition of EGTA [4] (table 1, legend).

2.3. *Reagents and salines*

The physiological saline contained (mM): NaCl, 130; KCl, 4.8; Na_2HPO_4, 1.0; $CaCl_2$, 1.0; $MgCl_2$, 1.0; Tes, 10; glucose, 15; pH 7.4 at 37°C, gassed with 100% O_2. During enzyme treatment and storage of the smc the saline contained 10 mg/ml BSA (Sigma fraction V). For fluorimetry, the BSA concentration was reduced to 0.8 mg/ml, while the extracellular $[Ca_i^{2+}]$ was increased to 2.5 mM. Extracellular $[Na^+]$ was reduced below 10 mM by replacement with bis(2-hydroxyethyl)dimethylammonium chloride (Kodak). Fura-2/AM (Molecular Probes, OR, USA) was added to smc suspensions as a 2 mM solution in DMSO. Ionomycin (Calbiochem) was used as a 1 mM solution in DMSO.

3. RESULTS

3.1. *Loading smc with fura-2*

Cells separated from the loading solution retained a peak at ~340 nm (fig.1D); their fluorescence was much more intense than that of non-loaded cells (fig.1A).

3.2. *Effects of ionomycin (fig.2)*

To demonstrate the responses of intracellular fura-2 to $[Ca_i^{2+}]$, the specific Ca^{2+} ionophore, ionomycin [6], was used to perturb $[Ca_i^{2+}]$. When ionomycin was added to smc in 1 mM Ca_o^{2+}, the degree of Ca^{2+} saturation of intracellular fura-2 increased (fig.2A). Alternatively, smc were

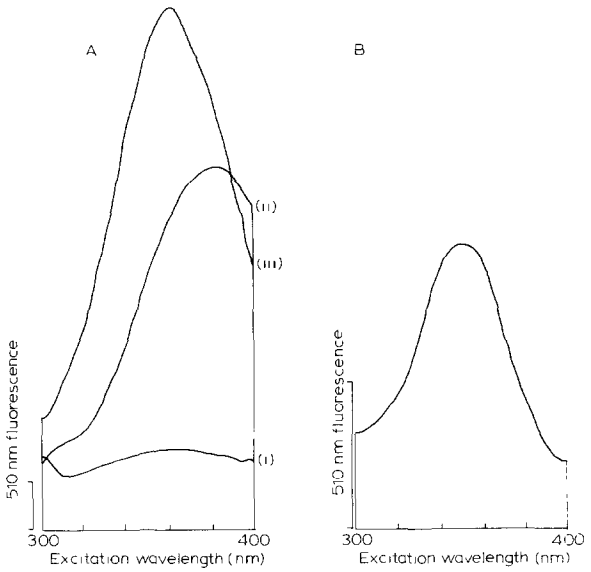

Fig.1. Loading smooth muscle cells with fura-2/AM. (A) (i) Excitation spectrum of non-loaded smc in 1% BSA saline. (ii) Addition of 4 μM fura-2/AM. (iii) During incubation at 37°C for 1 h, fura-2/AM ester was hydrolysed to fura-2 free acid and excitation maximum shifted towards 340 nm, characteristic of Ca^{2+}-saturated fura-2. (B) Retention of free-acid peak by smc separated from loading medium. Ordinate scale indicated by vertical bar. smc contained 20–50 μM fura-2 free acid, estimated relative to fura-2 standards following lysis. Intracellular volume was estimated as 90% of the packed cell volume obtained by microhaematocrit centrifugation.

equilibrated in $[Ca_o^{2+}]$ of about 80 nM (fig.2B, trace a). Ionomycin then allowed Ca^{2+} to leave the cell (fig.2B, traces b–d).

3.3. *Responses of fura-2-loaded smc to carbachol or KCl (fig.3)*

100 μM carbachol rapidly reduced 510 nm emission excited at 380 nm (fig.3A, trace a), which fell within about 5 s to a minimum, corresponding to an increase in $[Ca_i^{2+}]$ to 1.4-times resting levels (fig.3A, trace a), while 510 nm emission excited at 340 nm was enhanced (fig.3A, inset). Elevated $[Ca_i^{2+}]$ persisted for more than 4 min, in the continuous presence of the agonist. Depolarization of the smc by the addition of 50 mM KCl elevated $[Ca_i^{2+}]$ by approx. 1.6-fold, the stimulated level again being maintained for at least 3 min (fig.3B, trace a).

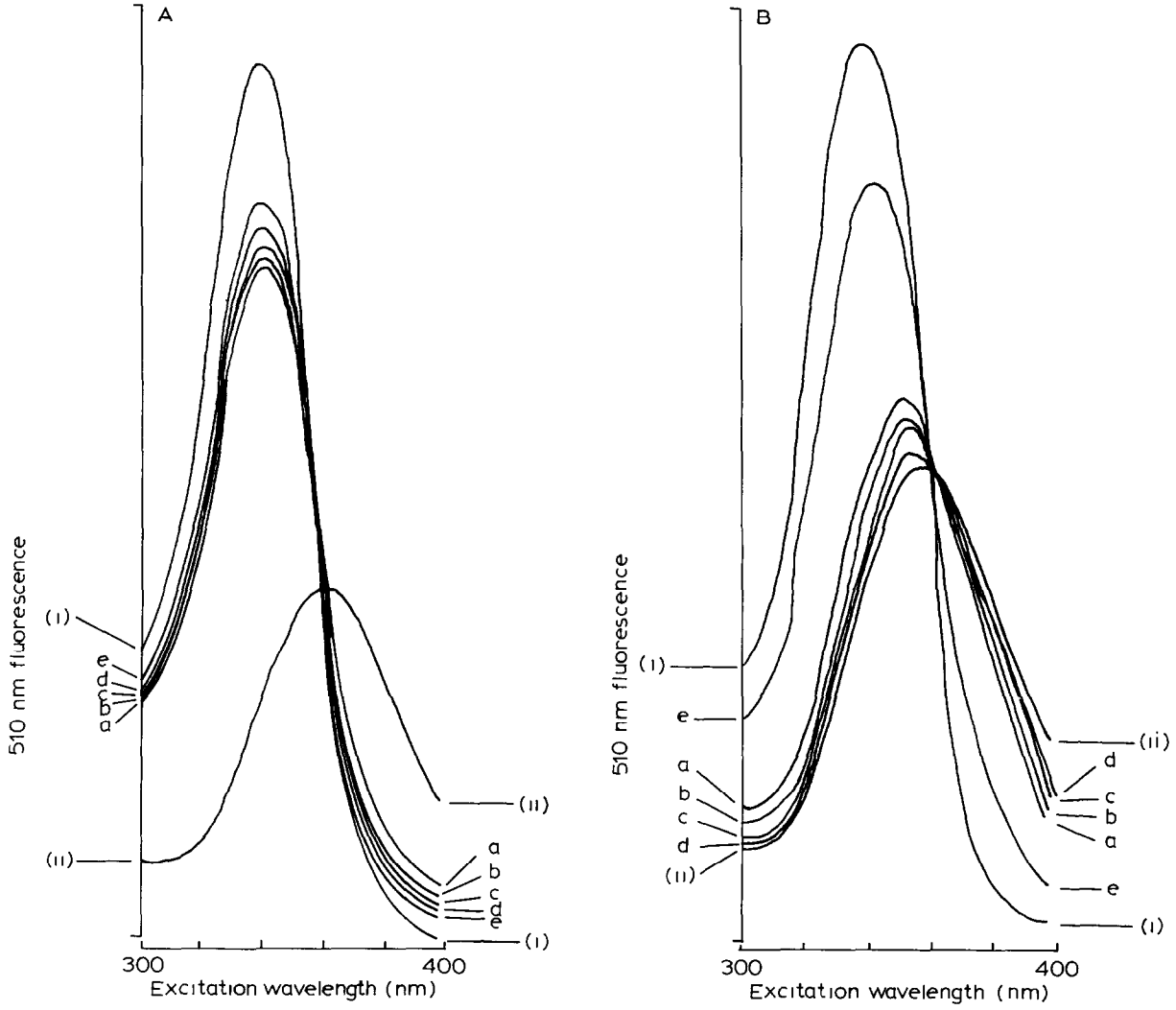

Fig.2. Effect of ionomycin on fura-2 loaded smc. (A) $[Ca_o^{2+}]$ = 1.0 mM. (a) Excitation spectrum of fura-2-loaded smc. (b) Addition of 400 nM ionomycin. (c,d) Successive recordings. (e) New, stable (> 10 min) level of $[Ca_i^{2+}]$. (B) $[Ca_o^{2+}]$ < 80 nM (total extracellular $[Ca^{2+}]$ = 1.0 mM, plus 2.5 mM EGTA). (a) Spectrum of smc equilibrated for 15 min at 37°C at low $[Ca_o^{2+}]$. (b) Addition of 400 nM ionomycin. (c,d) Successive recordings. (e) Restoration of $[Ca_o^{2+}]$ by addition of 6 mM $CaCl_2$. Cells equilibrated for 10 min. In both A and B: (i) Addition to (e) of 50 μM digitonin (5 mM solution in 60% ethanol). (ii) Lysed cells in 10 mM EGTA, pH 8.3 (in A) or 30 mM EGTA, pH 8.3 (in B).

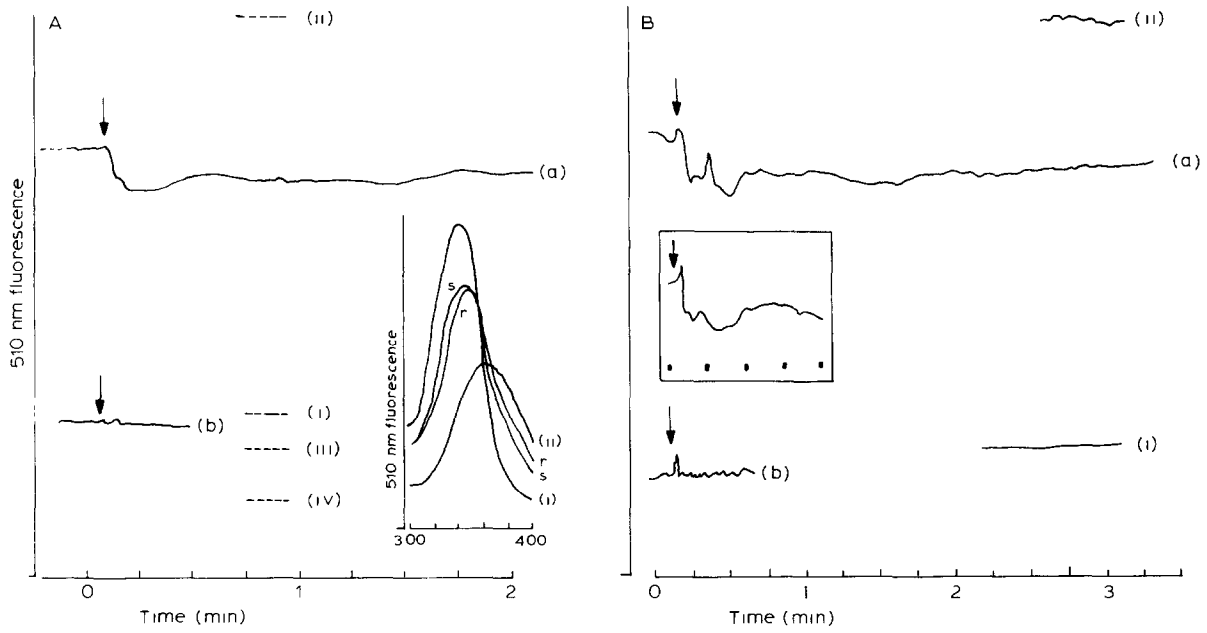

Fig.3. Responses of fura-2 loaded smc to elevated $[K_o^+]$ or to 100 μM carbachol. (A) (a) Response of loaded smc to carbachol (arrow). (b) Non-loaded smc. Both traces recorded at fixed 380 nm excitation wavelength. (i) Lysis with 50 μM digitonin. (ii) Addition to lysed cells of 10 mM EGTA, pH 8.3. (iii) Autofluorescence of non-loaded cells. (iv) Background fluorescence from 1% BSA saline. Inset: excitation spectra of resting (r) and stimulated (s) fura-2-loaded smc. (B) Response to high $[K_o^+]$. (a) Effect of 50 mM K_o^+ on fura-2-loaded smc. Inset: response of a separate preparation of smc. (b) Response of non-loaded cells. Traces recorded at fixed 380 nm excitation. (i,ii) As in A. Continuous recordings during stimulation were obtained by injecting carbachol or KCl into the cuvette via a tube, followed immediately by 50 μl air; the mixing artefact was negligible.

Table 1

Effects of reduced plasmalemmal $[Na_o^+]/[Na_i^+]$ gradient on $[Ca_i^{2+}]$ of fura-2-loaded smooth muscle cells

	Normal $[Na_o^+]$ = 130 mM				Low $[Na_o^+]$ < 10 mM
	Normal $[Ca_o^{2+}]$ = 2.5 mM		Low $[Ca_o^{2+}]$ < $[Ca_i^{2+}]$		Normal $[Ca_o^{2+}]$ = 2.5 mM (n = 7)
	Controls (n = 13)	100 μM ouabain (n = 8)	Controls (n = 4)	100 μM ouabain (n = 3)	
$[Ca_i^{2+}]$ (nM)	107 ± 26	305 ± 170[a]	74 ± 12	72 ± 14	256 ± 44[b]

[a] Significantly different from normal $[Ca_o^{2+}]$ control; p = 0.01; paired t = 3.5
[b] Significantly different from normal $[Ca_o^{2+}]$ control; 0.002 > p > 0.001; paired t = 5.74

All $[Ca_i^{2+}]$ values have been corrected for the effects of background fluorescence due to extracellular fura-2; corrected $[Ca_i^{2+}]$ was independent of this component of the signal. The amount of extracellular fura-2 was unaffected by ouabain treatment (t = 0.9) or by low-Na^+ saline (t = 0.1)

3.4. *Effects of reduced $[Na_o^+]/[Na_i^+]$ gradient on $[Ca_i^{2+}]$ of fura-2-loaded smc (table 1)*

Treatment with 100 μM ouabain elevated $[Ca_i^{2+}]$ progressively over at least 20 min. After 15 min incubation, $[Ca_i^{2+}]$ of smc was elevated approx. 3-fold. Ouabain did not elevate $[Ca_i^{2+}]$ of smc suspended in saline with $[Ca_o^{2+}] < [Ca_i^{2+}]$. Reduction of $[Na_o^+]$ to below 10 mM elevated $[Ca_i^{2+}]$ more than 2-fold.

4. DISCUSSION

Isolated taenia coli smc maintain a resting $[Ca_i^{2+}]$ similar to values reported for striated muscle fibres [7,8], to aequorin results from vascular smooth muscle [5], and to our data on taenia smc loaded with quin2 [9]. As reported for single visceral smc [10] and for vascular smooth muscle strips [5], stimulation of smc by agonist (carbachol) or high K_o^+ depolarization rapidly elevated $[Ca_i^{2+}]$. In our system $[Ca_i^{2+}]$ remained high for at least 3 min, in the continued presence of the stimulus. The relative elevation of $[Ca_i^{2+}]$ is apparently less than 2-fold, in agreement with recent aequorin results [11].

Reuter et al. [1] proposed that a specific Na^+/Ca^{2+} exchange process was important in regulating smooth muscle $[Ca_i^{2+}]$. Inhibition by ouabain of the Na^+/K^+-ATPase, or replacement of Na_o^+ with an organic cation, would both be expected to reduce the plasmalemmal $[Na_o^+]/[Na_i^+]$ gradient. The observed elevation of $[Ca_i^{2+}]$ in either case suggests that the normal steep $[Na_o^+]/[Na_i^+]$ gradient contributes to the maintenance of low $[Ca_i^{2+}]$ in taenia smc, favouring Ca^{2+} extrusion in exchange for extracellular Na^+. Reduced Ca^{2+} extrusion apparently allows net Ca^{2+} influx, since $[Ca_i^{2+}]$ elevation by ouabain depended on the availability of extracellular Ca^{2+}. Taenia coli $[Na_i^+]$ is about 13 mM [13], so that a reduction of $[Na_o^+]$ below 10 mM in our experiments might be expected to favour Na_i^+/Ca_o^{2+} exchange. However, Na_i^+/Ca_o^{2+} exchange in squid axon is inhibited by Ca_i^{2+} buffers (e.g. quin2) [14], and may depend on $[Ca_i^{2+}]$ reaching micromolar levels. It is not clear, therefore, whether the 2–3-fold elevation of $[Ca_i^{2+}]$ observed in taenia smc involved a linked Na_i^+/Ca_o^{2+} exchange, i.e. a 'reverse' mode of operation of a specific Na^+/Ca^{2+} antiporter, or whether it was wholly due to inward leakage of extracellular Ca^{2+}, although there is a clear demonstration of the importance of the normal $[Na_o^+]/[Na_i^+]$ gradient, maintained by Na^+/K^+-ATPase activity, for the regulation of normal resting $[Ca_i^{2+}]$.

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