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SODIUM–CALCIUM EXCHANGE IN CULTURED BOVINE PULMONARY ARTERY ENDOTHELIAL CELLS

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SUMMARY

1. Intracellular free calcium ($[Ca^{2+}]_i$) was measured in cultured bovine pulmonary artery endothelial cell monolayers loaded with the fluorescent calcium indicator Fura-2.

2. Resting $[Ca^{2+}]_i$ was 112 ± 10 nM. Application of ouabain (20 μM) was without effect on $[Ca^{2+}]_i$ for periods of up to 1 h. Monensin (10 μM) increased resting $[Ca^{2+}]_i$ to 145 ± 32 nM over approximately 2 min. In the presence of ouabain (20 μM), 10 μM-monensin increased $[Ca^{2+}]_i$ to 146 ± 15 nM.

3. Removal of extracellular sodium was without effect in resting cells or cells exposed to ouabain alone. However, in the presence of monensin, replacement of extracellular Na^+ with Li^+ resulted in a prompt increase in $[Ca^{2+}]_i$ to a peak of 280 ± 37 nM, which then returned towards resting levels. When Na^+ was removed in the presence of both ouabain and monensin, $[Ca^{2+}]_i$ reached a peak of 585 ± 53 nM.

4. When extracellular Na^+ was replaced with K^+, to achieve simultaneous Na^+ removal and depolarization, $[Ca^{2+}]_i$ reached a peak of 568 ± 63 nM, compared with a peak of 462 ± 38 nM when Li^+ was used as a Na^+ substitute in paired experiments. The transient increase in $[Ca^{2+}]_i$ evoked by sodium removal peaked earlier when K^+ was used as the sodium substitute, showing that depolarization increased the rate of calcium influx into the cell when sodium was removed from the bathing medium.

5. Removal of extracellular K^+ had no effect on the low-Na^+-evoked increase in $[Ca^{2+}]_i$.

6. Returning extracellular Na^+ during the increase in $[Ca^{2+}]_i$ resulting from Na^+ removal increased the rate of return of $[Ca^{2+}]_i$ towards basal levels. In the absence of Na^+, $[Ca^{2+}]_i$ took 41 ± 5 s to decline from 400 to 200 nM, and this was reduced to 26 ± 6 s ($n = 4$, S.E.M.) when Na^+ was returned to the bathing solution.

7. These results indicate endothelial cells possess a voltage-dependent Na^+–Ca^{2+} exchange mechanism in the surface membrane. However, this mechanism does not appear to be of primary importance in the maintenance of resting $[Ca^{2+}]_i$ since cells were able to restore a low $[Ca^{2+}]_i$ in the absence of extracellular Na^+. The evidence

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for the existence of a Na^+–Ca^{2+} exchanger in the surface membrane of endothelial cells and the possibility that this mechanism may contribute to calcium entry and/or extrusion during agonist-evoked responses is discussed.

INTRODUCTION

Despite the importance of changes in intracellular calcium ($[Ca^{2+}]_i$) in mediating the release of endothelium-derived factor (EDRF) (Singer & Peach, 1982; Long & Stone, 1985; Luckhoff & Busse, 1986; Peach, Singer, Izzo & Loeb, 1987), the processes that are responsible for calcium homeostasis across the surface membrane of endothelial cells remain poorly understood. Agonist-evoked increases in $[Ca^{2+}]_i$ consist of two phases; the first being transient and which has been ascribed to the release of Ca^{2+} from intracellular stores and the second a maintained (Hallam & Pearson, 1986; Colden-Stanfield, Schilling, Ritchie, Eskin, Navarro & Kunze, 1987; Hallam, Jacob & Merritt, 1989) or oscillating plateau (Sage, Adams & van Breemen, 1989) that may arise from a net increase in Ca^{2+} influx across the surface membrane.

Long-term intracellular Ca^{2+} homeostasis is achieved by the balance of Ca^{2+} entry and extrusion mechanisms across the surface membrane. While short-term regulation can be supplied by intracellular stores, the calcium content of these stores is ultimately determined by the surface membrane. A calcium ATPase and Na^+–Ca^{2+} exchange are the only mechanisms that have been described that can extrude Ca^{2+} across the surface membrane. In a recent study of endothelial cells, the voltage dependence of resting Ca^{2+} was shown to be consistent with a simple pump–leak model (Cannell & Sage, 1989). This study provided no evidence for the existence of a Na^+–Ca^{2+} exchange mechanism in the surface membrane. A similar conclusion was reached by Laskey & van Breemen (1989) who replaced Na^+ with K^+ and found that this manoeuvre (which would be expected to depolarize the cell) decreased $[Ca^{2+}]_i$. However, it has been reported that putative blockers of Na^+–Ca^{2+} exchange inhibit EDRF release (Winquist, Bunting & Schofield, 1985). While the selectivity of Na^+–Ca^{2+} exchange blockers is uncertain, this result was interpreted in terms of the blocker decreasing $[Ca^{2+}]_i$ by reducing Ca^{2+} influx via the exchanger. Since the activity of the exchanger is expected to be steeply dependent on $[Na^+]_i$, it is possible that changes in $[Ca^{2+}]_i$ due to the exchanger will not become apparent until $[Na^+]_i$ is sufficiently elevated. Agonist application may increase Na^+ influx via non-selective surface membrane channels (Johns, Lategan, Lodge, Ryan, van Breemen & Adams, 1987; Bregestovski, Bakhramov, Danilov, Moldobaeva & Takeda, 1988) and thereby increase $[Na^+]_i$. It has been suggested that such an increase in $[Na^+]_i$, coupled with a Na^+–Ca^{2+} exchanger, could contribute Ca^{2+} to the plateau phase of the agonist response (Cannell & Sage, 1989). While this mechanism is compatible with reported effects of Na^+–Ca^{2+} exchange blockers, Laskey & van Breemen (1989) found no evidence for a Na^+–Ca^{2+} exchange activity during the plateau of an agonist-evoked elevation in $[Ca^{2+}]_i$. In view of the controversy concerning the existence of a Na^+–Ca^{2+} exchanger in endothelial cells, we have investigated the effects of ionic substitutions on $[Ca^{2+}]_i$ measured using Fura-2. Conditions were chosen to optimize Ca^{2+} entry by a Na^+–Ca^{2+} exchange mechanism. A preliminary account of some of these results has been presented (Adams, van Breemen, Cannell & Sage, 1989).

METHODS

Procedures for measuring $[Ca^{2+}]_i$ in cultured endothelial cells have been described previously (Sage *et al.* 1989). Briefly, endothelial cells were obtained from a continuous cell culture line obtained from calf pulmonary artery without proteolytic digestion and cultured in Ryan Red medium as previously described (Ryan, 1984; Ryan & Maxwell, 1986). Eleventh or twelfth passage cells were harvested from flasks using a rubber policeman, seeded onto quartz cover-slips and grown to confluence (48–72 h).

Cover-slips were washed once in physiological saline (PSS) of composition (in mM): 135 NaCl, 5 KCl, 0·75 $MgCl_2$, 10 Na–HEPES, pH 7·4 at 22 °C and then the cells were loaded with Fura-2 by incubation with 0·5 μM-Fura-2/AM in PSS for 30 min at 37 °C. Cover-slips were then stored in PSS at room temperature until use (< 1 h after loading). This loading procedure yielded cells with a relatively uniform cytosolic distribution of Fura-2 as assessed by epifluorescence microscopy (Nikon Diaphot microscope fitted with a Zeiss × 100 Neofluor oil immersion objective, excitation wavelength 360 nm).

Fura-2 fluorescence at 505 nm was recorded with a Spex Flurolog 1681 spectrofluorimeter with excitation wavelengths of 340 and 380 nm. Autofluorescence from each cover-slip was measured prior to loading the cells with Fura-2. This autofluorescence (at 340 and 380 nm) was subtracted from the fluorescence records to allow calculation of $[Ca^{2+}]_i$ after determination of R_{min} and R_{max} at the end of each experiment. Data were acquired by personal computer and the 340/380 ratio converted to $[Ca^{2+}]_i$ as previously described (Sage *et al.* 1989). The cuvette inside the spectrofluorimeter was modified to allow the saline to be changed without the need for removal or drainage, thereby minimizing artifacts in the fluorescence record.

Sodium substitution was achieved by equimolar replacement of NaCl by LiCl or KCl. Ouabain was diluted from a 20 mM stock solution in 140 mM-NaCl and monensin from a 10 mM stock in ethanol. When repetitive low-sodium manoeuvres were carried out, the preparation was exposed to monensin and ouabain in control PSS for 5 min between challenges.

Ouabain and monensin were obtained from Sigma (St Louis, MO, USA), and Fura-2 acetoxymethyl ester was from Molecular Probes (Eugene, OR, USA). All other chemicals were reagent grade.

RESULTS

It is well known that inhibition of the Na^+–K^+-ATPase by cardiotonic steroids increases $[Na^+]_i$ in most cell types. In cardiac muscle, squid axon and barnacle muscle, sodium pump inhibition increases $[Ca^{2+}]_i$, an effect that is believed to be mediated by Na^+–Ca^{2+} exchange (for review see Blaustein, 1984). The increase in $[Na^+]_i$ promotes Na^+ extrusion, and therefore calcium entry, as well as inhibiting Na^+ entry and calcium extrusion by the exchanger. Thus, irrespective of the direction of operation of the exchanger, an increase in $[Na^+]_i$ should increase net calcium influx into the cell if a Na^+–Ca^{2+} exchange mechanism is present.

Figure 1*A* shows that application of 20 μM-ouabain was without effect on $[Ca^{2+}]_i$. Similar results were observed in six preparations and no changes in $[Ca^{2+}]_i$ were observed for exposures to ouabain lasting up to 1 h (data not shown). The lack of effect of ouabain cannot be attributed to the absence of Na^+–K^+-ATPase as there is evidence for an active Na^+ pump from both flux (Nakagawa, Takamatsu, Toyoda, Sawada, Tsuji & Iyichi, 1987) and electrophysiological (Daut, Merke, Nees & Newman, 1988) studies. Since no change in $[Ca^{2+}]_i$ was observed, the sodium gradient across the cell membrane was reversed by removing external Na^+. This should force a Na^+–Ca^{2+} exchanger into the Na^+ efflux–Ca^{2+} influx mode of operation and thereby increase $[Ca^{2+}]_i$ (Chapman, 1974; Allen, Eisner, Lab & Orchard, 1983). However, as can be seen in Fig. 1*A*, this manoeuvre was without effect.

An alternative method for increasing $[Na^+]_i$ is to apply monensin, a Na^+ ionophore. Figure 1*B* shows that application of 10 μM-monensin resulted in an increase in $[Ca^{2+}]_i$ which reached a peak of 145 ± 32 nM from a basal level of 108 ± 30 nM (S.E.M., $n = 4$) in approximately 2 min. After application of monensin,

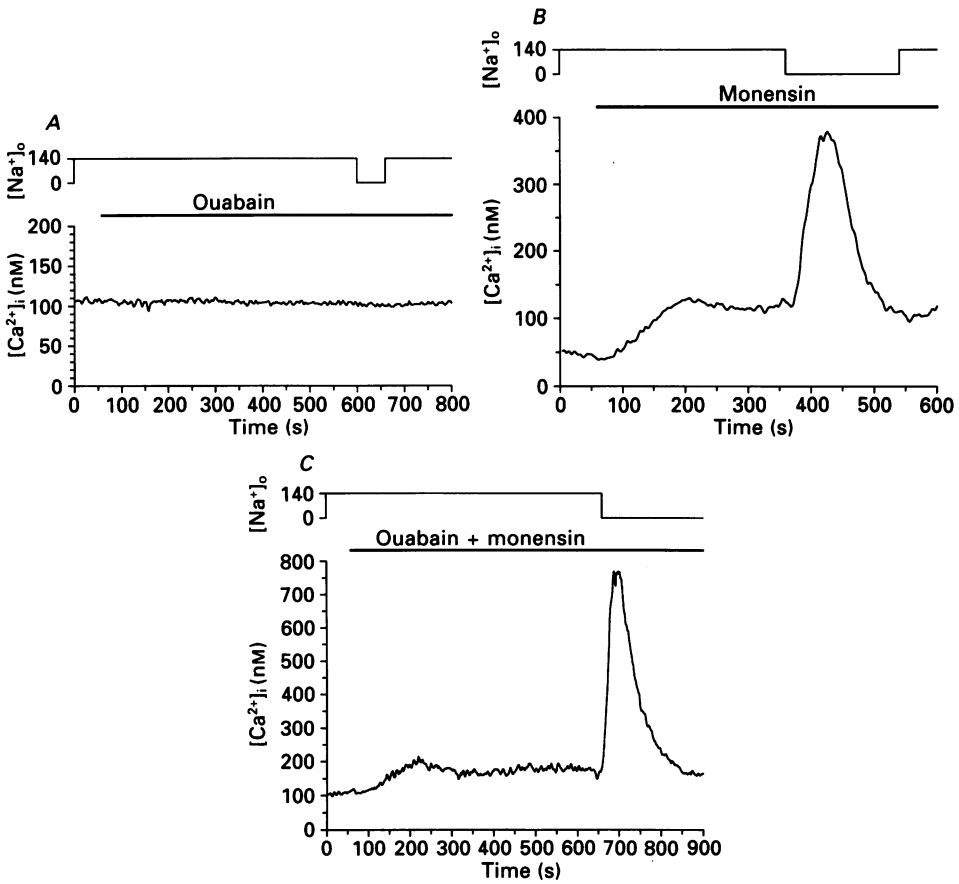

Fig. 1. Effects of ouabain and monensin on resting $[Ca^{2+}]_i$ in Fura-2-loaded bovine pulmonary artery endothelial cell monolayers. Ouabain and/or monensin were simultaneously applied as indicated by the horizontal bar in each panel. In each panel, external sodium was isosmotically replaced with Li^+ at the time shown by the upper trace. *A*, application of ouabain (10 μM) was without effect on $[Ca^{2+}]_i$. Subsequent sodium removal had no effect in the presence of ouabain also. *B*, effect on monensin (10 μM) on $[Ca^{2+}]_i$. Removal of external sodium in these conditions resulted in a transient increase in $[Ca^{2+}]_i$. *C*, combined effect of ouabain (20 μM) and monensin (10 μM) on $[Ca^{2+}]_i$.

removal of extracellular Na^+ resulted in an increase in $[Ca^{2+}]_i$ to a peak of 280 ± 37 nM (S.E.M., $n = 4$) after approximately 70 s. $[Ca^{2+}]_i$ then declined with a half-time of 38 ± 6 s to a level similar to that observed prior to the manoeuvre. This result is consistent with the presence of an internal sodium-dependent calcium entry pathway such as Na^+–Ca^{2+} exchange. The decrease in $[Ca^{2+}]_i$ during the period of exposure to

the zero Na^+ solution has also been observed in cardiac muscle (Chapman, 1974; Allen *et al.* 1983) and suggests that there must be a Na^+-independent calcium extrusion mechanism (such as a calcium ATPase) in the surface membrane of the cell. In addition, it is likely that Na^+ leaves the cell during the exposure to the zero Na^+ solution and this will lead to a loss of calcium entry via the exchanger.

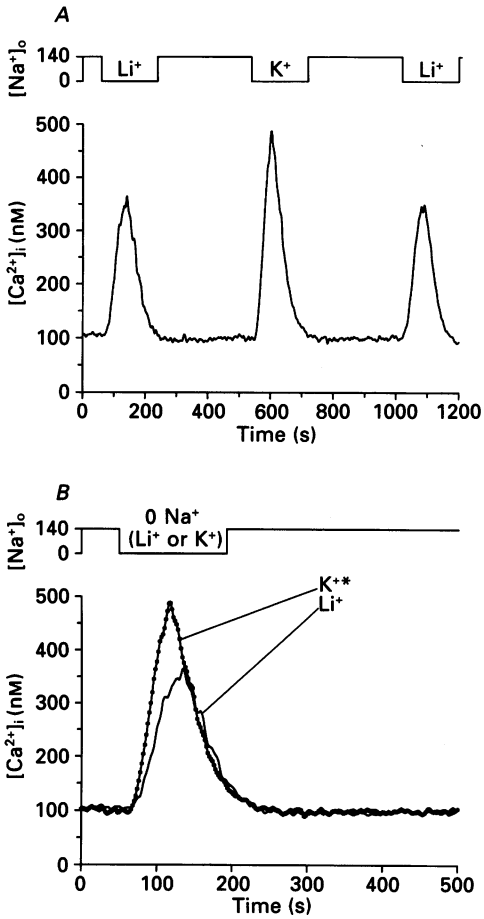

Fig. 2. Effect of depolarization on the rise in $[Ca^{2+}]_i$ evoked by Na^+ removal. The Fura-2-loaded monolayer was pre-treated with ouabain (20 μM) and monensin (10 μM). *A*, extracellular Na^+ was isosmotically replaced with Li^+ or K^+ as indicated by the upper trace to achieve simultaneous Na^+ removal and depolarization. Depolarization resulted in a greater increase in $[Ca^{2+}]_i$ when sodium was removed. *B*, superimposed records comparing the time course of the rise in $[Ca^{2+}]_i$ evoked by Li^+ (curve with no symbols) or K^+(*).

Application of monensin (10 μM) together with ouabain (20 μM) (Fig. 1*C*) resulted in an elevation in $[Ca^{2+}]_i$ which was qualitatively similar to that observed after application of monensin alone (Fig. 1*B*). The elevation in $[Ca^{2+}]_i$ immediately after the application of monensin and ouabain was 48 ± 8 nM (S.E.M., $n = 8$). This increase

in $[Ca^{2+}]_i$ was slightly greater than observed in the presence of monensin alone. The removal of extracellular Na^+ after the application of monensin and ouabain resulted in an increase in $[Ca^{2+}]_i$ to a peak of 585 ± 53 mM (S.E.M., $n = 24$) which was significantly greater than after application of monensin alone (Student's t test,

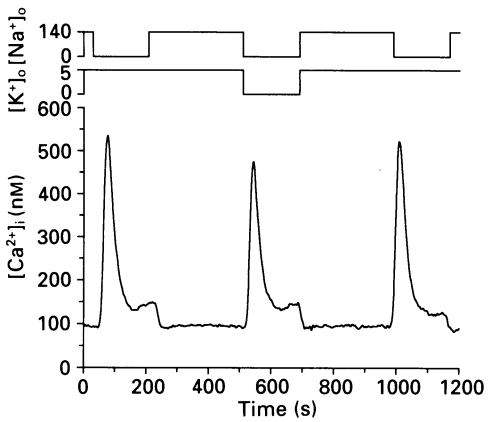

Fig. 3. Removal of extracellular K^+ does not inhibit the increase in $[Ca^{2+}]_i$ evoked by Na^+ removal. The monolayer was pre-treated as described for Fig. 2. Extracellular Na^+ and K^+ were isosmotically replaced with Li^+ as indicated by the upper traces. The absence of potassium did not abolish the transient increase in $[Ca^{2+}]_i$ resulting from sodium removal.

$0{\cdot}02 > 0{\cdot}02\ P > 0{\cdot}01$). Since the Na^+–K^+-ATPase should oppose the increase in $[Na^+]_i$ induced by monensin, it is likely that the $[Na^+]_i$ would be higher in these conditions than when monensin alone was applied. Thus the increased resting level of $[Ca^{2+}]_i$ and the greater amplitude of the $[Ca^{2+}]_i$ transient resulting from the low-Na^+ exposure can both be explained by a greater $[Na^+]_i$ stimulation of calcium entry by a Na^+–Ca^{2+} exchange mechanism.

If this internal Na^+-dependent calcium influx pathway reflects an electrogenic Na^+–Ca^{2+} exchange mechanism, then membrane depolarization should promote Ca^{2+} influx. To achieve simultaneous depolarization and Na^+ removal, extracellular Na^+ was replaced with K^+ (instead of Li^+). Figure 2 shows that, in the presence of ouabain and monensin, replacement of Na^+ with K^+ resulted in a greater elevation in $[Ca^{2+}]_i$ than when Na^+ was replaced with Li^+. In nine such paired experiments, the peak $[Ca^{2+}]_i$ attained was 568 ± 63 nM in K^+ compared with 462 ± 38 nM (S.E.M.) in Li^+. Depolarization of endothelial cells in the absence of an elevated $[Na^+]_i$ decreases $[Ca^{2+}]_i$ (Cannell & Sage, 1989; Laskey, Adams, Johns, Rubanyi & van Breemen, 1990), an observation quite unlike that shown in Fig. 2. However, depolarization will favour calcium entry via a Na^+–Ca^{2+} exchanger, especially when $[Na^+]_i$ is increased. As shown in Fig. 2*B*, the peak of the transient increase in $[Ca^{2+}]_i$ evoked by sodium removal occurred earlier when K^+ was used as the sodium substitute, which was due to an increase in the maximum rate of rise of $[Ca^{2+}]_i$. The maximum rate of rise of $[Ca^{2+}]_i$ was 10·5 nM/s in the presence of 140 mM-K^+, compared to 6·5 nM/s when Li^+ was used as the sodium substitute. These results are therefore consistent with the

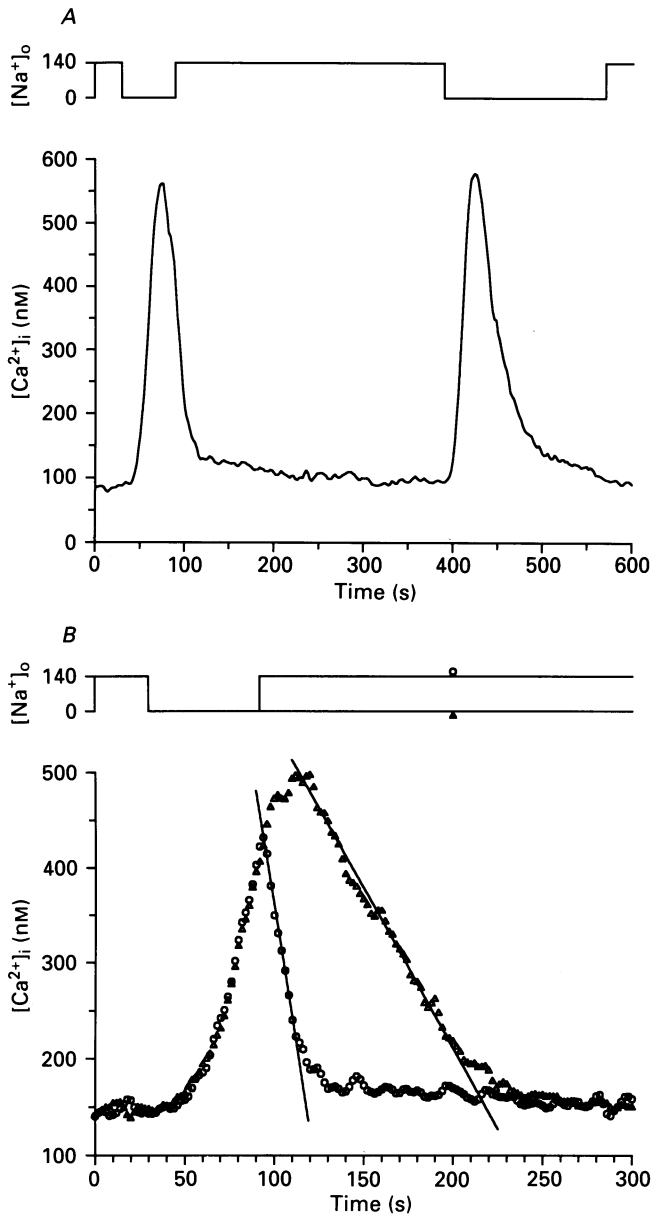

Fig. 4. *A*, restoration of extracellular Na^+ abbreviates the increase in $[Ca^{2+}]_i$ resulting from Na^+ removal. Conditions as described for Fig. 2. *B*, superimposed traces showing the effect of readdition of Na^+ during the increase in $[Ca^{2+}]_i$ evoked by Na^+ removal.

presence of an electrogenic Na^+–Ca^{2+} exchange mechanism in the surface membrane. There was also a slight increase in the rate of decline of $[Ca^{2+}]_i$ when K^+ was used as the sodium substitute, as might be expected from consideration of the effects of depolarization on sodium efflux via Na^+–Ca^{2+} exchange and leak pathways for

sodium and calcium. However, a more direct interpretation of the time course of the decline of $[Ca^{2+}]_i$ is not possible without further information on the time course of the loss of internal sodium (see below).

It has recently been reported that in salamander rod outer segments Na^+–Ca^{2+} exchange is inhibited by removal of extracellular K^+ (Cervetto, Lagnado, Perry, Robinson & McNaughton, 1989). Figure 3 shows simultaneous removal of Na^+ and K^+ did not abolish the transient increase in $[Ca^{2+}]_i$. In thirteen paired experiments, peak $[Ca^{2+}]_i$ in the presence of K^+ was 538 ± 18 nM compared with 510 ± 26 nM (S.E.M.) in the absence of K^+. The difference was not significant (Student's t test, $P \gg 0{\cdot}05$). This result suggests that the endothelial cell Na^+–Ca^{2+} exchanger is unlike that of the rod outer segment and is more like that of cardiac muscle, which also operates in the absence of extracellular K^+ (Crespo, Grantham & Cannell, 1990; Yasui & Kimura, 1990). Another feature visible in Fig. 3 was that $[Ca^{2+}]_i$ was increased throughout the period of exposure to zero Na^+ solution. While this result was seen in only seven of twenty-one experiments, it suggests that Na^+–Ca^{2+} exchange can also extrude Ca^{2+} from the cell, as expected from studies on other tissues (Blaustein, 1984).

The transient increase in $[Ca^{2+}]_i$ that resulted from sodium removal could be reversed by returning sodium to the bathing solution. As shown in Fig. 4, returning sodium to the bathing solution during the $[Ca^{2+}]_i$ transient resulted in a prompt decline in $[Ca^{2+}]_i$. The rate of decline of $[Ca^{2+}]_i$ in the absence of sodium was 3·4 nM/s, and this rate was increased to 11·9 nM/s by returning sodium to the bathing solution. In four experiments, the time taken for $[Ca^{2+}]_i$ to decline from 400 to 200 nM was 41 ± 5 s (S.E.M.) in the absence of sodium, compared to 26 ± 6 s when sodium was reintroduced to the bathing solution. Interpretation of the rate of decline of $[Ca^{2+}]_i$ is complicated by the possibility that not all the internal sodium is lost at the peak of the $[Ca^{2+}]_i$ transient. If this were the case, then the rate of decline will reflect a large Ca^{2+}-ATPase-mediated calcium efflux in parallel with a smaller calcium influx via the Na^+–Ca^{2+} exchange (which declines to zero in the steady state). The increased rate of decline in $[Ca^{2+}]_i$ observed in the presence of external sodium is then explainable by the reversal of the direction of calcium transport by the exchanger, so that calcium efflux is mediated by both a Ca^{2+}-ATPase and the Na^+–Ca^{2+} exchange.

DISCUSSION

We have demonstrated that an increase in $[Ca^{2+}]_i$ can be evoked by removal of external sodium when $[Na^+]_i$ is sufficiently elevated. A previous study, in which increases in $[Ca^{2+}]_i$ in response to external sodium replacement were not observed, concluded that there was no Na^+–Ca^{2+} exchange mechanism present in these cells (Laskey & van Breemen, 1989). In contrast, in another study (Winquist *et al.* 1985), a putative Na^+–Ca^{2+} exchange blocker was found to inhibit endothelium-dependent relaxation. Our studies provide a reconciliation of these disparate data, since we have shown that an elevation of $[Na^+]_i$ is required to disclose the presence of the Na^+–Ca^{2+} exchanger in endothelial cells.

A novel result of the present study was the finding that a high concentration of ouabain (20 μM) was without effect on $[Ca^{2+}]_i$. It is unlikely that the lack of effect of

ouabain was due to the absence of Na^+–K^+-ATPase since both flux measurements using radioactive tracers (Nakagawa *et al.* 1987) and electrophysiological measurements (Daut *et al.* 1988) support the existence of a ouabain-inhibitable Na^+–K^+-ATPase. While the lack of effect of ouabain on $[Ca^{2+}]_i$ might be taken as evidence *against* the presence of Na^+–Ca^{2+} exchange in these cells, it is possible that the expected stimulation of calcium entry (or inhibition of calcium extrusion) by the Na^+–Ca^{2+} exchange is offset by changes in membrane potential. More specifically, the inhibition of Na^+ pump current will lead to depolarization of the cell (Daut *et al.* 1988) which will lead to a decrease in calcium entry and should decrease $[Ca^{2+}]_i$ (Cannell & Sage, 1989). This effect may be offset by the increase in $[Na^+]_i$ which results from Na^+–K^+-ATPase inhibition which will promote calcium entry (or inhibit calcium extrusion) via the Na^+–Ca^{2+} exchange (Chapman, 1974; Allen *et al.* 1983), the combination of these effects leading to little or no change in $[Ca^{2+}]_i$. A similar combination of effects has been noted in cardiac muscle. Depolarization (which should promote calcium entry via the exchanger) does not increase $[Ca^{2+}]_i$ unless $[Na^+]_i$ is increased (Eisner, Lederer & Vaughan-Jones, 1983) and when the exchanger is blocked, depolarization decreases $[Ca^{2+}]_i$ (Cannell, Eisner, Lederer & Valdeolmillos, 1986). In squid axon, depolarization also decreases $[Ca^{2+}]_i$ only when $[Na^+]_i$ is low (Requena, Mullins, Whittembury & Brinley, 1986). In human platelets, $[Ca^{2+}]_i$ is not increased by the application of ouabain (Schaeffer & Blaustein, 1989), and the lack of effect of ouabain or reducing external sodium on $[Ca^{2+}]_i$ has led to controversy over the existence of Na^+–Ca^{2+} exchange in these cells also (Brass, 1984; Sage & Rink, 1986; Zimlichman, Goldstein, Zimlichman & Keister, 1987). However, sodium removal in platelets (in the presence of ouabain) increases $[Ca^{2+}]_i$ and this has been taken as direct evidence for Na^+–Ca^{2+} exchange in these cells (Schaeffer & Blaustein, 1989). With these results in mind, we conclude that the inability of cardiotonic steroids to increase $[Ca^{2+}]_i$ cannot be used as evidence against the presence of a Na^+–Ca^{2+} exchanger in the surface membrane.

In our experiments, removal of external sodium in the presence of ouabain did not increase $[Ca^{2+}]_i$, an observation which might also, in the view of the above discussion, be taken as evidence against the presence of Na^+–Ca^{2+} exchange. However, it is important to note that the activity of the exchanger is critically dependent on both $[Ca^{2+}]_i$ and $[Na^+]_i$, so that removal of external sodium may not disclose the presence of the exchanger if $[Ca^{2+}]_i$ and $[Na^+]_i$ are kept low by other mechanisms (see below). To ensure an increase in $[Na^+]_i$ we applied monensin, which resulted in an increase in $[Ca^{2+}]_i$. Under these conditions, the changes in $[Ca^{2+}]_i$ in response to the removal of external sodium and the elevated basal $[Ca^{2+}]_i$ were consistent with the presence of a voltage-dependent Na^+–Ca^{2+} exchange mechanism in the surface membrane. However, the increase of $[Ca^{2+}]_i$ was of limited extent, even when monensin and ouabain were applied together and relaxed towards baseline levels, so that there must be an additional mechanism for extruding calcium from the cytoplasm. A similar result has been observed in cardiac muscle, where the increase in $[Ca^{2+}]_i$ in response to removal of external sodium also relaxes towards baseline levels (Chapman, 1974; Allen *et al.* 1983). In our experiments, however, the relaxation of $[Ca^{2+}]_i$ was nearly always complete whereas in cardiac muscle $[Ca^{2+}]_i$ does not return to baseline levels. In cardiac muscle, the regulation of $[Ca^{2+}]_i$ in the absence of

Na^+–Ca^{2+} exchange is thought to be mediated by a sarcolemmal Ca^{2+}-ATPase (Chapman, 1974; Caroni & Carafoli 1981, 1983; Allen *et al.* 1983). We therefore infer that the relaxation of $[Ca^{2+}]_i$ is more complete in endothelium than cardiac muscle because the relative contribution of the Ca^{2+}-ATPase to calcium homeostasis across the surface membrane is much greater in endothelium than in cardiac muscle. If this is the case, then the inability of ouabain to increase $[Ca^{2+}]_i$ via the exchanger may be explained by the Ca^{2+}-ATPase extruding any calcium brought into the cell by Na^+–Ca^{2+} exchange (as well as the effects of a ouabain-induced depolarization; see above). If the resting sodium influx is small, then this mechanism would prevent large increases in $[Na^+]_i$ during sodium pump inhibition also, and may explain why removing sodium had no effect in these conditions. In support of this idea, Nakagawa *et al.* (1987) found that ouabain caused a slight increase in ^{45}Ca influx and decrease in efflux, which they attributed to a Na^+–Ca^{2+} exchanger. If these changes are due to Na^+–Ca^{2+} exchange, then the ^{45}Ca fluxes would be associated with net sodium efflux via the exchanger and would then help compensate for the loss of Na^+–K^+-ATPase activity. In addition, there is a Na^+–K^+–Cl-co-transport mechanism in endothelial cells (Brock, Brugnara, Canessa & Gimbrone, 1986; O'Donnell, 1989) which may help limit the increase in intracellular Na^+ in the presence of ouabain. Under resting conditions, up to 70% of Na^+ influx may be extruded by the bumetanine-sensitive Na^+–K^+–Cl-co-transporter (O'Donnell, 1989) so that the inhibition of the Na^+–K^+-ATPase may not result in a large increase in $[Na^+]_i$. Without an increase in $[Na^+]_i$, the Na^+–Ca^{2+} exchanger may be unable to transport calcium into the cell at a high enough rate to overcome other calcium buffering systems (as discussed above). However, further study is required to clarify this point.

In other tissues, an increase in $[Ca^{2+}]_i$ stimulates both calcium influx and calcium efflux by the exchanger (Blaustein, 1984). If this is also the case for endothelial cells, then the agonist-evoked release of calcium from intracellular stores will stimulate the exchanger, and thereby make the exchanger more sensitive to changes in $[Ca^{2+}]_i$ and $[Na^+]_i$. It is therefore possible that the role of the Na^+–Ca^{2+} exchanger may become more prominent during agonist-induced increases in $[Ca^{2+}]_i$, especially if the agonist also increases $[Na^+]_i$. These considerations may explain the reported inhibition of agonist-evoked smooth muscle relaxation by putative Na^+–Ca^{2+} exchange blockers (Winquist *et al.* 1985) even though the activity of the exchanger is not evident under resting conditions.

However, it has recently been reported that depolarization during the plateau of the agonist-evoked response decreases $[Ca^{2+}]_i$ (Laskey *et al.* 1990). Nevertheless, depolarization of resting cells decreases $[Ca^{2+}]_i$ (Cannell & Sage, 1989), whereas $[Ca^{2+}]_i$ was still elevated with respect to basal levels when the cells were depolarized in the presence of bradykinin (Laskey *et al.* 1990). Thus these results do not exclude a possible contribution of a Na^+–Ca^{2+} exchange mechanism to agonist-evoked responses. Since the cell is able to regulate $[Ca^{2+}]_i$ in the absence of Na^+–Ca^{2+} exchange, any contribution of the exchanger to agonist-evoked increases in $[Ca^{2+}]_i$ may not be large. It will be important to determine the extent to which the agonist in question elevates cytosolic $[Na^+]$ before rejecting any possible contribution.

The inability of ouabain to alter $[Ca^{2+}]_i$ in these cells may be of some clinical importance since cardiotonic steroids are widely used as cardiac inotropic agents.

Since EDRF production may be (at least partly) regulated by $[Ca^{2+}]_i$, the finding that $[Ca^{2+}]_i$ does not change in the presence of ouabain suggests that EDRF release from the endothelium will not change during the administration of such compounds. Our data also emphasize that the sensitivity of calcium-regulated cellular processes to cardiotonic steroids will vary, depending on the relative importance of the Na^+–Ca^{2+} exchange in regulating $[Ca^{2+}]_i$ in different tissues, and that the levels of $[Ca^{2+}]_i$ and $[Na^+]_i$ will be critically important in determining this contribution.

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