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EFFECT OF SODIUM–POTASSIUM PUMP INHIBITION AND LOW SODIUM ON MEMBRANE POTENTIAL IN CULTURED EMBRYONIC CHICK HEART CELLS

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SUMMARY

1. When the Na^+–K^+ pump of cultured embryonic chick heart cells was inhibited by addition of ouabain with or without removal of external K^+, the membrane potential rapidly depolarized to −40 mV and the Na^+ content approximately doubled within 3 min.

2. After this, exposure to an $[Na^+]_o$ of 27 mM caused a fall in Na^+ content, a gain in Ca^{2+} content and a hyperpolarization. The hyperpolarization was ~ 25 mV in a $[K^+]_o$ of 0 or 5·4 mM after 3 min of pump inhibition. After ~ 10 min of pump inhibition, the same hyperpolarization was observed in a $[K^+]_o$ of 5·4 mM but in K^+-free solution the hyperpolarization increased to ~ 44 mV.

3. Varying $[K^+]_o$ during the 10 min period of Na^+–K^+ pump inhibition showed that the increase in hyperpolarization was associated with the period of exposure to K^+-free solution rather than the $[K^+]_o$ at the time of lowering $[Na^+]_o$.

4. Changes in Na^+ and Ca^{2+} content induced by exposure to an $[Na^+]_o$ of 27 mM in K^+-free solution were similar at 3 and 10 min. This and the above observations suggest that the increased hyperpolarization was due to an increased membrane resistance.

5. 10 mM-Cs^+ reduced the low-$[Na^+]_o$ hyperpolarization by 26% but did not significantly affect the movements of Na^+ and Ca^{2+}. 1 mM-La^{3+} reduced the low-$[Na^+]_o$ hyperpolarization by 15%: it also totally blocked the rise in Ca^{2+} content and partially blocked the fall in Na^+ content. 1 mM-Ba^{2+} reduced the low-$[Na^+]_o$ hyperpolarization by 20%.

6. Raising $[Ca^{2+}]_o$ from 2·7 to 13·5 mM produced similar but smaller hyperpolarizations (~ 6 mV after 3 min pump inhibition). High $[Ca^{2+}]_o$ caused a rise in Ca^{2+} content but no significant drop in Na^+ content. The hyperpolarization in high $[Ca^{2+}]_o$ was insensitive to verapamil (20 μM) and 10 mM-Cs^+.

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7. We conclude from the disparities between the magnitudes of the hyperpolarizations and the changes in ion contents that Na^+–Ca^{2+} exchange cannot be unequivocally identified as electrogenic solely from the low-$[Na^+]_o$ hyperpolarizations.

INTRODUCTION

The question of whether or not Na^+–Ca^{2+} exchange is electrogenic in cardiac muscle has several important consequences for the functioning of the exchange. First, an electrogenic exchange can contribute to the transmembrane current flow and hence the action potential, perhaps even leading to oscillatory behaviour (Fischmeister & Vassort, 1981): a role for Na^+–Ca^{2+} exchange currents has been reviewed recently by Noble (1984). The second consequence is that, thermodynamically, the direction in which an electrogenic exchange runs is dependent on membrane potential (E_m) so that the direction of the exchange could vary during the cardiac cycle. Na^+–Ca^{2+} exchange could therefore be involved in both the import and export of Ca^{2+} across the sarcolemma; whether or not the exchange contributes significantly to either of these processes is determined by its kinetic properties. The third consequence is an indirect one: if the exchange were electroneutral then its stoicheiometry would be 2:1 and, thermodynamically, there would be insufficient potential in the transmembrane Na^+ electrochemical gradient for Na^+–Ca^{2+} exchange to be able to maintain the low diastolic level of cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$). Again, the same reservation applies here: even if the stoicheiometry of the exchange is greater than 2:1 the role of Na^+–Ca^{2+} exchange in maintaining the diastolic $[Ca^{2+}]_i$ still depends on its kinetic properties.

The electrogenicity of Na^+–Ca^{2+} exchange in heart muscle has proven difficult to establish. Eisner & Lederer (1985) in their recent review concluded that although much of the evidence is equivocal and indirect, it does suggest an electrogenic exchange. The strongest evidence comes from recent experiments on cardiac sarcolemmal vesicles and isolated myocytes. In cardiac sarcolemmal vesicles, Reeves & Hale (1984) measured a 3:1 stoicheiometry thereby implying electrogenicity. In isolated internally perfused cells, Kimura, Noma & Irisawa (1986) have measured a Na^+–Ca^{2+} exchange current: there have also been other reports of currents which appear to be due to Na^+–Ca^{2+} exchange (Uehara & Hume, 1985; Hume & Uehara, 1986; Mechmann & Pott, 1986). It is important, however, to confirm the electrogenic nature of Na^+–Ca^{2+} exchange by experiments with intact heart cells.

In this article we evaluate an approach for demonstrating electrogenic Na^+–Ca^{2+} exchange in intact cells. The approach is to show a change in E_m, or equivalently transmembrane current, associated with Na^+–Ca^{2+} exchange. Two extensive reports have appeared using this approach on preparations of intact cells: Coraboeuf, Gautier & Guiraudou (1981) measured hyperpolarizations associated with Na^+–Ca^{2+} exchange in dog Purkinje fibres and Mentrard, Vassort & Fischmeister (1984) measured transmembrane currents associated with Na^+–Ca^{2+} exchange in frog atrial trabeculae. In both cases the evidence linking the electrical changes to the exchange was somewhat circumstantial and neither investigation completely eliminated the possibility that the observed changes could have been due to passive ionic movements. Furthermore, Lederer, Sheu, Vaughan-Jones & Eisner (1984) have shown that

conductance changes are at least in part responsible for the current generated by exposure to low $[Na^+]_o$.

Although the experimental protocol is similar to that of Coraboeuf *et al.* (1981) we reach a different conclusion, namely that the hyperpolarizations induced by exposure to low $[Na^+]_o$ are at least in part due to uncoupled electrodiffusive ion movements so that we cannot conclude that Na^+–Ca^{2+} exchange is electrogenic. In the following article (Jacob, Lieberman & Liu, 1987) we show that under slightly different conditions it is possible to elicit a hyperpolarization that is unequivocally due to an electrogenic Na^+–Ca^{2+} exchange.

The properties of the cultured heart cell provide a good compromise between naturally occurring preparations and internally perfused isolated cells or vesicles. On the one hand they are in a natural physiological state particularly with respect to their intracellular constituents; on the other hand they have very little extracellular diffusion delay. Consequently, Na^+–Ca^{2+} exchange can be rapidly stimulated and Na^+ and Ca^{2+} content changes can be followed with relative ease. An active Na^+–Ca^{2+} exchange has been reported to exist in these cells (e.g. Wakabayashi & Goshima, 1981; Barry & Smith, 1982; Murphy, Wheeler, LeFurgey, Jacob, Lobaugh & Lieberman, 1986).

Preliminary reports of this work have appeared in abstract form (Jacob, Murphy & Lieberman, 1982, 1983).

METHODS

Cell culture

Cell suspensions were obtained by disaggregation of thirty 11-day-old embryonic chick hearts. Finely minced heart was serially exposed to 0·05% trypsin in Ca^{2+}- and Mg^{2+}-free Hank's salt solution with disaggregation being aided by gentle trituration and agitation. The first exposure was a brief wash with cold trypsin solution; subsequent exposures were for 10 min at 37 °C. After each exposure, the entire disaggregating solution was aspirated and fresh solution added. Cells released during the cold wash and the first three exposures to warm trypsin were discarded. Cells released during the following four exposures were collected by adding the disaggregating solution to an equal volume of ice-cold R medium (see below) to deactivate the trypsin. The resulting cell suspensions were combined, filtered through a double layer of 60 μm mesh Nitex cloth and centrifuged at 140 *g* for 6 min. The supernatant was aspirated and the cells resuspended in a small volume of ice-cold R medium.

Before culture, fibroblast contamination was reduced by a preferential attachment method (Blondel, Roijen & Chevenal, 1971). Ten plastic culture dishes (100 mm diameter) were set up with warm F medium (see below) and the cell suspension was divided equally between these dishes and incubated for 1 h. After aspirating the cell suspension, the dishes were washed once with cold R medium. Cells from the aspiration and the wash were combined, centrifuged at 140 *g* for 6 min, resuspended in a small volume of F medium, counted with a haemocytometer (using trypan blue exclusion to assess cell viability) and then diluted to give a suspension of 1×10^7 cells ml^{-1}. The yield from thirty hearts was $\sim 8 \times 10^7$ viable cells.

Cells were cultured either conventionally as a confluent layer or as a polystrand (Horres, Lieberman & Purdy, 1977). For confluent layer cultures, $1{\cdot}5 \times 10^6$ cells per 35 mm culture dish were incubated for 3 or 4 days in F medium. The polystrand consists of cells grown for 3 days in F medium and 1–2 days in R medium on a 20 μm diameter nylon monofilament which is itself wound thirty-five times around a U-shaped silver-wire clip: the two arms of the U are 35 mm long and 10 mm apart. The cells grow around the nylon to form seventy spontaneously beating sheaths, each up to 5 mm in length and with an outer strand diameter typically between 80 and 120 μm.

Solutions

Bicarbonate-buffered experimental solutions. The control solution was a modified Earle's balanced salt solution (MEBSS) containing (in mM): Na^+, 145; K^+, 5·4; Mg^{2+}, 0·8; Ca^{2+}, 2·7; Cl^-, 129; HCO_3^-, 26; $H_2PO_4^-$, 0·8; SO_4^{2-}, 0·8; dextrose, 5; bovine serum albumin, 1·4 g l^{-1}. Solutions with a $[K^+]_o$ of 0 and 0·54 mM were made by equimolar substitution of K^+ by Na^+. Low-$[Na^+]_o$ solutions were made by equimolar substitution of Na^+ by tetramethylammonium (TMA). A solution with a $[Ca^{2+}]_o$ of 13·5 mM was made by addition of $CaCl_2$; the HCO_3^- concentration was lowered to 15 mM. The pH was adjusted to 7·40 ± 0·05.

HEPES–Tris-buffered experimental solutions. K^+-free solution contained (in mM): Na^+, 124; Mg^{2+}, 0·8; Ca^{2+}, 2·7; Cl^-, 129; $H_2PO_4^-$, 0·8; SO_4^{2-}, 0·8; HEPES, 10; Tris, 8; dextrose, 5; bovine serum albumin, 1·4 g l^{-1}. Low-$[Na^+]_o$ solutions were made by equimolar substitution of Na^+ by TMA. A solution with a $[Ca^{2+}]_o$ of 13·5 mM was made by addition of $CaCl_2$. In experiments with a $[Ca^{2+}]_o$ of 13·5 mM or in the presence of Ba^{2+}or La^{3+}, SO_4^{2-} was replaced by Cl^- and $H_2PO_4^-$ was omitted. The pH was adjusted to 7·40 ± 0·05.

Cs^+, Ba^{2+} and La^{3+} were added as chloride salts.

Culture solutions. Ca^{2+}- and Mg^{2+}-free Hank's solution (in mM): Na^+, 141; K^+, 5·4; Cl^-, 141; $H_2PO_4^-$, 0·44; HPO_4^{2-}, 0·35; HCO_3^-, 4·2; dextrose, 5. R and F medium: the same inorganic salts and dextrose as the MEBSS plus 60 % (v/v) M199 and 5 % (v/v) fetal bovine serum. The F medium also contained 2 % (v/v) chick embryo extract.

Rinse solution. In mM: choline, 123; Mg^{2+}, 0·8; Cl^-, 125; HEPES, 5; Tris, 4.

Materials

Sources of materials were as follows: fetal bovine serum, Hyclone Laboratories. Trypsin (1:300) and M199, GIBCO. HEPES (*N*-2-hydroxyethylpiperazine sulphonic acid), Research Organics. Bovine serum albumin (fraction V), Tris (tris-hydroxymethylaminomethane), ouabain and tetrodotoxin, Sigma Chemical Co. Amiloride was a generous gift from Dr E. Cragoe of Merck, Sharp and Dohme. Verapamil hydrochloride was a gift from Knoll Pharmaceutical.

Electrophysiology

Polystrands were placed in a rapid-flow chamber similar to that described by Horres, Lieberman & Purdy (1977), and perfused at 10–20 ml min^{-1}. The perfusate could be changed with a half-time of ~ 5 s by means of a change-over valve placed close to the bath. The solutions were thermostatically controlled at 37 ± 0·5 °C.

Membrane potential was measured with micro-electrodes pulled from thin-walled glass tubing (i.d. 0·75 mm; o.d. 1 mm) with a glass filament. They were filled with 3 M-KCl and bevelled to 10–20 MΩ using the thick-slurry bevelling technique (Lederer, Spindler & Eisner, 1979). The reference was a free-flowing calomel electrode placed downstream from the preparation.

Content measurement

Experiments on confluent layer cultures were carried out in a glove port incubator at 37 °C, 95 % relative humidity and 5 % CO_2. Experiments were ended by rinsing three times in ice-cold rinse solution. Ionic contents were extracted in 0·75 N-HNO_3 and protein was extracted in 1 N-NaOH. Ionic contents were determined by atomic absorption spectrophotometry and protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Details are given in Murphy, Aiton, Horres & Lieberman (1983).

Statistics

Means are quoted ± the standard error. *n* is normally the number of observations. However, where the result is expressed as a ratio, the value of *n* attributed to the ratio is the value of *n* for the numerator or denominator used in calculating the ratio, whichever was less. Differences between means were tested by one- or two-tailed *t* test. When more than one drug was tested in one experiment, significant variation between the means was tested by one-way analysis of variance (ANOVA) with the quoted probability referring to the F value (Wallenstein, Zucker & Fleiss, 1980).

Experimental protocol

All experiments started with inhibition of the Na^+–K^+ pump either by addition of ouabain (0·1 mM) or by removal of external K^+, or both. In many experiments, $[K^+]_o$ was altered at the

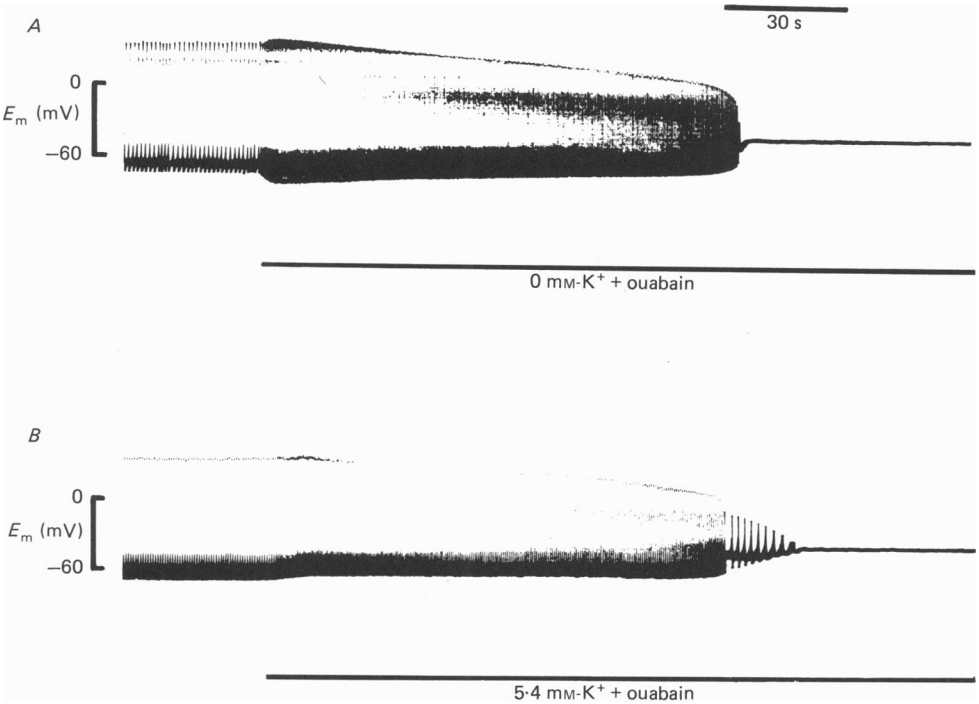

Fig. 1. Effect of ouabain (0·1 mM) on E_m. *A*, external K^+ removed simultaneously with the addition of ouabain. *B*, $[K^+]_o = 5·4$ mM.

time of pump inhibition and was then kept constant for the remainder of the experiment. To stimulate Na^+–Ca^+ exchange, cells were exposed to low-$[Na^+]_o$ or to high-$[Ca^{2+}]_o$ solutions either at 3 min or between 9 and 12 min after pump inhibition. Addition of drugs (including cations such as Cs^+ or Ba^{2+}) and changes in the type of buffer were usually effected 1 min prior to exposure to low $[Na^+]_o$ or high $[Ca^{2+}]_o$. Cells were cultured once a week and experiments were performed after 4 or 5 days in culture. In most cases comparisons were limited to those made between results obtained in a single day. We specifically state when comparisons are made between results from different weeks, or when mean values were obtained by averaging results obtained on different weeks.

RESULTS

Response of membrane potential to Na^+–K^+ pump inhibition

Na^+–K^+ pump inhibition by 0·1 mM-ouabain, with or without removal of external K^+, increased the rate of beating (Fig. 1). In K^+-free solution there was also an initial slight hyperpolarization of the maximum diastolic potential. During the rapid beating phase, the maximum diastolic potential gradually declined until E_m became quiescent at ~ -40 mV. When pump inhibition was accomplished solely by removal of external K^+, the cells took between 5 and 8 min to become quiescent: in the presence of ouabain they took only 1·5–2 min. Once E_m was quiescent, it gradually hyperpolarized at a rate which varied from week to week between 8 and 30 mV h^{-1} so that, in some cases, E_m had reattained the normal resting potential (~ -70 mV) at the end of an hour. This hyperpolarization could be due to an increasing

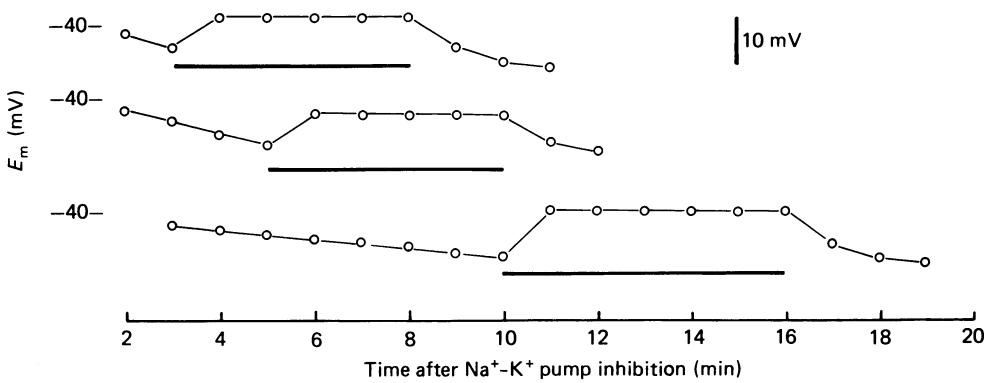

Fig. 2. The effect of Ba^{2+} on the hyperpolarization of E_m after Na^+–K^+ pump inhibition by exposure to K^+-free solution plus ouabain (0·1 mM). The long horizontal bars indicate exposure to 1 mM-Ba^{2+}.

permeability to K^+ (P_K). Consistent with this was the fact that the hyperpolarization was blocked by 1 mM-Ba^{2+} (a blocker of P_K, e.g. Cohen, Falk & Mulrine, 1983; DiFrancesco, Ferroni & Visentin, 1984) when added at various times after pump inhibition (Fig. 2).

Exposure to varying low-$[Na^+]_o$ solutions

When strands were exposed to various low external Na^+ concentrations (HEPES–Tris buffer) after 9–12 min, in K^+-free solution without ouabain, the dependence of hyperpolarization on $[Na^+]_o$ exhibited saturation for $[Na^+]_o < 10$ mM (Fig. 3) perhaps due to a non-linear current–voltage relationship. To maximize the chances of detecting changes in Na^+–Ca^{2+} exchange current we avoided this region of saturation in subsequent experiments by using an $[Na^+]_o$ of 27 mM for the low-$[Na^+]_o$ solution. After 10 min exposure to K^+-free solution plus ouabain, the low-$[Na^+]_o$ hyperpolarization was $49·3 \pm 1·2$ mV ($n = 3$) and this was not significantly affected (F test, $P > 0·25$) by 16 μM-tetrodotoxin (TTX) or 1 mM-amiloride in the presence of which the hyperpolarizations were $43·0 \pm 4·0$ mV ($n = 3$) and $45·7 \pm 0·7$ mV ($n = 3$) respectively: amiloride almost completely blocks Na^+–H^+ exchange in this preparation at 0·1 mM (Piwnica-Worms, Jacob, Horres & Lieberman, 1985).

Exposure to $[Na^+]_o$ of 27 mM with varying $[K^+]_o$

These hyperpolarizations could be due to an electrogenic Na^+–Ca^{2+} exchange but they could also be due to an electroneutral Na^+–Ca^{2+} exchange causing a rise in $[Ca^{2+}]_i$ which could modulate the cationic membrane permeability. In particular, any increase in P_K would cause a hyperpolarization since the K^+ reversal potential, E_K, is very negative, approximately −80 mV, when $[K^+]_o = 5·4$ mM (calculated from unpublished content data) and nominally infinitely negative when $[K^+]_o = 0$. Another possibility is that lowering the Na^+ reversal potential, E_{Na}, by partial removal of external Na^+ will cause E_{Na} to shift in a negative direction and could cause a shift in E_m towards E_K if there was any significant permeability to Na^+. To reduce the possibility of these effects, we repeated the experiment in a $[K^+]_o$ of 5·4 mM plus

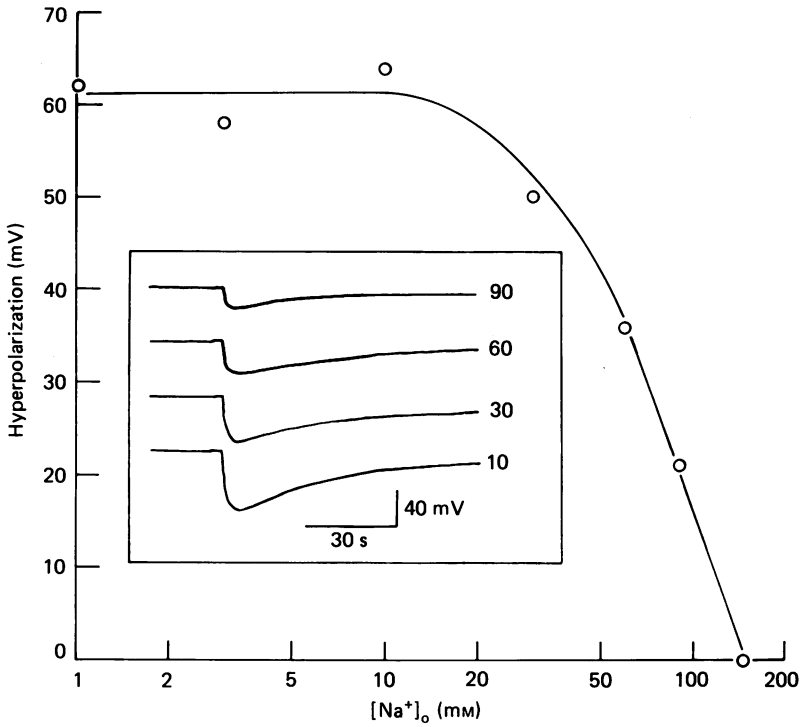

Fig. 3. The dependence of the low-$[Na^+]_o$ hyperpolarization on the value to which $[Na^+]_o$ was lowered. Hyperpolarizations were measured after 9–12 min in K^+-free solution. Inset shows records for 10, 30, 60 and 90 mM-$[Na^+]_o$.

ouabain. Exposure to an $[Na^+]_o$ of 27 mM after 9–12 min of Na^+–K^+ pump inhibition hyperpolarized E_m by ~ 22 mV (actual values and standard errors for this section are given in Table 1). This reduction in hyperpolarization was consistent with E_K playing a role. We also exposed strands to an $[Na^+]_o$ of 27 mM after 3 min in K^+-free solution plus ouabain, assuming that $[Na^+]_i$ would be lower at 3 min than at 9–12 min: the hyperpolarization was ~ 25 mV (Table 1) and this was consistent with there being less driving force for Na^+–Ca^{2+} exchange at 3 min than at 9–12 min.

Two facts argued against the explanations outlined above. First, preliminary measurements of Na^+ content showed little change between 3 and 10 min (see below). Secondly, the low-$[Na^+]_o$ hyperpolarization after 3 min in a $[K^+]_o$ of 5·4 mM plus ouabain was ~ 22 mV i.e. at 3 min the low-$[Na^+]_o$ hyperpolarization was virtually independent of the transmembrane K^+ gradient.

To evaluate how critical was the complete removal of external K^+, we repeated these experiments in a $[K^+]_o$ of 0·54 mM: the hyperpolarization behaved in the same way as in K^+-free solution (Table 1).

Results obtained from experiments with HEPES–Tris- or HCO_3-buffered solutions were not statistically different and were therefore averaged. The effects of $[K^+]_o$ and time on the low-$[Na^+]_o$ hyperpolarizations are summarized in Table 1 and typical traces are shown in Fig. 4. As can be seen from Fig. 4, the hyperpolarization often exhibited a notch which varied in magnitude from week to week.

TABLE 1. Hyperpolarizations of Na^+–K^+ pump-inhibited preparations by low $[Na^+]_o$ or high $[Ca^{2+}]_o$

$[K^+]_o$ (mM)	3 min (mV)	9–12 min (mV)
After exposure to an $[Na^+]_o$ of 27 mM		
0	25·1 ± 1·6* (10)	43·7 ± 1·9* (9)
0·54	25·4 ± 1·1* (19)	43·0 ± 3·5* (6)
5·4	22·1 ± 1·1‡ (6)	22·4 ± 1·3‡ (5)
After exposure to a $[Ca^{2+}]_o$ of 13·5 mM		
0	6·3 ± 0·2* (3)	15·1 ± 1·8† (5)
5·4	6·8* (2)	6·8 ± 0·3† (3)

Times refer to the period of Na^+–K^+ pump inhibition by ouabain before exposure to an $[Na^+]_o$ of 27 mM or an $[Ca^{2+}]_o$ of 13·5 mM. $[K^+]_o$ refers to the $[K^+]$ following the point when ouabain was added. The value of n for each experiment is indicated in brackets.

* Average of measurements made in HCO_3^--buffered solutions.

† Average of measurements made in HEPES–Tris-buffered solutions.

‡ Average of measurements made in both types of buffer.

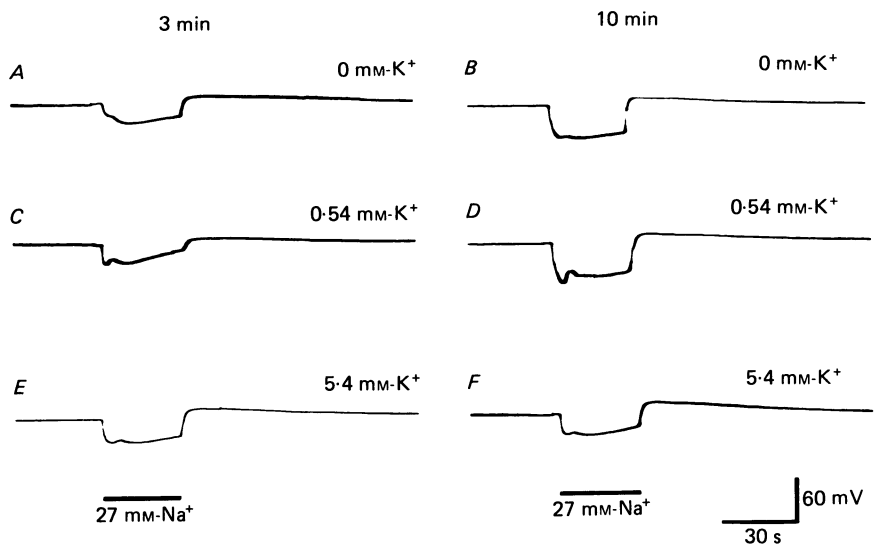

Fig. 4. Records of the hyperpolarization caused by lowering $[Na^+]_o$ to 27 mM after varying times of Na^+–K^+ pump inhibition by ouabain (0·1 mM) in the presence of various external K^+ concentrations.

The dependence on $[K^+]_o$ of the magnitude of the low-$[Na^+]_o$ hyperpolarization at 9–12 min could be due to two factors. It could depend either on $[K^+]_o$ at the time of exposure to low $[Na^+]_o$ (irrespective of $[K^+]_o$ during the preceding period) or it could depend on $[K^+]_o$ during the previous 9–12 min (irrespective of $[K^+]_o$ at the moment of exposure to low $[Na^+]_o$). To test this, $[K^+]_o$ was changed during the course of the experiment: strands were pump inhibited in a $[K^+]_o$ of 5·4 mM plus ouabain for 9·25 min and then exposed to K^+-free solution plus ouabain for varying periods of time before lowering $[Na^+]_o$ to 27 mM. The results (Fig. 5) show that the important factor was $[K^+]_o$ during the period before the strands were exposed to an $[Na^+]_o$ of 27 mM. We deduce from this that the variation in hyperpolarization with time was

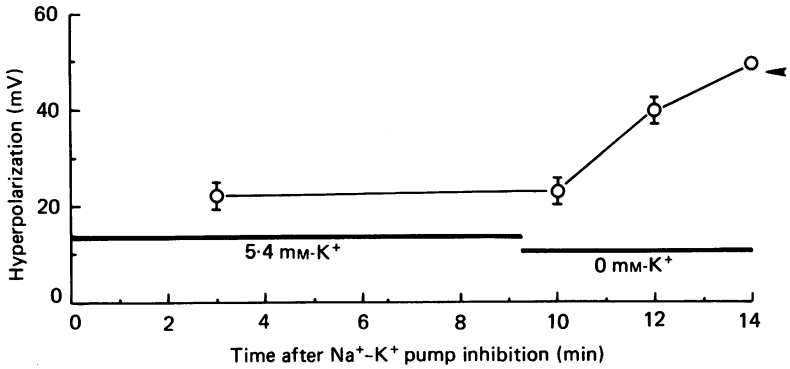

Fig. 5. Effect of changing $[K^+]_o$ on the low-$[Na^+]_o$ hyperpolarization. The hyperpolarization was elicited by lowering $[Na^+]_o$ to 27 mM after various durations of Na^+–K^+ pump inhibition by ouabain (0·1 mM). $[K^+]_o$ was changed from 5·4 to 0 mM at 9·25 min. Each point is the mean of three observations. The arrow on the right indicates the value of the hyperpolarization obtained after 10 min in K^+-free solution plus ouabain (0·1 mM).

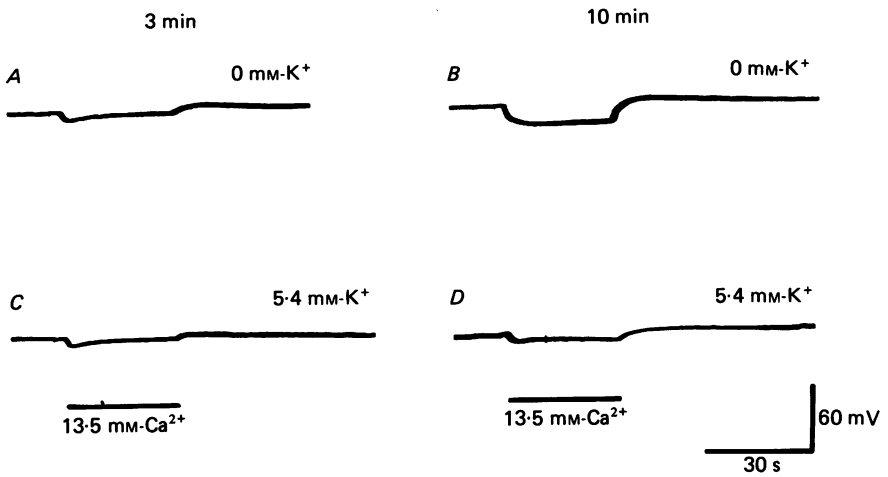

Fig. 6. Records of hyperpolarizations caused by raising $[Ca^{2+}]_o$ to 13·5 mM after varying times of Na^+–K^+ pump inhibition by ouabain in the presence of various external K^+ concentrations.

due to a change in membrane resistance (R_m, see below for other evidence) and was not an intrinsic property of Na^+–Ca^{2+} exchange.

Exposure to a $[Ca^{2+}]_o$ of 13·5 mM

We tried to stimulate Na^+–Ca^{2+} exchange by raising $[Ca^{2+}]_o$ from 2·7 to 13·5 mM. The resulting hyperpolarizations were much less than those produced by exposure to an $[Na^+]_o$ of 27 mM but the effects of time and $[K^+]_o$ were very similar (Table 1 and Fig. 6). Verapamil at 10 μM had no effect ($P > 0·8$) on the hyperpolarization at 9–12 min in K^+-free solution ($14·7 \pm 1·4$ mV, $n = 3$).

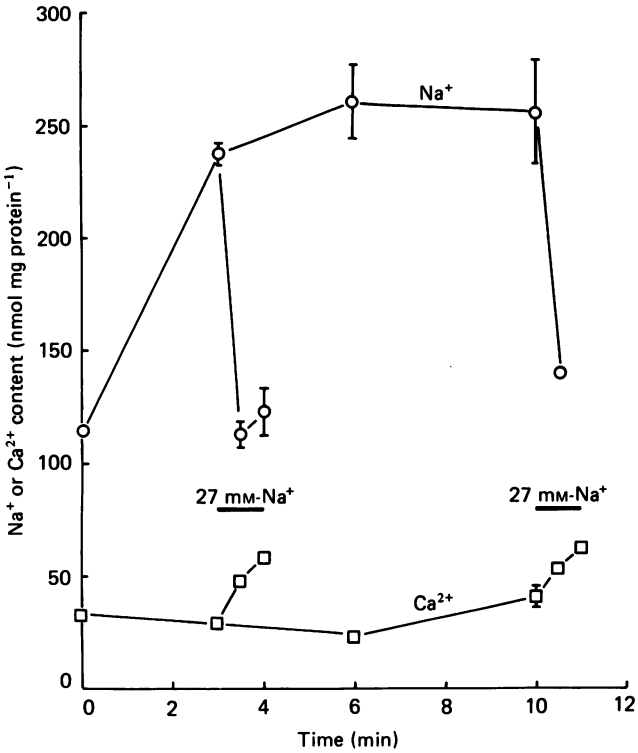

Fig. 7. Changes in Na^+ (○) and Ca^{2+} (□) content when confluent layer cultures were exposed to K^+-free solution plus ouabain (0·1 mM) followed by an $[Na^+]_o$ of 27 mM either between 3 and 4 min or between 10 and 11 min after Na^+–K^+ pump inhibition. Each point is the average of content determinations from three culture dishes. The Na^+ and Ca^{2+} contents were measured on separate cultures. Where not indicated, the standard error lies within the symbol size.

Effects of Cs^+, Ba^{2+} and La^{2+} on low-$[Na^+]_o$ hyperpolarizations

In this section the results were obtained in K^+-free solution plus ouabain. To test for an involvement of P_K in the hyperpolarization we tested the effect of Cs^+ which has been reported to block intracellular Ca^{2+}-activated K^+ channels (Isenberg, 1977). The presence of 10 mM-Cs^+, added at 2 min, did not alter E_m at 3 min (control: 40·4±0·9 mV, $n = 3$; with Cs^+: 39·9±0·5 mV, $n = 3$) but significantly ($P < 0·025$) reduced the low-$[Na^+]_o$ hyperpolarization at 3 min from 32·7±2·6 mV ($n = 3$) to 24·2±1·0 mV ($n = 3$). Cs^+ did not affect ($P > 0·9$) the high-$[Ca^{2+}]_o$ hyperpolarization (9·6±1·2 mV, $n = 3$; with Cs^+: 9·8±1·1 mV, $n = 3$).

Ba^{2+} has also been reported to block K^+ permeability (e.g. Cohen *et al.* 1983) and at 1 mM it significantly ($P < 0·0005$) reduced the low-$[Na^+]_o$ hyperpolarization from 39·0±0·6 mV ($n = 3$) to 31·3±0·7 mV ($n = 3$). The significance of this is obscured by the fact that Ba^{2+} has been reported to block Na^+–Ca^{2+} exchange (Trosper & Philipson, 1983).

Since La^{3+} has been reported to block Na^+–Ca^{2+} exchange (e.g. Trosper & Philipson, 1983) it should block any hyperpolarization due to an electrogenic

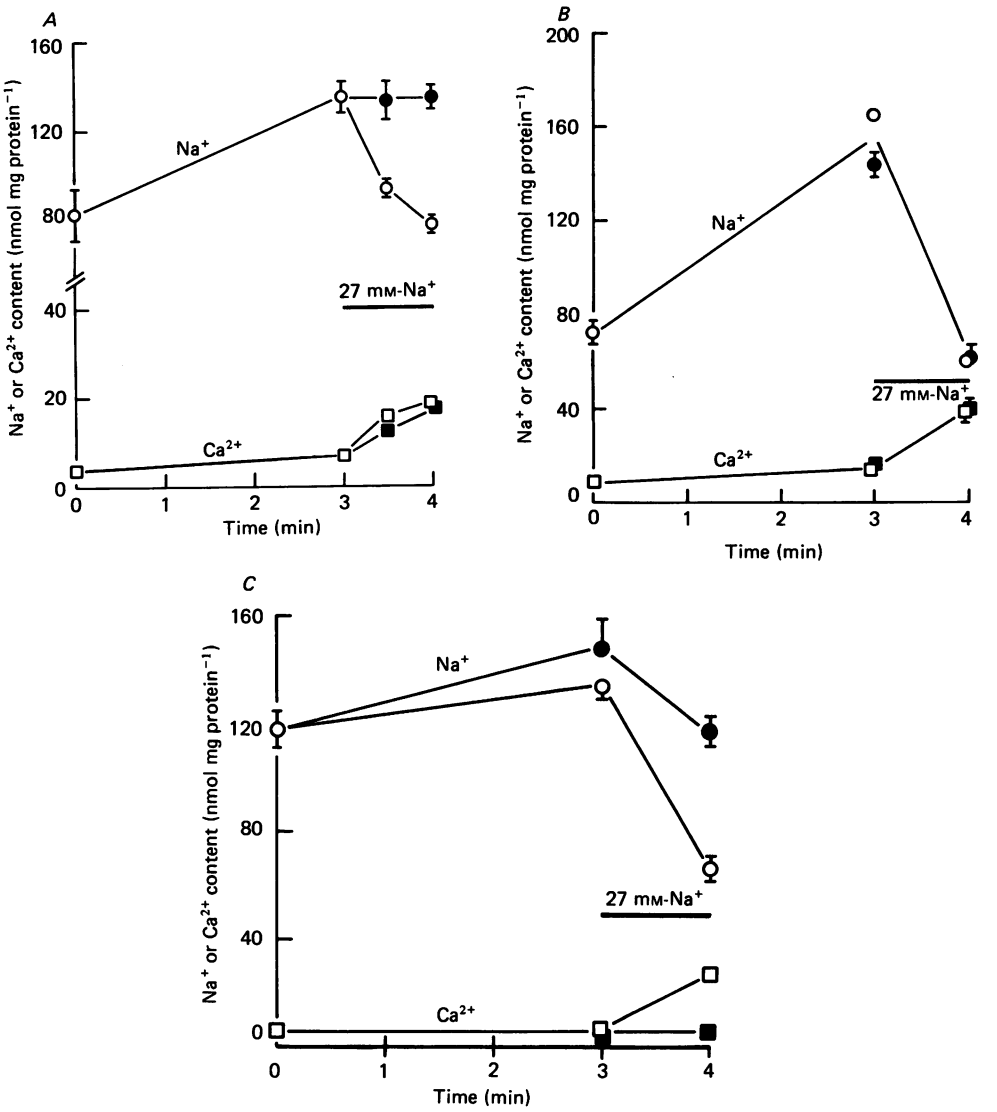

Fig. 8. Na^+ contents (○,●) and Ca^{2+} contents (□,■) of confluent layer cultures after 3 min in K^+-free solution plus ouabain (0·1 mM) followed by exposure to an $[Na^+]_o$ of 27 mM. Each point is the average of content determinations from between three and six culture dishes. Where not indicated, the standard error lies within the symbol size. *A*, comparison of the effect of exposure to an $[Na^+]_o$ of 27 mM (○,□) or a $[Ca^{2+}]_o$ of 13·5 mM (●,■). *B*, effect of Cs^+ (10 mM) added at 2 min. ○ and □ measured in the absence of Cs^+; ● and ■ measured in the presence of Cs^+. Cs^+ appeared to cause a small but significant ($P < 0·01$) depression in the Na^+ content at 3 min, but did not do so ($P > 0·4$) in another experiment. *C*, effect of La^{3+} (1 mM) added at 2 min. ○ and □ measured in the absence of La^{3+}; ● and ■ measured in the presence of La^{3+}.

exchange. In fact, the effect of 1 mM-La^{3+} on the low-$[Na^+]_o$ hyperpolarization was slight (control: $25{\cdot}5 \pm 1{\cdot}0$ mV, $n = 3$; with La^{3+}: $21{\cdot}8 \pm 1{\cdot}0$ mV, $n = 4$, $P < 0{\cdot}025$) and, curiously, it appeared to potentiate the accompanying contracture since it proved very difficult to maintain stable impalements during these experiments. This potentiation by La^{3+} has been directly observed by Coraboeuf *et al.* (1981) and Mead & Clusin (1985).

Na^+ and Ca^{2+} content changes

Using the same protocol as that employed for the electrical experiments, we measured changes in Na^+ and Ca^{2+} contents on confluent layer cultures exposed to low $[Na^+]_o$. These cultures exhibit movements of Na^+ and Ca^{2+} which can be shown to correlate very closely with those measured on polystrands (Jacob *et al.* 1987). Fig. 7 shows that lowering $[Na^+]_o$ caused a loss of Na^+ and a gain of Ca^{2+}. These movements were similar when measured at 3 or at 10 min after pump inhibition implying that the increased low-$[Na^+]_o$ hyperpolarization seen after 10 min in K^+-free solution was not caused by an increased Na^+–Ca^{2+} exchange activity. Assuming a cell volume of 8·3 μl mg protein^{-1} (Gaynes, Lobaugh & Lieberman, 1985), $[Na^+]_i$ is approximately 30 mM and E_{Na} is approximately 3 mV. Thus, the movement of Na^+ out of the cells was counter to the Na^+ electrochemical gradient, thereby confirming that the movement of Na^+ was not electrodiffusive.

Other discrepancies between electrical and content measurements were also found. Although an appreciable hyperpolarization was recorded on raising $[Ca^{2+}]_o$ to 13·5 mM, there was no significant drop in Na^+ content (Fig. 8*A*). (In the following article (Jacob *et al.* 1987) the drop in Na^+ content measured under similar circumstances but with more measurements was found to be significant although it was still small.) Similarly, 10 mM-Cs^+ had virtually no effect on the movements of Na^+ and Ca^{2+} (Fig. 8*B*) although it significantly attenuated the hyperpolarizations. Conversely, 1 mM-La^{3+} partially blocked the fall in Na^+ content and totally blocked the rise in Ca^{2+} content (Fig. 8*C*) although it only had a marginal effect on the hyperpolarization.

DISCUSSION

We considered several approaches to determining the electrogenicity of Na^+–Ca^{2+} exchange in intact cells. The first was to stimulate Na^+–Ca^{2+} exchange and measure its stoicheiometry by comparing changes in Na^+ and Ca^{2+} content. Besides the problem of ensuring that Na^+ and Ca^{2+} cross the sarcolemma exclusively by Na^+–Ca^{2+} exchange, this method suffers from insufficient accuracy. For example, calculating an error for the data of Bridge & Bassingthwaite (1983) shows that their estimate of 3·0 for the stoicheiometry bears an error of $\pm 1{\cdot}4$.

The second approach is to show that Na^+–Ca^{2+} exchange can be reversed by varying the membrane potential (E_m). As discussed by Eisner & Lederer (1985), it is not sufficient to show that E_m merely affects the rate of Na^+–Ca^{2+} exchange since an electroneutral exchange may still have potential-sensitive steps in the over-all electroneutral cycle. The absolute criterion of a change in E_m causing a reversal of the exchange is difficult to prove when the movements of Na^+ and Ca^{2+} cannot be

clearly divided between those via Na^+–Ca^{2+} exchange and those via other (possibly potential-sensitive) pathways.

If Na^+–Ca^{2+} exchange is at thermodynamic equilibrium then the relationship between the transmembrane electrochemical gradients for Na^+ and Ca^{2+} ($\tilde{\mu}_{Na}$ and $\tilde{\mu}_{Ca}$) can be used to determine the stoicheiometry. Sheu & Fozzard (1982) used this approach to derive what they termed an energy coupling ratio ($\tilde{\mu}_{Na}/\tilde{\mu}_{Ca}$) of $\sim 2{\cdot}5$: this would equal the stoicheiometry if it were measured when the exchange was at equilibrium. However, as has been pointed out by Axelsen & Bridge (1985), and as Sheu & Fozzard (1985) acknowledge in relation to their results, Na^+–Ca^{2+} exchange is unlikely to be at equilibrium during such experiments. Thus the accuracy of such a study rests on the undetermined relationship between the thermodynamic driving force for the exchange and its velocity.

The approach we finally chose was that of trying to show a change in E_m that could be unequivocally associated with Na^+–Ca^{2+} exchange. In particular, we tried to identify Na^+–Ca^{2+} exchange as the cause of the hyperpolarization produced by exposing Na^+–K^+ pump-inhibited preparations to low $[Na^+]_o$.

Exposure of Na^+–K^+ pump-inhibited preparations to low-$[Na^+]_o$ stimulated Na^+–Ca^{2+} exchange as revealed by the changes in Na^+ and Ca^{2+} contents (see also Murphy *et al.* 1986). If the hyperpolarization accompanying these movements had been sufficiently large to drive E_m negative to the Na^+ and K^+ reversal potentials (E_{Na} and E_K) then this would have confirmed the electrogenic nature of the exchange. However, although the hyperpolarization was appreciable it was never large enough to fulfil this criterion since $E_K \sim -80$ mV in a $[K^+]_o$ of 5·4 mM.

Besides an electrogenic exchange, there are three possible explanations for the low-$[Na]_o$ hyperpolarizations. The first is that there is a significant membrane permeability to Na^+ in which case removal of external Na^+ will cause E_{Na} to shift in a negative direction so that E_m will shift towards E_K. The second is that an electroneutral Na^+–Ca^{2+} exchange causes a rise in $[Ca^{2+}]_i$ which in turn causes an increase in the membrane permeability to K^+: as above, this will cause E_m to shift towards E_K. The third possibility applies when external Na^+ is completely removed in which case the reversal potential for a non-specific cation channel would be negative to E_m and activation of this channel by a rise in $[Ca^{2+}]_i$ would cause a hyperpolarization. This mechanism appears to be involved in the low-$[Na^+]_o$-induced outward current observed in sheep Purkinje fibres (Lederer, Sheu, Eisner & Vaughan-Jones, 1983).

Several observations suggest that passive diffusive movements generate at least part of the low-$[Na^+]_o$ hyperpolarization. The partial block of the hyperpolarization by Cs^+ is one of these observations. Originally, Cs^+ was reported to block $[Ca^{2+}]_i$-activated P_K (Isenberg, 1977) although recently it has been reported to block the pace-maker current, i_f (DiFrancesco, 1982). Either explanation implies the same interpretation of our results since both articles report some effect of Cs^+ in the region of -60 mV, i.e. the potential to which E_m was hyperpolarizing on exposure to low $[Na^+]_o$. This explanation is consistent with the results of Mead & Clusin (1984) who reported a Cs^+-sensitive current in cultured chick embryo heart cells on removal of external Na^+: they tentatively related this current to i_f. An alternative explanation for the action of Cs^+ could be that it was blocking Na^+–Ca^{2+} exchange. However,

monovalent cations in general do not affect Na^+–Ca^{2+} exchange (Philipson & Nishimoto, 1981; Slaughter, Sutko & Reeves, 1983) in agreement with our observation that Cs^+ did not affect the movements of Na^+ and Ca^{2+} in the confluent layer cultures.

The notch that was often seen in the record of low-$[Na^+]_o$ hyperpolarizations may indicate that two mechanisms (with different time courses) were involved in generating the hyperpolarization. The variability of this notch precluded its detailed investigation. However, the notch is virtually absent when similar experiments are performed in a $[K^+]_o$ of 24 mM in which the driving force for electrodiffusive transmembrane K^+ movements is much reduced (Jacob *et al.* 1987). Thus the initial phase of the low-$[Na^+]_o$ hyperpolarization seen in low $[K^+]_o$ may be due to electrodiffusive ion movements.

If the low-$[Na^+]_o$ hyperpolarization is due to an electrogenic Na^+–Ca^{2+} exchange, its magnitude should decline as $[Na^+]_i$, and hence the thermodynamic gradient for Na^+–Ca^{2+} exchange, declines. Although such a decline was seen (e.g. Fig. 3), consistent with an electrogenic exchange, it does not prove the point. The hyperpolarization could be due to a $[Ca^{2+}]_i$-modulated conductance with $[Ca^{2+}]_i$ being gradually regulated back to its original value.

Since the magnitude of the hyperpolarization is the product of the exchange current and the membrane resistance one might even expect that if the hyperpolarization were caused entirely by an electrogenic exchange then blocking a component of the passive membrane permeability would cause an *increase* in the low-$[Na^+]_o$ hyperpolarization. The same argument applies to Ba^{2+} which, like Cs^+, caused a decrease in hyperpolarization despite the fact that it is a blocker of P_K (Cohen *et al.* 1983; DiFrancesco *et al.* 1984) and does not attenuate Na^+–Ca^{2+} exchange in cultured chick heart cells under conditions similar to those reported here (Jacob *et al.* 1986).

In contrast to the action of Cs^+, La^{3+} significantly blocked the movements of Na^+ and Ca^{2+} induced by exposure to low $[Na^+]_o$ but had less effect than Cs^+ on the hyperpolarization.

Finally, exposure to a $[Ca^{2+}]_o$ of 13·5 mM caused a significant hyperpolarization but failed to induce a significant drop in Na^+ content. Taking these observations in conjunction, there is a disparity between the magnitude of the Na^+–Ca^{2+} exchange and the accompanying hyperpolarization. The inference is that the hyperpolarization is, to a significant extent, not due to an electrogenic exchange in this preparation.

The insensitivity of the low-$[Na^+]_o$ hyperpolarization to TTX or amiloride suggests that neither the fast Na^+ channel nor Na^+–H^+ exchange is involved (either directly or indirectly) in the hyperpolarization. Apparently, exposure to a low $[K^+]_o$ (0 or 0·54 mM) for several minutes causes an increase in R_m: this was indicated by the fact that although movements of Na^+ and Ca^{2+} induced by exposure to a low $[Na^+]_o$ at 3 or 9–12 min were very similar, the hyperpolarizations were not. Moreover, the effect of $[K^+]_o$ depended on the $[K^+]_o$ preceding the exposure to low $[Na^+]_o$ rather than the $[K^+]_o$ at the time of exposure. Given the rapid clearance of extracellular space in these preparations (half-time $\sim$ 7 s; Piwnica-Worms, Jacob, Horres & Lieberman, 1983) and the relatively small changes in $[K^+]_i$ after 10 min exposure to a $[K^+]_o$ of 0 or 5·4 mM plus ouabain (R. Jacob, unpublished observation, based on K^+ content measurements), the effect must be mediated by a change in R_m. This effect may be

related to the time-dependent conductance change seen in sheep cardiac Purkinje fibres exposed to Na^+- and K^+-free medium (Carmeliet, 1982).

Hyperpolarizations induced by lowering $[Na^+]_o$ in the presence or absence of Na^+–K^+ pump inhibition have been reported for sheep heart Purkinje fibres (Ellis, 1977; Bers & Ellis, 1982), sheep ventricular trabeculae (Sheu & Fozzard, 1982), dog Purkinje fibres (Coraboeuf *et al.* 1981), guinea-pig and ferret ventricular trabeculae (Chapman, McGuigan, Rodrigo & Yates, 1983) and cultured embryonic chick ventricular cells (Barry & Hasin, 1983). However, in no instance was E_m reported to hyperpolarize negative to E_{Na} and E_K.

Coraboeuf *et al.* (1981) explicitly investigated the electrogenicity of Na^+–Ca^{2+} exchange in heart muscle and argued that the exchange was electrogenic on the basis of the sensitivity of the low-$[Na^+]_o$ hyperpolarizations to various agents. But consideration must be given to the ambiguities in the role of the agents. For example, 0·4 mM-La^{3+} (and higher concentrations) did not attenuate the hyperpolarization and this was interpreted as consistent with reports that La^{3+} does not block Na^+–Ca^{2+} exchange in intact heart muscle (e.g. Katzung, Reuter & Porzig, 1973). However, these reports relate to the effect of La^{3+} on Na^+–Ca^{2+} exchange when it is mediating Ca^{2+} efflux. Our results show that La^{2+} does block Na^+–Ca^{2+} exchange when it is mediating Ca^{2+} uptake (see also Barry & Smith, 1982; Jacob *et al.* 1987) and La^{3+} has also been reported to block currents tentatively identified as Na^+–Ca^{2+} exchange currents (Mentrard *et al.* 1984; Uehara & Hume, 1985): La^{3+} should therefore block hyperpolarizations due to an electrogenic exchange. In the light of our results, the lack of effect of La^{3+} indicates that the hyperpolarizations might not have been due to an electrogenic exchange.

Equivalent to recording a hyperpolarization, Mentrard *et al.* (1984) recorded an outward current associated with exposure of frog atrial muscle to low $[Na^+]_o$. They tentatively identified this current as due to Na^+–Ca^{2+} exchange because its voltage dependence matched that predicted by the model of Mullins (1976). But, as pointed out by Eisner & Lederer (1985), the Mullins model was derived assuming the exchange to be at equilibrium in the absence of an applied potential: this is unlikely to be the case in the experiments of Mentrard *et al.* (1984). Furthermore, the actual current–voltage relationship for the exchange may well be different from that predicted by Mullins (Eisner & Lederer, 1985) and measurement of the exchange current may be complicated by conductance changes (Lederer *et al.* 1984). Finally, Mentrard *et al.* (1984) assumed that after lowering $[Na^+]_o$, $[Na^+]_i$ and $[Ca^{2+}]_i$ did not change significantly during the time course of their measurements. According to Chapman & Tunstall (1984) and Chapman *et al.* (1984) this assumption is not valid since measurements on frog atrial trabeculae show that the intracellular Na^+ activity (a^i_{Na}) falls and tension rises very soon after lowering $[Na^+]_o$. We therefore interpret the results of Coraboeuf *et al.* (1981) and Mentrard *et al.* (1984) in the same way as our own: the hyperpolarization (or the current) caused by lowering $[Na^+]_o$ may in part be caused by an electrogenic Na^+–Ca^{2+} exchange, but other processes may also be involved. Although an electrogenic Na^+–Ca^{2+} exchange is by no means inconsistent with our results the present experiments do not identify Na^+–Ca^{2+} exchange as electrogenic.

We conclude that the resulting hyperpolarization cannot be unequivocally attri-

buted to an electrogenic exchange because of a sensitivity to Cs^+, a relative insensitivity to La^{3+} and because of disparities between the degrees of hyperpolarization and net trans-sarcolemmal movements of Na^+ and Ca^{2+}. In the following article (Jacob *et al.* 1987) we report experiments in which the K^+ gradient and passive K^+ permeability are altered so that the hyperpolarization induced by exposure to low $[Na^+]_o$ can be unambiguously attributed to electrogenic Na^+–Ca^{2+} exchange.

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