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ELECTROGENIC SODIUM–CALCIUM EXCHANGE IN CULTURED EMBRYONIC CHICK HEART CELLS

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SUMMARY

1. The membrane potential (E_m) of cultured chick embryonic heart cells depolarized to −36 mV after inhibition of the Na^+–K^+ pump by 0·1 mM-ouabain in a $[K^+]_o$ of 24 mM: this was accompanied by a rise in Na^+ content of ~ 65 % in 3 min. Lowering $[Na^+]_o$ to 27 mM then caused a fall in Na^+ content, a rise in Ca^{2+} content and a small hyperpolarization of ~ 5 mV. The fall in Na^+ content indicated a movement of Na^+ which was in the opposite direction to the Na^+ electrochemical gradient (a countergradient movement).

2. In the presence of 10 mM-Cs^+ or 1 mM-Ba^{2+} the hyperpolarization was ~ 10 or ~ 30 mV, respectively. A 30 mV hyperpolarization took E_m negative to the reversal potentials for K^+, Na^+ and Cl^- as measured by ion-selective micro-electrodes.

3. The decay of the intracellular Na^+ activity, a^i_{Na}, in an $[Na^+]_o$ of 27 mM followed a simple exponential time course (time constant, 36 s). The initial rate depended on the value to which $[Na^+]_o$ was lowered in a manner suggesting a simple competitive inhibition of the exchange by external Na^+.

4. The low-$[Na^+]_o$ hyperpolarization was unaffected by amiloride (0·1 or 1 mM) or verapamil (20 μM). Both La^{3+} (1 mM) and Mn^{2+} (20 mM) blocked the hyperpolarization sufficiently to prevent E_m hyperpolarizing negative to the reversal potentials for K^+, Na^+ and Cl^-.

5. Re-establishing $[Na^+]_o$ caused a rise in Na^+ content and a countergradient drop in Ca^{2+} content. The effects of verapamil (20 μM), amiloride (0·1 and 1 mM), dichlorobenzamil (0·1 mM), quinidine (1 mM), Mn^{2+} (20 mM) and La^{3+} (1 mM) were tested on the movements of Na^+ and Ca^{2+} both during exposure to an $[Na^+]_o$ of 27 mM and on re-establishing $[Na^+]_o$. The only consistent and substantial effects were the attenuation by La^{3+} and Mn^{2+} of Na^+ and Ca^{2+} movements during exposure to an $[Na^+]_o$ of 27 mM. However, neither La^{3+} nor Mn^{2+} affected the movements of Na^+ and Ca^{2+} on re-establishing $[Na^+]_o$.

6. We conclude that cultured embryonic chick heart cells contain a Na^+–Ca^{2+} exchange evidenced by the ability to cause movements of Na^+ and Ca^{2+} which are counter to their respective electrochemical gradient and which are accompanied by downhill movements of the counter ion. In the presence of Ba^{2+} (which presumably blocks K^+ permeability) the exchange is accompanied by a hyperpolarization which

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takes E_m negative to the reversal potentials of the major ions. This shows that Na^+–Ca^{2+} exchange is electrogenic.

INTRODUCTION

In the preceding article (Jacob, Lieberman, Murphy & Piwnica-Worms, 1987) we attempted to demonstrate the electrogenicity of Na^+–Ca^{2+} exchange by examining the changes in membrane potential (E_m) induced by exposure to low $[Na^+]_o$ in the presence of low $[K^+]_o$ and ouabain. We concluded that the hyperpolarizations induced by exposure to low $[Na^+]_o$ could not be considered as an absolute test of the electrogenicity of Na^+–Ca^{2+} exchange.

There are two ways to proceed from this point. The first would be to block every alternative pathway to Na^+–Ca^{2+} exchange before lowering $[Na^+]_o$: the use of drug cocktails and ion substitutions to attain this end is becoming more frequent and has recently been successful in the measurement of a Na^+–Ca^{2+} exchange current in isolated heart cells (Kimura, Noma & Irisawa, 1986). The second approach, which we have opted for, is to seek a physiological criterion (rather than a pharmacological one) that unambiguously eliminates ionic movements via electrodiffusive pathways as a potential source of the hyperpolarization. The condition we have chosen is that the hyperpolarization should take E_m negative to the reversal potentials for the major ions, namely Na^+, K^+ and Cl^-. If such a hyperpolarization can be demonstrated then it would clearly imply the existence of an electrogenic process because ion flow through electrodiffusive pathways cannot drive E_m beyond the range of these reversal potentials.

Preliminary reports of this work have appeared in abstract form (Jacob, Murphy & Lieberman, 1983; Jacob, Liu, Murphy & Lieberman, 1984; Jacob, Liu & Lieberman, 1985; Liu, Jacob & Lieberman, 1985)

METHODS

Culture techniques, recording of membrane potential, content determinations on confluent layer cultures and statistical analysis were made as described in the preceding article (Jacob *et al.* 1987).

Solutions

The control solution was either a HCO_3^--buffered modified Earle's balanced salt solution (Jacob *et al.* 1987) or a HEPES–Tris-buffered modified Earle's balanced salt solution (MEBSS) with the following composition (in mM): Na^+, 144; K^+, 5·4; Mg^{2+}, 0·8; Ca^{2+}, 2·7; Cl^-, 154; $H_2PO_4^-$, 0·8; SO_4^{2-}, 0·8; HEPES, 10; Tris, 8; dextrose, 5; bovine serum albumin, 1·4 g l^{-1}. A solution with a $[K^+]_o$ of 24 mM was made by equimolar substitution of Na^+ for K^+. A solution with an $[Na^+]_o$ of 27 mM was made by equimolar substitution of tetramethylammonium (TMA) for Na^+. A solution with a $[K^+]_o$ of 24 mM and $[Ca^{2+}]_o$ of 13·5 mM was made by addition of $CaCl_2$: SO_4^{2-} and $H_2PO_4^-$ were omitted from this solution and it was always HEPES–Tris buffered. SO_4^{2-} was also omitted from solutions to which Ba^{2+} was added and both SO_4^{2-} and $H_2PO_4^-$ were omitted from solutions to which La^{3+} was added.

The following modifications were made in the ion-selective micro-electrode experiments. In the HEPES–Tris-buffered solution with a $[K^+]_o$ of 24 mM, Cl^- was the same as in the MEBSS (128 mM) and methanesulphonate (28 mM) was used to balance the anion concentration. The resulting solution was slightly hypertonic to the HCO_3^--buffered solution with a $[K^+]_o$ of 24 mM (295 *versus* 280 mosM). If $BaCl_2$ was added to the HEPES–Tris-buffered with a $[K^+]_o$ of 24 mM then its osmolarity was 300 mosM. When using solutions with an $[Na^+]_o$ of 27 mM and containing

20 mM-Mn^{2+} (as $MnCl_2$), which were hypertonic (350 mosM), TMACl was added to the solution with a $[K^+]_o$ of 24 mM between 2 and 3 min to prevent volume changes complicating the measurements of the intracellular Na^+ activity, a^i_{Na}. The concentration of bovine serum albumin was lowered to 1 g l^{-1}.

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

Ion-selective micro-electrodes

Ion-selective micro-electrodes were drawn from 1 mm diameter borosilicate glass tubing (i.d. 0·5 mm) with an internal glass filament. After drying by heating, electrodes were silanized by exposure to hexamethyldisilazane vapour at 200 °C for 15 min. K^+ electrodes were backfilled with ~ 10 μl of Corning 477317 ion-exchange resin and left at least 5 h. A filling solution of 0·5 M-KCl was then added and the tips were immersed in this filling solution for at least 1 h before use. Electrodes typically had a resistance $> 1 \times 10^{10}$ Ω but were bevelled using the thick-slurry bevelling technique (Lederer, Spindler & Eisner, 1979) to a resistance of ~ 5×10^9 Ω. K^+ electrodes were calibrated at 36–38 °C by the 'unorthodox method' (Thomas, 1978) using KCl solutions of 10 mM, 100 mM, 155 mM and 1M, and mixed solutions of NaCl and KCl with a total cation concentration of 155 mM and KCl concentrations of 5, 10, 20, 50 and 100 mM (the balance being made up by NaCl). Selectivity of K^+:Na^+ was ~ 50:1. The electrode response to pure KCl solutions ranged from 59 to 62 mV decade^{-1}. If the pre- and post-calibrations in the high-K^+ region (100–150 mM) differed in offset by > 2 mV or in slope by > 2 mV decade^{-1} then the readings were rejected; otherwise the post-calibration curve was used.

Cl^- electrodes were made in a similar fashion except that Corning resin 477913 was used and the filling solution was 100 mM-KCl. In addition, the end portion of a broken-tipped micro-electrode containing filling solution was inserted down the barrel so that it reached within 100 μm of the tip of the ion-selective electrode (Walker, 1980): this shortened the effective resin column length and so decreased the electrical response time. Before use, tips were immersed for 1 h in the filling solution. Resistance was typically between 1×10^{10} and 1×10^{11} Ω before bevelling and between 1×10^9 and 5×10^9 Ω afterwards. The electrodes were calibrated using KCl solutions of 5, 10, 50 and 155 mM and the slope ranged from 56 to 62 mV decade^{-1}. The $Cl:HCO_3^-$ selectivity was 25:1. As with the K^+ electrode, pre- and post-calibration curves were measured and the same criterion was used for rejection of data.

Na^+ electrodes were made the same way as the Cl^- electrodes except that a neutral carrier cocktail (Fluka 71176) was used; the filling solution was 100 mM-NaCl. Electrodes were stored in air and used without pre-soaking the tip in filling solution. Before bevelling, resistance was typically $> 1 \times 10^{11}$ Ω and afterwards was between 1×10^{10} and 5×10^{10} Ω. Electrodes were calibrated according to the method of Dagostino & Lee (1982). The selectivity of Na^+:K^+ was 50:1 and of Na^+:Ca^{2+} was 1:2. To correct for interference by intracellular K^+ (approximately 100 mM), 2 mM was subtracted from the measured values of a^i_{Na}.

The signal from the ion-selective micro-electrode was buffered by an amplifier using an Analog Devices AD515L with an input impedance of 1×10^{15} Ω. The signal was then fed into the non-inverting input of a differential amplifier; the inverting input of this amplifier was fed by the E_m signal after it had passed through a variable frequency low-pass first-order filter. The filter frequency was adjusted to minimize deviations of the difference signal caused by action potentials i.e. it matched the frequency response of the E_m-sensing electrode to that of the ion-selective micro-electrode. Typical electrical time constants were 1–10 ms for the K^+ electrodes, 10–50 ms for the Cl^- electrode and 50 ms for the Na^+ electrode. The chemical response times for the ion-selective micro-electrodes were < 5 s. Impalements of the polystrand by the E_m-sensing electrode and the ion-selective micro-electrode were ~ 0·5 mm apart.

After exposure of polystrands to Cl^--free solution for 15 min, the Cl^- electrodes still registered an intracellular Cl^- activity, a^i_{Cl}, of ~ 10 mM. Since the $^{36}Cl^-$ efflux rate constant in Cl^--free solution is 0·67 min^{-1} (Piwnica-Worms, Jacob, Horres & Lieberman, 1983), by 15 min all the intracellular Cl^- should have been washed out. Although Cl^- electrodes are known to be sensitive to HCO_3^-, removal of HCO_3^- from the Cl^--free solution did not affect the residual activity. Presumably these cells contain other anions to which the Cl^- electrode is sensitive: this problem has been encountered by others (Vaughan-Jones, 1979; Spitzer & Walker, 1980; Baumgarten & Fozzard, 1981). The measured a^i_{Cl} was corrected by subtraction of the residual apparent activity.

Content determinations on polystrands

Experiments on polystrands were carried out using the same apparatus as that used for the electrophysiology and were ended by rinsing the strands for 30 s in ice-cold rinse solution. Preparations were then dried and weighed. The weight of the nylon support thread was subtracted to yield the tissue dry weight. Preparations were then extracted in 1 N-HNO_3 and the contents determined by atomic absorption spectrophotometry (see Piwnica-Worms, Jacob, Horres & Lieberman, 1985, for details).

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, tetrodotoxin, quinidine hydrochloride and hexamethyldisilazane, Sigma Chemical Co. Amiloride and dichlorobenzamil were generous gifts from Dr E. Cragoe of Merck, Sharp and Dohme. Verapamil hydrochloride was a gift from Knoll Pharmaceutical.

Protocol

Experiments started with exposure of the cells to a $[K^+]_o$ of 24 mM plus 0·1 mM-ouabain. Hyperpolarizations or content changes were elicited by exposing cells either to an $[Na^+]_o$ of 27 mM or to a $[Ca^{2+}]_o$ of 13·5 mM 3 min after raising $[K^+]_o$ and adding ouabain. Whenever the effects of drugs were investigated, the hyperpolarization or the content changes were compared with drug-free measurements made during the same experiment. In some cases content results were determined from one experiment in which case the accompanying value of n refers to the number of culture dishes or polystrands used to determine each point. In other cases, results were obtained by averaging values from several individual experiments (each value itself being obtained by averaging within one experiment measurements from several culture dishes) and the value of n refers to the number of experiments over which the average was taken.

RESULTS

Electrophysiological data

Low-$[Na^+]_o$ hyperpolarization. In previous experiments, stimulation of Na^+–Ca^{2+} exchange by exposure to an $[Na^+]_o$ of 27 mM after 3 min of Na^+–K^+ pump inhibition by ouabain in a $[K^+]_o$ of 5·4 mM caused a shift in E_m from ~ -40 mV to ~ -65 mV (Jacob *et al.* 1987). Although this hyperpolarization was consistent with an electrogenic exchange, the K^+ reversal potential (E_K), calculated from the K^+ contents was ~ -80 mV so that the hyperpolarization could have been due to passive ionic movements. To increase the possibility of E_m hyperpolarizing beyond E_K this experiment was repeated in a $[K^+]_o$ of 24 mM plus 0·1 mM-ouabain. This value of $[K^+]_o$ was chosen for two reasons. Firstly, in the presence of ouabain, E_m in a $[K^+]_o$ of 24 mM is approximately the same as in a $[K^+]_o$ of 0 or 5·4 mM, i.e. ~ -40 mV (Lieberman, Horres, Aiton & Shigeto, 1982). Secondly, although the relation between E_m and log $[K^+]_o$ is fairly constant in the range $[K^+]_o$ = 0–20 mM, it is approximately Nernstian for $[K^+]_o \geqslant 24$ mM (Lieberman *et al.* 1982) suggesting that when $[K^+]_o$ = 24 mM, $E_m \simeq E_K$. By coupling this last observation with the fact that $[Na^+]_i$ rises by only a modest amount after Na^+–K^+ pump inhibition (Jacob *et al.* 1987), implying that the Na^+ reversal potential, E_{Na}, is much more positive than E_m when $[Na^+]_o$ = 27 mM, we may infer that even a modest hyperpolarization should carry E_m negative to the most negative ionic reversal potential, which is E_K.

Exposing strands to a $[K^+]_o$ of 24 mM plus 0·1 mM-ouabain resulted in a rapid

depolarization with E_m settling to $-36{\cdot}6 \pm 0{\cdot}9$ mV ($n = 9$) after 3 min. At this time, exposure to an $[Na^+]_o$ of 27 mM caused only a small hyperpolarization of $5{\cdot}2 \pm 0{\cdot}3$ mV ($n = 3$, Fig. 1). There are two possible reasons why this hyperpolarization was so much less than that seen in a $[K^+]_o$ of 5·4 mM. The hyperpolarizations could in both

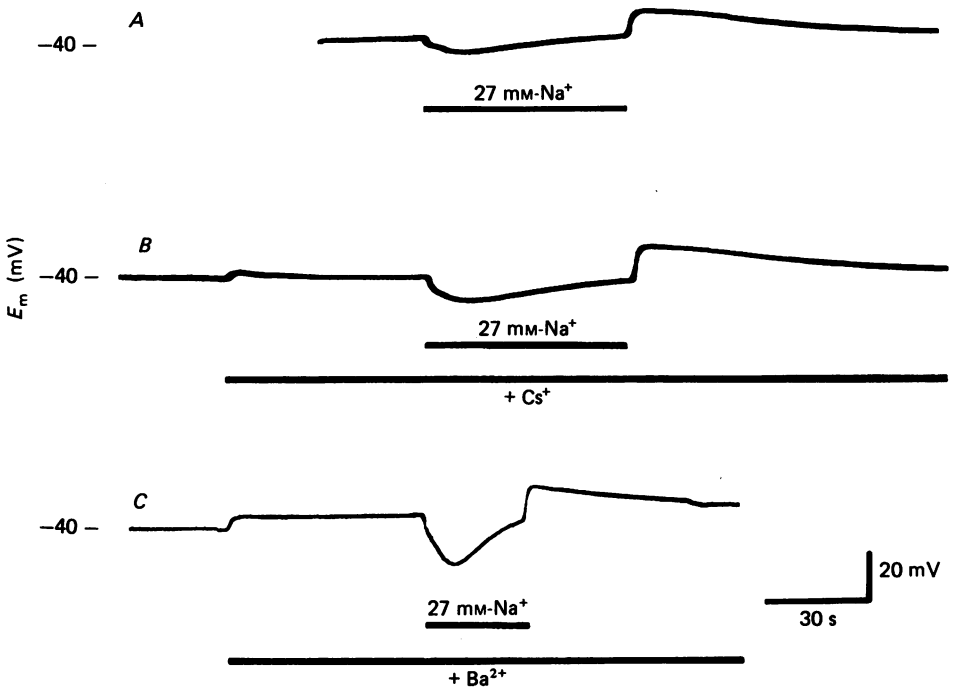

Fig. 1. Records of the hyperpolarizations caused by lowering $[Na^+]_o$ to 27 mM after 3 min in a $[K^+]_o$ of 24 mM plus ouabain (0·1 mM). *A*, control. *B*, 10 mM-Cs^+ present from 2 min onwards. *C*, 1 mM-Ba^{2+} present from 2 min onwards.

cases be due to electrodiffusive movements e.g. due to an increase in electrodiffusive K^+ permeability (P_K), but in a $[K^+]_o$ of 24 mM E_K is much closer to E_m than in a $[K^+]_o$ of 5·4 mM. Alternatively, the hyperpolarization could be due to an electrogenic exchange but because $[K^+]_o$ is high, the membrane conductance is high and so the resulting hyperpolarization is small. One way to distinguish between these two alternatives is to add agents that block electrodiffusive cation pathways. According to the first hypothesis, an appropriate blocker should further decrease the hyperpolarization (cf. the effects of Cs^+ and Ba^{2+} reported in the preceding article; Jacob *et al.* 1987). According to the second hypothesis, a blocker should increase the hyperpolarization since it will decrease the membrane conductance.

Low-$[Na^+]_o$ hyperpolarization in the presence of Cs^+ and Ba^{2+}. The effect of Cs^+ was tested because it has been reported to block K^+ channels (Isenberg, 1977*a*) and Na^+ channels (e.g. DiFrancesco, 1982). When 10 mM-Cs^+ was added at 2 min it caused a small transient depolarization (Fig. 1), presumably due to electrodiffusion, but the presence of Cs^+ did not affect the E_m at 3 min ($36{\cdot}5 \pm 0{\cdot}4$ mV, $n = 3$): the hyper-

polarization was potentiated ($P < 0.005$) to 9.6 ± 0.7 mV, $n = 3$ (Fig. 1), suggesting that Na^+–Ca^{2+} exchange is electrogenic. However, measurement of a^i_K at 3 min showed that E_K was -47.0 ± 0.3 mV ($n = 7$) so that E_m was not hyperpolarizing beyond E_K.

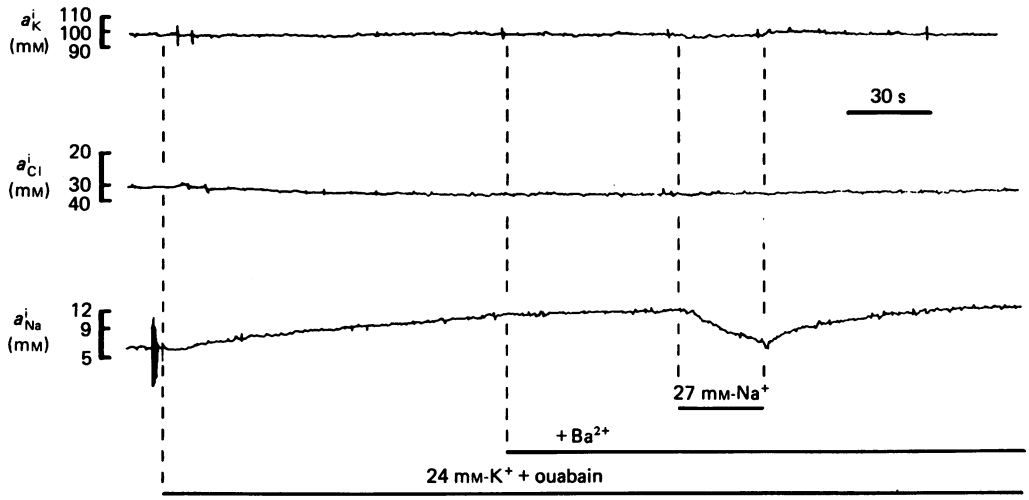

Fig. 2. Records of a^i_K, a^i_{Cl} and a^i_{Na} during exposure to a $[K^+]_o$ of 24 mM plus ouabain (0·1 mM) with $[Na^+]_o$ being lowered to 27 mM at 3 min for 30 s. 1 mM-Ba^{2+} was present from 2 min onwards. Records for each ion were obtained in separate experiments.

Recently, Ba^{2+} has been reported to block P_K (Cohen, Falk & Mulrine, 1983; DiFrancesco, Ferroni & Visentin, 1984) and there is less controversy over its role than for Cs^+. Ba^{2+} has been reported to block Na^+–Ca^{2+} exchange (see Discussion) but content measurements showed that it has no effect under these conditions (see below). Addition of 1 mM-Ba^{2+} at 2 min caused a small depolarization so that at 3 min E_m was -32.5 ± 2.3 mV ($n = 3$). The low-$[Na^+]_o$ hyperpolarization increased from 3.9 ± 1.3 mV ($n = 3$) in the absence of Ba^{2+} to 29.8 ± 2.5 mV ($n = 3$) in the presence of Ba^{2+} (Fig. 1) so that the most negative E_m reached was -62.3 ± 1.5 mV ($n = 3$), very significantly ($P < 0.0005$) negative to the previously measured E_K. To check for any involvement of residual Na^+–K^+ pump activity, the experiment was repeated with the concentration of ouabain being raised to 1·1 mM at 2 min: E_m hyperpolarized to -57.3 ± 1.5 mV ($n = 3$), still well beyond E_K ($P < 0.005$). When E_m and E_K were measured simultaneously in the presence of Ba^{2+}, E_m hyperpolarized significantly ($P < 0.005$, paired t test) negative to E_K by 17.0 ± 2.9 mV ($n = 4$).

Because of the possibility of an intracellular Ca^{2+}-activated Cl^- conductance (e.g. Owen, Segal & Barker, 1984) a^i_{Cl} was measured after 3 min in a $[K^+]_o$ of 24 mM plus ouabain and the Cl^- reversal potential, E_{Cl}, was determined to be -34.3 ± 1.3 mV ($n = 7$) so that the hyperpolarizations could not be accounted for by a Cl^- conductance change. When exposed to an $[Na^+]_o$ of 27 mM for 30 s at 3 min there was a small drop in a^i_{Cl}, of 2.5 ± 0.6 mM ($n = 3$).

When E_m was maximally hyperpolarized the Na^+ reversal potential, E_{Na} (calcu-

lated from a^i_{Na}) was 23·5±0·8 mV (n = 13) thus completing the trio of observations that E_m hyperpolarized negative to E_K, E_{Na} and E_{Cl}. Records of intracellular activity measurements are shown in Fig. 2. Note that the decline in a^i_{Na} during the 30 s exposure to low $[Na^+]_o$ could account for the decay of the concomitant hyperpolarization since the gradient for Na^+–Ca^{2+} exchange is being dissipated as $[Na^+]_i$ falls.

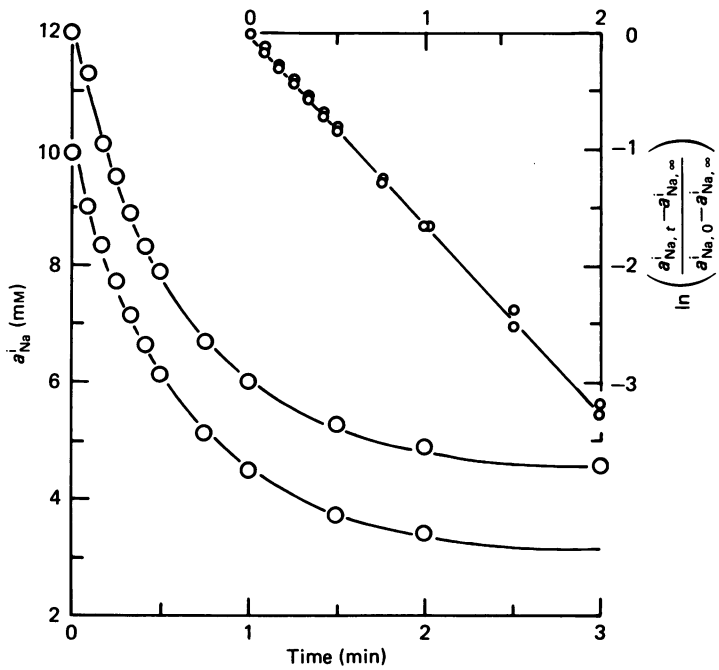

Fig. 3. The exponential decay of a^i_{Na} on lowering $[Na^+]_o$ to 27 mM after 3 min exposure to a $[K^+]_o$ of 24 mM plus ouabain (0·1 mM). 1 mM-Ba^{2+} was present from 2 min onwards. The inset (upper right) shows a semilogarithmic plot of the decay. The values of a^i_{Na} at time (t) = ∞ ($a^i_{Na,\infty}$) were set at 4·6 and 3·15 mM for the upper and lower curves respectively. The time constant for the decay is 36 s.

Movements of Na^+ recorded by ion-selective micro-electrode. In two instances, the decline of a^i_{Na} on exposure to low $[Na^+]_o$ was followed for long enough to establish that the decay followed a simple exponential time course (Fig. 3). We also measured the rate of decay as a function of varying values of low $[Na^+]_o$ (Fig. 4*A*): the rate of Na^+ efflux showed no saturation with respect to the lowering of $[Na^+]_o$. In cardiac sarcolemmal vesicles, the effect of $[Na^+]_o$ on Ca^{2+} uptake suggests a site on the Na^+–Ca^{2+} exchanger which can bind either one Ca^{2+} ion or one or two Na^+ ions (Reeves & Sutko, 1983). This was indicated by a Hill plot with two linear segments characteristic of multisite competitive inhibition. Our data do not show this type of behaviour. The Dixon plot was linear when $[Na^+]_o$ was plotted on the abscissa (Fig. 4*B*) and the Hill plot was a straight line with a slope close to 1 (Fig. 4*C*). The apparent inhibition constant, K_i, for inhibition of Na^+–Ca^{2+} exchange by external Na^+ was equal to 22 mM.

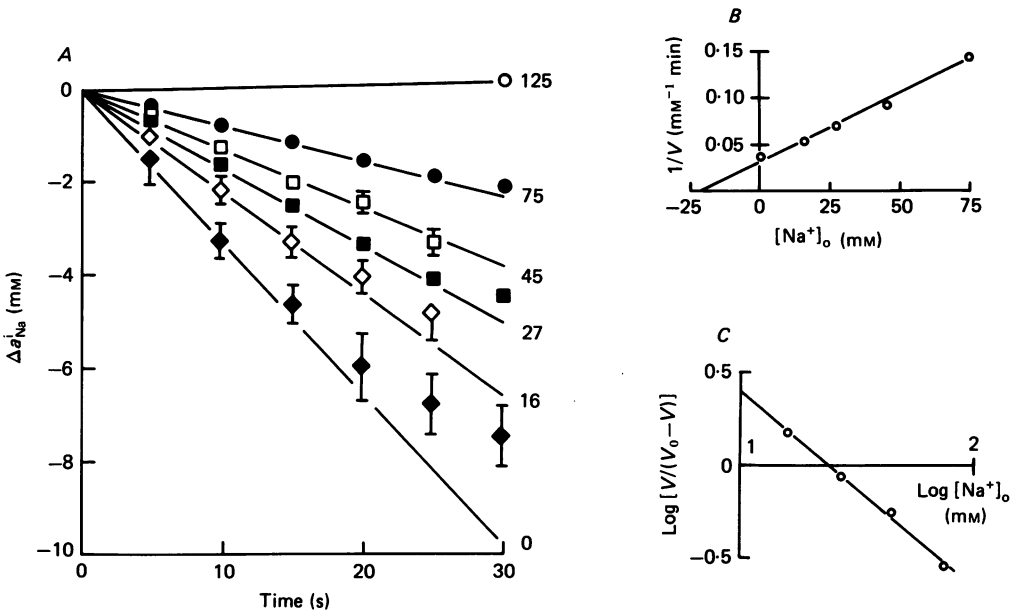

Fig. 4. *A*, the decay of a^i_{Na} on lowering $[Na^+]_o$ to various values (indicated by the numbers on the right in mM) after 3 min in a $[K^+]_o$ of 24 mM plus ouabain (0·1 mM). 1 mM-Ba^{2+} was present from 2 min onwards. Each point is the mean of three determinations: where not shown, the standard error lies within the symbol size. The regression lines were fitted for the first 10 s for $[Na^+]_o$ = 0 mM, for the first 15 s for $[Na^+]_o$ = 16 mM, and for the first 20 s for $[Na^+]_o$ = 27–75 mM. *B*, Dixon plot of the data. The intercept (i.e. the apparent K_i) is 22 mM and the correlation coefficient is 0·994. The maximum rate of decrease of a^i_{Na} (V_0) calculated from this plot is 30·9 mM min^{-1}. *C*, Hill plot of the data. *V* is the rate of decrease of a^i_{Na} determined from the slope of the regression line: V_0 is the rate of decrease of a^i_{Na} in Na^+-free solution, calculated from the Dixon plot above. A correction was made for the slight increase in a^i_{Na} when $[Na^+]_o$ was kept constant at 125 mM. The slope of the Hill plot is 1·06±0·05 and the correlation coefficient is 0·997.

The initial rate of decay of a^i_{Na} calculated for the first 15 s in an $[Na^+]_o$ of 27 mM was 12·0±0·8 mM min^{-1} (n = 12) which corresponds to an initial efflux rate of 29±2 pmol cm^{-2} s^{-1}, calculated using a volume-to-surface ratio of $1{\cdot}06 \times 10^{-4}$ cm (Horres, Lieberman & Purdy, 1977). To assess the correlation between activity and content measurements, $[Na^+]_i$ values were calculated from a^i_{Na} for various times during the experiment (Table 1).

High-$[Ca^{2+}]_o$ hyperpolarization. Exposure to a $[Ca^{2+}]_o$ of 13·5 mM at 3 min caused a very small hyperpolarization of 1·2 mM (n = 2) that was only slightly larger (3·6±0·3 mV, n = 3) in the presence of 1 mM-Ba^{2+}.

Effects of various agents on the low-$[Na^+]_o$ hyperpolarizations. The effects of various agents on the low-$[Na^+]_o$ hyperpolarization were investigated. In each case, 1 mM-Ba^{2+} was present from 2 min onwards.

Cultured heart cells have a very active Na^+–H^+ exchange (Piwnica-Worms *et al.* 1985; Frelin, Vigne & Lazdunski, 1985). To check for possible involvement of Na^+–H^+ exchange (presumably an indirect involvement since Na^+–H^+ exchange is electroneutral), hyperpolarizations were measured in the presence of amiloride.

Amiloride (0·1 mM) added at 3 min had no effect (control: 21·6 ± 0·6 mV, $n = 3$; with amiloride: 20·1 ± 0·9 mV, $n = 3$; $P > 0·1$) even though this dose rapidly inhibits Na^+–H^+ exchange by 98% (Piwnica-Worms *et al.* 1985). Similarly, 1 mM-amiloride added at 2 min (i.e. 1 min prior to and during exposure to low $[Na^+]_o$) had no significant effect (control: 17·7 ± 1·2 mV, $n = 5$; with amiloride: 14·6 ± 0·8 mV, $n = 3$; $P > 0·2$).

TABLE. 1. Comparison of $[Na^+]_i$ (mM) calculated from contents and activities in polystrand and confluent layer cultures of heart cells

Time (min)	0	3	3·5	4
From confluent layer contents	10·5 ± 0·8 (16)	17·0 ± 1·2 (16)	10·6 ± 1·0 (6)	7·7 ± 0·7 (15)
From polystrand contents	8·9 ± 0·6 (6)	13·3 ± 0·4 (6)	8·5 ± 0·4 (3)	6·8 ± 0·6 (3)
From polystrand activities	8·1 ± 0·4 (15)	15·2 ± 0·4 (15)	8·8 ± 0·4 (12)	—

Contents and activities were measured in control solution (0 min), after 3 min in a $[K^+]_o$ of 24 mM plus 0·1 mM-ouabain (3 min) and after a further 0·5 and 1 min in an $[Na^+]_o$ of 27 mM (3·5 and 4 min). Volumes used in calculating the activities were 4·29 ± 0·39 μl mg dry wt.$^{-1}$ for the polystrand (calculated from Horres *et al.* 1977) and 8·32 ± 0·82 μl mg protein^{-1} (Gaynes, Lobaugh & Lieberman, 1985) for the confluent layer cultures. The errors of the volume measurements were not compounded into the errors quoted above. Activities were converted into concentrations using an activity coefficient of 0·735, calculated using the extended Debye–Hückel equation (Robinson & Stokes, 1965).

Verapamil (10 μg ml^{-1}) did not affect the hyperpolarization (control: 21·6 ± 0·6 mV, $n = 5$; with verapamil: 22·8 ± 0·8, $n = 3$; $P = > 0·1$).

Both La^{3+} and Mn^{2+} have been reported to block Na^+–Ca^{2+} exchange (see Discussion) and when added simultaneously with the low-$[Na^+]_o$ solution they both blocked the hyperpolarization sufficiently so that E_m no longer hyperpolarized negative to E_K (for La^{3+} see Fig. 5; for Mn^{2+} see Fig. 7). In the presence of La^{3+} (1 mM) the hyperpolarization was reduced ($P < 0·01$) from 18·3 ± 1·6 mV ($n = 5$) to 7·5 ± 0·2 mV ($n = 3$) while Mn^{2+} reduced ($P < 0·005$) the hyperpolarization from 17·7 ± 1·2 mV ($n = 3$) to 7·5 ± 0·2 mV ($n = 3$). As reported previously (Jacob *et al.* 1987), La^{3+} appeared to potentiate the low-$[Na^+]_o$ contracture since it proved very difficult to maintain an impalement in its presence.

Content data

Na^+ and Ca^{2+} content changes in polystrands. Changes in the Na^+ and Ca^{2+} contents of polystrands were measured to confirm the existence of Na^+–Ca^{2+} exchange following exposure to an $[Na^+]_o$ of 27 mM. Fig. 6*A* shows the average of results obtained from three cultures. In two experiments, Ba^{2+} was added in the usual manner. In one of these experiments, the drop in Na^+ content between 3 and 4 min was measured in the presence and absence of Ba^{2+}: since there was no significant difference ($P > 0·1$) results with and without Ba^{2+} were pooled. Although the fall in Na^+ content during 1 min of exposure to an $[Na^+]_o$ of 27 mM was consistent from week to week, the rise in Ca^{2+} content was variable (5·4 ± 2·1, $n = 3$; 2·9 ± 1·4, $n = 3$;

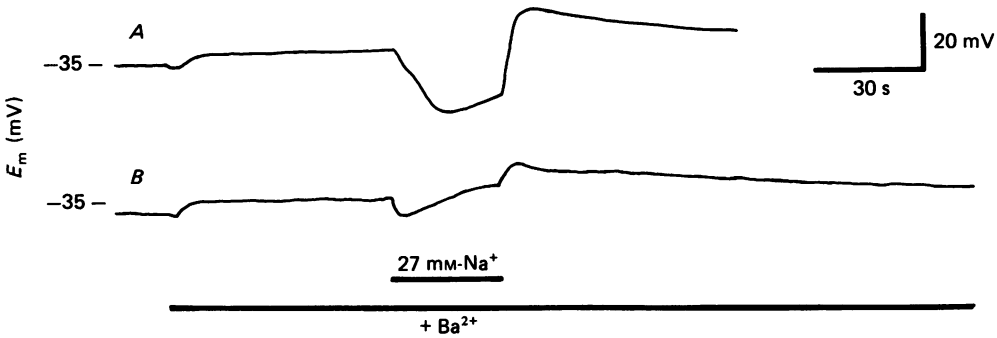

Fig. 5. Effect of La^{3+} on the hyperpolarization caused by lowering $[Na^+]_o$ to 27 mM after 3 min in a $[K^+]_o$ of 24 mM plus ouabain (0·1 mM). 1 mM-Ba^{2+} was present from 2 min onwards for both traces. *A*, control. *B*, 1 mM-La^{3+} present during the exposure to low $[Na^+]_o$.

16·0 ± 2·2, $n = 4$, all in nmol mg dry wt.$^{-1}$: significant at $P < 0·05$, $P < 0·1$ and $P < 0·005$, respectively). Accordingly the errors for the averaged Ca^{2+} content values are large leading to a large error for the increase in Ca^{2+} content between 3 and 4 min even though significant increases in Ca^{2+} content were recorded in the individual experiments. Because E_{Na} is positive to E_m during these experiments, the fall in Na^+ content is due to an outward movement of Na^+ which is counter to its electrochemical gradient.

In one of these experiments, changes in Na^+ content were measured not only after 1 min exposure to an $[Na^+]_o$ of 27 mM but also after 1 min exposure to a $[Ca^{2+}]_o$ of 13·5 mM. The decrease in Na^+ content induced by exposure to the high-$[Ca^{2+}]_o$ solution was only 28 ± 19% ($n = 3$) of that induced by exposure to the low-$[Na^+]_o$ solution.

In another experiment, Ca^{2+} content was recorded at 5 min i.e. 1 min after $[Na^+]_o$ had been restored to its normal value. During this phase, Ca^{2+} content fell from 43·1 ± 1·0 ($n = 4$) to 29·2 ± 2·6 ($n = 4$) nmol mg dry wt.$^{-1}$ ($P < 0·005$) representing a net outward movement of Ca^{2+} against its electrochemical gradient.

$[Na^+]_i$ may be calculated from the Na^+ content using a volume of 4·29 ± 0·39 μl mg dry wt.$^{-1}$ (recalculated from the data of Horres *et al.* 1977). The average values of $[Na^+]_i$ calculated in this manner are shown in Table 1.

Na^+ and Ca^{2+} content changes in confluent layer cultures. Similar results were obtained when confluent layer cultures were put through the same manoeuvres as polystrands (Fig. 6*B*). Some of the contents were measured in the presence of Ba^{2+} but since Ba^{2+} did not significantly affect the movements of Na^+ and Ca^{2+} (see below) these data were pooled. As with polystrands, exposure to low $[Na^+]_o$ caused a countergradient efflux of Na^+ and when $[Na^+]_o$ was subsequently re-established there was a countergradient efflux of Ca^{2+} ($P < 0·025$). The average ratio of the changes in Na^+ and Ca^{2+} contents, measured after 0·5 or 1 min exposure to an $[Na^+]_o$ of 27 mM, was 3·3 ± 0·4 ($n = 20$) whilst the average ratio measured during the 1 min after re-establishing $[Na^+]_o$ was 6 ± 1 ($n = 7$).

When confluent layer cultures were exposed to a $[Ca^{2+}]_o$ of 13·5 mM for 0·5 or 1 min,

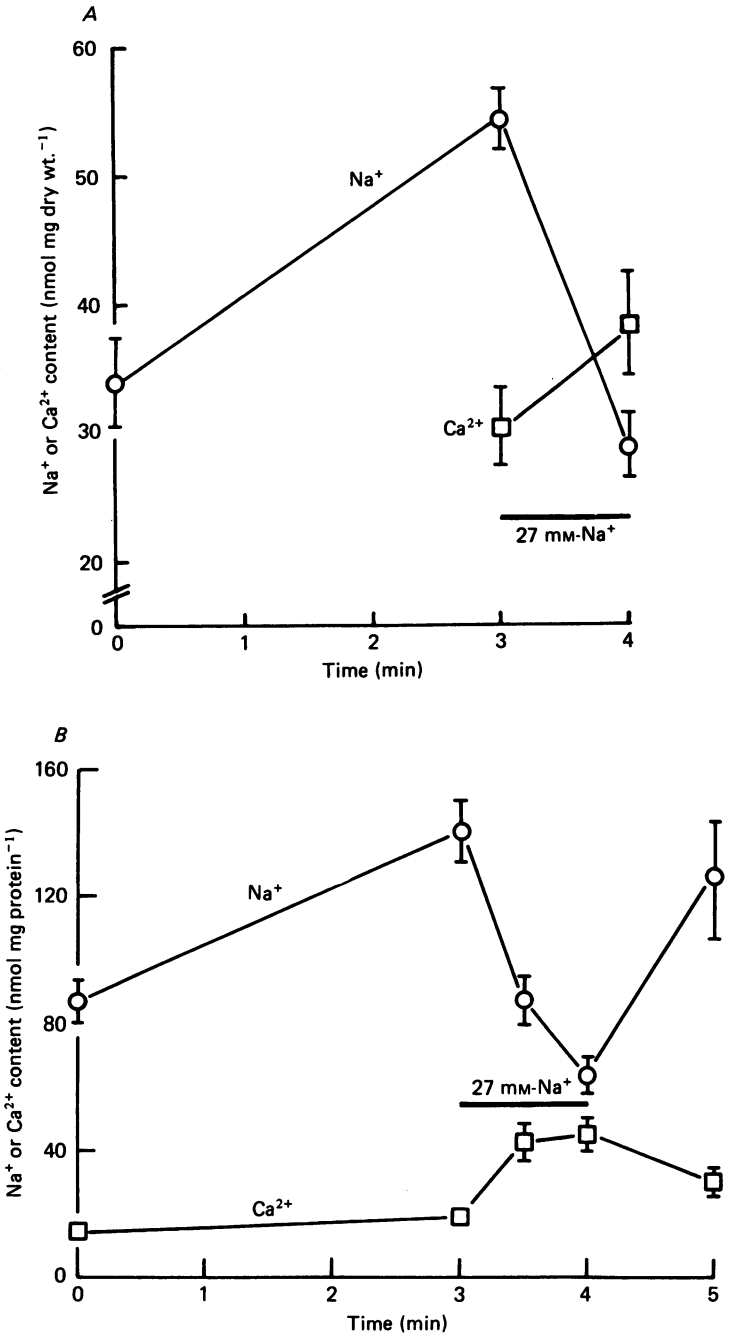

Fig. 6. Na^+ and Ca^{2+} content changes measured on exposure to a $[K^+]_o$ of 24 mM plus ouabain (0·1 mM) with $[Na^+]_o$ being lowered to 27 mM between 3 and 4 min. ○, Na^+ content; □, Ca^{2+} content. Where not indicated, the standard error lies within the symbol size. *A*, measurements on polystrands ($n = 3$). *B*, measurements on confluent layer cultures. $n = 6$ or 7 for the 3·5 and 5 min points: $n = 13$–16 for the other points. The fall in Ca^{2+} content between 4 and 5 min was significant at $P < 0·005$ (paired t test).

the drop in Na^+ content was $25 \pm 11\%$ ($n = 5$) of that induced by exposure to low $[Na^+]_o$: the rise in Ca^{2+} content, however, was comparable ($81 \pm 16\%$, $n = 5$) with that induced by exposure to low $[Na^+]_o$.

Effects of various agents on Na^+ and Ca^{2+} movements. The effects of various agents on Na^+–Ca^{2+} exchange were tested (see Discussion for references to the actions of

TABLE 2. Effects of various agents on movements of Na^+ and Ca^{2+} during Na^+–Ca^{2+} exchange in confluent layer cultures

	Ca^{2+} entry mode		Ca^{2+} exit mode	
	Na^+ movement	Ca^{2+} movement	Na^+ movement	Ca^{2+} movement
Verapamil (20 μM)	100±5 (4)	91±8 (4)	97±8 (4)	104±3 (4)
Amiloride (0·1 mM)	102±5 (4)	72±3*** (3)	52±8*** (3)	97±26 (3)
Amiloride (1 mM)	102±12 (4)	70±6*** (4)	80±15 (3)	103±13 (3)
Quinidine (1 mM)	81±6** (5)	64±7*** (5)	66±14 (3)	18±30* (3)
Ba^{2+} (1 mM)	91±8 (3)	85±14 (3)	—	—
Mn^{2+} (20 mM)	45±5*** (4)	−7±9*** (3)	83±12 (3)	113±15 (3)
La^{3+} (1 mM)	31±9*** (4)	−6±5*** (4)	98±12 (4)	149±26 (3)
La^{3+} (50 μM)	55±7*** (3)	42±8*** (3)	—	—
Tetrodotoxin (16 μM)	—	—	106±18 (3)	90±3** (3)

The results are expressed as a percentage of the movement observed in the absence of any agent. To allow for the multiple comparisons a modified t statistic was calculated using the Bonferroni method (Wallenstein, Zucker & Fleiss, 1980). The values of n (in parentheses) refer to the number of individual experiments: within each experiment, three to six culture dishes were used to determine each content value. Movements of Na^+ and Ca^{2+} in the Ca^{2+} entry mode were measured during a 1 min exposure to an $[Na^+]_o$ of 27 mM after 3 min of Na^+–K^+ pump inhibition in a $[K^+]_o$ of 24 mM plus ouabain (0·1 mM). $[Na^+]_o$ was then re-established and movements of Na^+ and Ca^{2+} in the Ca^{2+} exit mode were made during the subsequent minute (i.e. between 4 and 5 min). Drugs were applied only during the period when the ion movements were being measured (i.e. either between 3 and 4 min or between 4 and 5 min). * $P < 0.1$; ** $P < 0.05$; *** $P < 0.01$.

these agents). The agents were chosen for the following reasons. Verapamil has been reported to block Ca^{2+} movements through the slow inward channel and, at higher concentrations, to block Na^+–Ca^{2+} exchange. Amiloride blocks Na^+–H^+ exchange and does so maximally in these cells at 0·1 mM: at higher concentrations (~ 1 mM) amiloride has been reported to block Na^+–Ca^{2+} exchange. Quinidine, La^{3+}, Ba^{2+} and Mn^{2+} have all been reported to block Na^+–Ca^{2+} exchange and TTX is a well-established blocker of Na^+ movements through the fast sodium channel. The results of adding these agents to confluent layer cultures are shown in Table 2 where the values of n refer to the number of separate experiments used to determine the percentages. In just one experiment, dichlorobenzamil (DCB) was tested: DCB is an amiloride analogue which has been reported to be more potent than amiloride as an inhibitor of Na^+–Ca^{2+} exchange. DCB at 0·1 mM had no significant effect on movements of Na^+ and Ca^{2+} during 1 min exposure to an $[Na^+]_o$ of 27 mM (values were 94 ± 10 and $85 \pm 9\%$ of control respectively, $n = 3$) and no significant effect on the Ca^{2+} loss during 1 min after re-establishing $[Na^+]_o$ ($84 \pm 20\%$ of control, $n = 3$): Na^+ uptake, however, was reduced to $63 \pm 10\%$ ($n = 3$) of its control value. The effect of Mn^{2+} on Na^+ movements was also tested using ion-selective micro-electrodes on

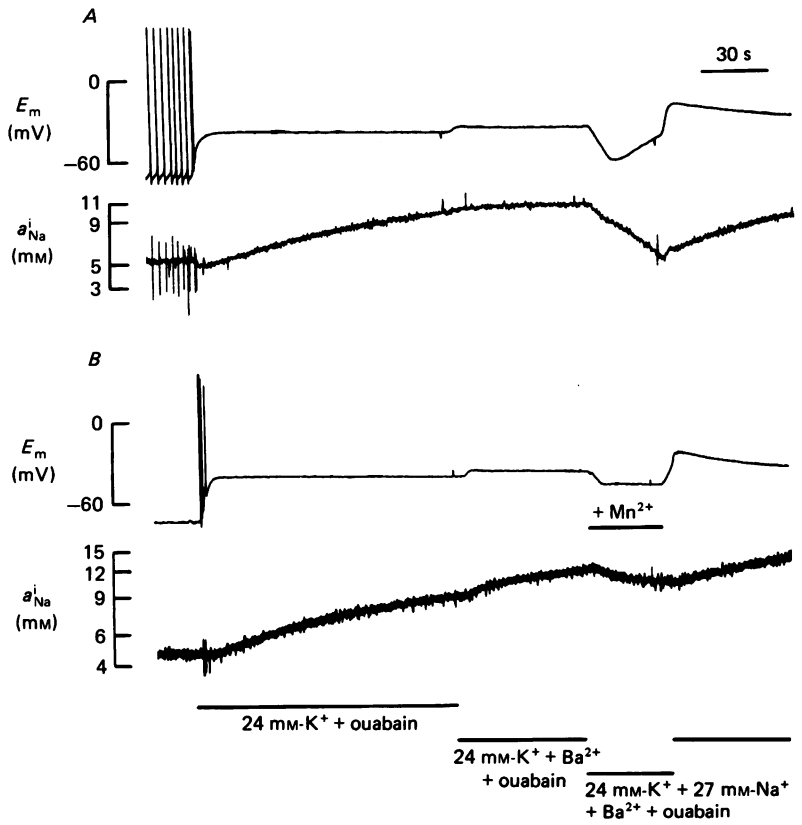

Fig. 7. Effect of 20 mM-Mn^{2+} on the decay of a^i_{Na} induced by lowering $[Na^+]_o$ to 27 mM after 3 min in a $[K^+]_o$ of 24 mM plus ouabain (0·1 mM). 1 mM-Ba^{2+} was present from 2 min onwards in both cases. *A*, effect of lowering $[Na^+]_o$ in the absence of Mn^{2+}. *B*, effect of lowering $[Na^+]_o$ with 20 mM-Mn^{2+} present during the exposure to low $[Na^+]_o$.

polystrands (Fig. 7). In the presence of 20 mM-Mn^{2+}, the drop in a^i_{Na} was 41 ± 4% ($n = 6$) of that in the absence of Mn^{2+}.

DISCUSSION

Electrogenicity of Na^+–Ca^{2+} exchange

In the previous article (Jacob *et al.* 1987) we reported that after Na^+–K^+ pump inhibition in a $[K^+]_o$ of 0 or 5·4 mM, exposure to low $[Na^+]_o$ caused a hyperpolarization. Disparities between the hyperpolarization and the accompanying changes in Na^+ and Ca^{2+} contents prevented us from concluding that the two observations were unequivocally linked. In this paper we have sought to demonstrate the electrogenicity of Na^+–Ca^{2+} exchange by altering the conditions so that a hyperpolarization could carry E_m negative to the reversal potentials for the major cations. This criterion eliminates ion movement by passive diffusion as the source of that part of the hyperpolarization which is negative to the most negative reversal potential.

To achieve this goal, we carried out experiments in a $[K^+]_o$ of 24 mM so that E_m

would initially be approximately equal to E_K. However, under these circumstances, exposure to an $[Na^+]_o$ of 27 mM produced only a small hyperpolarization ($\sim$ 5 mV), much less than that observed in low-$[K^+]_o$ solutions ($\sim$ 25 mV). If the hyperpolarization was due to an electrogenic exchange then increasing membrane resistance (R_m) by blocking cation channels should increase the hyperpolarization; this is what happened in the presence of 10 mM-Cs^+ although intracellular K^+ activity, a_K^i, measurements showed that E_m still did not hyperpolarize beyond E_K. If the action of Cs^+ is to block a K^+ channel, as has been reported by Isenberg (1979*a*), then these results strongly suggest the presence of an electrogenic exchange. However, if the action of Cs^+ is to block a Na^+ channel i.e. the pace-maker current, i_f, as has been reported by DiFrancesco (1982), then the hyperpolarization could still represent no more than a shift in E_m towards E_K with E_m getting closer to E_K in the presence of Cs^+ because of the reduced permeability to Na^+, P_{Na}.

The question was resolved by the use of Ba^{2+} in the presence of which E_m hyperpolarized negative to E_K, E_{Na} and E_{Cl}. Initially we hesitated to use Ba^{2+} because it blocks Na^+–Ca^{2+} exchange in cardiac sarcolemmal vesicles (Bartschat & Lindenmeyer, 1980; Trosper & Philipson, 1983). However, content measurements showed that Ba^{2+} did not have an inhibitory effect in these experiments. In cardiac muscle, the main action of Ba^{2+} is the block of i_{K1} (e.g. in calf Purkinje fibres, DiFrancesco *et al.* 1984) and i_{K1} has been identified as a Ca^{2+}-sensitive channel (in sheep Purkinje fibres, Isenberg, 1977*b*). The block of Ca^{2+}-sensitive K^+ channels by Ba^{2+} has also been substantiated in other preparations e.g. skeletal muscle (Vergara & Latorre, 1983) and pancreatic acinar cells (Iwatzuki & Petersen, 1985). Other components of K^+ permeability in cardiac muscle are also blocked by Ba^{2+} (Cohen *et al.* 1983) but the exact nature of the action of Ba^{2+} is not relevant to the interpretation of our results.

Although raising $[Ca^{2+}]_o$ after 3 min of pump inhibition caused a hyperpolarization, the magnitude of the hyperpolarization was small and so could not be positively identified as being due to an electrogenic Na^+–Ca^{2+} exchange. The small magnitude of the hyperpolarization was consistent with the small magnitude of the accompanying Na^+ content changes.

Two possible artifacts must be considered when identifying the low-$[Na^+]_o$ hyperpolarization as proof of an electrogenic Na^+–Ca^{2+} exchange. The first relates to the intermediate sensitivity of chick cardiac muscle to ouabain coupled with the high level of $[K^+]_o$ which will tend to inhibit ouabain binding. From measurements of ouabain binding to confluent layer cultures (Lobaugh & Lieberman, 1985) and assuming simple competitive inhibition between external K^+ and ouabain (Baker & Willis, 1970), we estimate that the fraction of unbound Na^+–K^+ pump sites in 0·1 mM-ouabain was no greater than 10%. It is possible that the reduction in $[Na^+]_o$ could have activated some of these unbound sites if external Na^+ competes weakly with external K^+ at the extracellular site, as suggested by Lindenmeyer, Schwartz & Thompson (1974). Using their constants (measured on beef brain ATPase) we calculate that lowering $[Na^+]_o$ to 27 mM would have resulted in no more than a 10% stimulation of the residual unbound sites. Assuming that the maximum velocity (V_{max}) for the Na^+–K^+ pump in polystrands is the same as that in confluent layer cultures ($\sim$ 150 nmol mg protein^{-1} min^{-1}, Lobaugh, 1986), then the Na^+ flux due to this effect would have been $\sim$ 0·3 pmol cm^{-2} s^{-1} in 0·1 mM-ouabain. This would not have had a significant effect for two reasons. First, the measured Na^+ flux was 100 times this value. Secondly, for this flux to have been responsible for the hyperpolarizations, R_m would have had to have been 0·5 MΩ cm^2 in the absence of Ba^{2+} and 3 MΩ cm^2 in the presence of Ba^{2+}. These are unphysiologically high values for R_m which, in cultured chick heart cells, ranges from 26 to 50 kΩ cm^2 for membrane potentials between −20 and

−60 mV (Ebihara & Mathias, 1985). Additional evidence for the lack of involvement of the Na^+–K^+ pump in these hyperpolarizations is provided by the small change in hyperpolarization when the experiment was repeated in 1·1 mM-ouabain. This elevenfold increase in ouabain concentration would have resulted in an approximately elevenfold decrease in the number of residual unbound sites.

Recently, an allosteric effect of external Na^+ on the binding of intracellular Na^+ to the inside Na^+–K^+ pump site has been reported by Karlish & Stein (1985) for pig kidney ATPase. We estimate that a low-$[Na^+]_o$ activation of the Na^+–K^+ pump by this mechanism would produce less effect than that discussed above.

The second potential artifact is that if K^+ is taken up when $[Na^+]_o$ is lowered then any K^+ depletion in the extracellular space would result in E_K being more negative than the value we calculate. In fact exposure to low $[Na^+]_o$ caused a slight decrease in K^+ of ~ 3% in polystrands and ~ 6% in confluent layer cultures (R. Jacob, unpublished observations) so that there could not have been any depletion artifact.

Measurements of Na^+ and Ca^{2+} in polystrands and confluent layer cultures

We have used two types of preparation, the polystrand and the confluent layer culture. The Na^+ content measurements on these two preparations produced very similar results when compared by converting to concentrations using the appropriate cell volumes (Table 1). When the errors in the volume determinations are taken into account, the $[Na^+]_i$ calculated for the two preparations are not significantly different. The polystrand and confluent layer cultures also responded in the same way to exposure to a $[Ca^{2+}]_o$ of 13·5 mM which in both cases caused a fall in Na^+ content that was ~ 25% of the fall caused by exposure to low $[Na^+]_o$.

The equality of contents does not extend to Ca^{2+} which for polystrands was usually in the region of 20–30 nmol mg dry wt.$^{-1}$ (R. Jacob, unpublished observation) compared to a mean content for confluent layer cultures of $14·5 \pm 1·8$ nmol mg protein^{-1}. Using a conversion of ~ 0·5 mg protein mg dry wt.$^{-1}$ (based on the volume determinations), this means that the polystrands contained about four times more Ca^{2+} per unit volume than did the confluent layer cultures. Qualitatively, however, the polystrand and confluent layer cultures exhibited the same behaviour with respect to Ca^{2+} content: exposure to low $[Na^+]_o$ caused an uptake of Ca^{2+} and subsequent re-establishment of $[Na^+]_o$ caused a fall in Ca^{2+} content. From this we may conclude that the basal level of Ca^{2+} did not affect Na^+–Ca^{2+} exchange, at least under the conditions which we used.

The $[Na^+]_i$ determined from contents agreed well with the $[Na^+]_i$ determined from a^i_{Na} (Table 1). Similarly, the percentage inhibition of Na^+ movement by 20 mM-Mn^{2+} determined from confluent layer culture contents (45 ± 5%) was not significantly different from that determined from the polystrand a^i_{Na} measurements (41 ± 4%). The good agreement between the Na^+ content and activity measurements shows that the content measurements are not biased by significant extracellular space contamination. Such contamination is most likely to occur with Na^+ since $[Na^+]_o \gg [Na^+]_i$ and the consequent error would have been particularly invidious because measurements were made in varying external Na^+ concentrations.

Na^+ and Ca^{2+} movements

The efflux of Na^+ which is induced by exposure to low $[Na^+]_o$ is counter to the Na^+ electrochemical gradient, ruling out a passive electrodiffusive movement as the mechanism. The only mechanism with sufficient potential energy to mediate this

efflux is Na^+–Ca^{2+} exchange (see below for a discussion of Na^+–H^+ exchange) and the concomitant increase in Ca^{2+} content supports this contention. Our results also confirm the bidirectional nature of the exchange since re-establishing the normal Na^+ gradient across the cell membrane caused Ca^{2+} to leave the cell against its electrochemical gradient, accompanied by an uptake of Na^+. This loss of Ca^{2+} and uptake of Na^+ was accompanied by a depolarization consistent with an electrogenic exchange. The presence of Na^+–Ca^{2+} exchange is also confirmed by the small but significant countergradient movement of Na^+ in response to raised $[Ca^{2+}]_o$. The decline in the hyperpolarization during the 30 s exposure to low $[Na^+]_o$ is consistent with the rapid decline in $[Na^+]_i$ leading to a decreasing gradient for Na^+–Ca^{2+} exchange.

It is tempting to use content changes to calculate a stoicheiometry and indeed the value we obtain from the ratio of Na^+ and Ca^{2+} movements caused by exposure to low $[Na^+]_o$ ($3{\cdot}3 \pm 0{\cdot}4$) agrees with the prevailing value of 3·0 (Reeves & Hale, 1984). However, we did not attempt to eliminate all alternative pathways for Na^+ and Ca^{2+} movements. This is highlighted by the fact that the 'stoicheiometry' calculated from the ratio of the Na^+ and Ca^{2+} movements following the re-establishment of $[Na^+]_o$ was 6 ± 1.

The kinetic properties of the exchanger do not appear to agree with what one might expect for an exchanger transporting three Na^+ ions per cycle. An exponential decay of a^i_{Na} in low $[Na^+]_o$ was also found in sheep cardiac Purkinje fibres (Ellis, 1977) and implies (with certain assumptions) that the efflux rate is linearly proportional to $[Na^+]_i$. The implied linear dependence of Na^+ flux on $[Na^+]_i$ is all the more surprising when one considers that E_m and probably $[Ca^{2+}]_i$ are also changing at the same time: it contrasts with the cubic dependence found in ferret and guinea-pig ventricular trabeculae (Chapman, Coray & McGuigan, 1983). Similarly, if the main effect of varying $[Na^+]_o$ is mediated by the competition of external Na^+ and Ca^{2+} ions (as appears to be the case with vesicles; Reeves & Sutko, 1983) then the slope of the Hill plot being close to 1 implies that the competition is on a one-to-one basis. However in each case the analysis only provides a lower limit to the number of Na^+ ions involved. Moreover, given the small number of points in the Hill plot, there is still room for more complex behaviour. Although our data shows no complex kinetic behaviour, such behaviour might be revealed in a more detailed study.

Countergradient net movements of Na^+ induced by exposure to low $[Na^+]_o$ or high $[Ca^{2+}]_o$ have been measured in other intact cardiac preparations and used to confirm the presence of Na^+–Ca^{2+} exchange (e.g. Ellis, 1977; Bridge & Bassingthwaite, 1983; Chapman *et al.* 1983). Similarly, a countergradient net Ca^{2+} movement driven by an inward Na^+ gradient has also been reported previously (Reuter & Seitz, 1968).

Na^+–H^+ exchange and the countergradient movement of Na^+

Cultured chick heart cells have a Na^+–H^+ exchange capable of mediating Na^+ fluxes as high as 54 pmol cm^{-2} s^{-1} (Piwnica-Worms *et al.* 1985). The rate of Na^+ transport which we measured on exposure to an $[Na^+]_o$ of 27 mM was 29 pmol cm^{-2} s^{-1}. We must therefore consider whether Na^+–H^+ exchange could have been involved in the countergradient movement of Na^+. There are two reasons why we may dismiss this possibility. First, amiloride at ten times the dose which rapidly inhibits 98 % of the Na^+–H^+ exchange in these cells (Piwnica-Worms *et al.* 1985) did not affect the movement of Na^+ caused by exposure to low $[Na^+]_o$. Secondly, because Na^+–H^+ exchange has a 1:1 stoicheiometry, a value of intracellular pH, pH_i, of greater than 7·6 would have been necessary

for Na^+–H^+ exchange to have been thermodynamically capable of extruding Na^+. Such an alkaline pH is unlikely since the resting pH_i of cultured chick heart cells has been reported to be 7·4 (Frelin, Vigne & Lazdunski, 1985) and after Na^+–K^+ pump inhibition pH_i, if anything, becomes more acid (Deitmer & Ellis, 1980). Furthermore, if pH_i did become sufficiently alkaline for Na^+–H^+ exchange to become thermodynamically capable of extruding Na^+ then the exchange would anyhow be inactivated because of the allosteric effect of pH_i on the rate of Na^+–H^+ exchange (Frelin *et al.* 1985).

Effects of various agents on Na^+–Ca^{2+} exchange

Ca^{2+} channel blocker. Verapamil (20 μM) was tested primarily to see to what extent Ca^{2+} uptake on exposure to low $[Na^+]_o$ might have been mediated by the slow inward channel. Verapamil had no effect on any of the Ca^{2+} or Na^+ movements. This result contrasts with reports that verapamil blocks Na^+–Ca^{2+} exchange in cardiac sarcolemmal vesicles (Bartschat, Cyr & Lindenmeyer, 1980; Ledvora & Hegyvary, 1983; Erdreich & Rahamimoff, 1984) but all these studies used considerably higher concentrations of the drug (100–465 μM).

Amiloride analogues. Amiloride at $\sim$ 1 mM has also been reported to block Na^+–Ca^{2+} exchange in a wide variety of preparations including cardiac sarcolemmal vesicles (Siegl, Cragoe, Trumble & Kaczorowski, 1984; Floreani & Luciani, 1984) and isolated cardiac myocytes (Altschuld, Hohl, Lamka & Brierly, 1984). Since 0·1 mM-amiloride almost totally inhibits Na^+–H^+ exchange in cultured chick heart cells (Piwnica-Worms *et al.* 1985), testing the effects of amiloride at 0·1 and 1 mM should allow us to differentiate its effects on the two exchangers. Neither concentration affected Na^+ loss in low-$[Na^+]_o$ solution but both significantly reduced Ca^{2+} uptake. This could indicate that in low $[Na^+]_o$, Na^+–H^+ exchange is operating as a leak pathway for Na^+ entry and supplying Na^+ to the inside of the cell for exchange with external Ca^{2+}. Blocking Na^+–H^+ exchange would reduce the amount of Na^+ available for exchange with Ca^{2+} and so reduce the Ca^{2+} uptake.

The partial inhibition by 0·1 mM-amiloride of the Na^+ uptake after $[Na^+]_o$ was re-established suggests that some of the Na^+ influx during this phase was via Na^+–H^+ exchange. We cannot explain why 1 mM-amiloride caused only an insignificantly small inhibition of Na^+ uptake.

The amiloride analogue dichlorobenzamil (DCB) is more potent than amiloride as an inhibitor of Na^+–Ca^{2+} exchange with a half-maximum inhibitory dose (IC_{50}) of about 10 μM (Siegl *et al.* 1984; Hume, Kaczorowski & Siegl, 1985). However, in our cells, even at 0·1 mM, DCB did not significantly inhibit Na^+–Ca^{2+} exchange.

Quinidine. Quinidine (1 mM) had a greater effect on Na^+–Ca^{2+} exchange than any of the other organic compounds tested although it by no means showed a clear-cut block of the exchange. Quinidine has been reported to block Na^+–Ca^{2+} exchange in canine cardiac sarcolemmal vesicles (Ledvora & Hegyvary, 1983). In cardiac cells quinidine blocks $[Na^+]_o$-dependent Ca^{2+} uptake (Chapman, Tunstall & Yates, 1984; Desilets & Horackova, 1984; Murphy, Wheeler, LeFurgey, Jacob, Lobaugh & Lieberman, 1986) and partially blocks a current which Mentrard, Vassort & Fischmeister (1984) associate with Na^+–Ca^{2+} exchange. But quinidine has many other effects including the blocking of several other trans-sarcolemmal pathways and the inhibition of Ca^{2+} uptake by mitochondria (Harrow & Dhalla, 1976) and by sarcoplasmic reticulum (Su & Libao, 1984). Since the magnitude of $[Na^+]_o$-dependent

Ca^{2+} uptake will depend on the degree to which $[Ca^{2+}]_i$ is buffered, the effects of quinidine on $[Na^+]_o$-dependent uptake cannot necessarily be attributed to a direct effect on Na^+–Ca^{2+} exchange (Chapman *et al.* 1984; Desilets & Horackova, 1984). Under the conditions reported here, quinidine is not an effective inhibitor of Na^+–Ca^{2+} exchange.

The variability of the results with these putative organic inhibitors and the often high concentrations required to obtain an effect indicate that they are very non-specific in their action. They are also fairly lipophilic and their ability to interact with the Na^+–Ca^{2+} exchanger may depend on its lipid environment.

Divalent and trivalent cations. Because of the extensive use of Ba^{2+} in the electrophysiological experiments and reports that Ba^{2+} inhibits Na^+–Ca^{2+} exchange in cardiac sarcolemmal vesicles (Bartschat & Lindenmeyer, 1980; Trosper & Philipson, 1983), we tested the effect of 1 mM-Ba^{2+} on the Na^+ and Ca^{2+} movements. The lack of effect of Ba^{2+} is not inconsistent with the vesicle data because Ba^{2+} appears to compete with Ca^{2+} (Bartschat & Lindenmeyer, 1980) and vesicle experiments are carried out in very low $[Ca^{2+}]_o$. Even with $[Ca^{2+}]_o = 50\ \mu M$, 1 mM-Ba^{2+} only inhibits Na^+–Ca^{2+} exchange by $\sim 50\%$ so that it is not surprising that Ba^{2+} had no effect in a $[Ca^{2+}]_o$ of 2·7 mM. At 1 mM-Ba^{2+}, we found no evidence of the Na^+–Ba^{2+} exchange reported to be present in sheep cardiac Purkinje fibres at 12 mM-Ba^{2+} (Deitmer & Ellis, 1978).

Both La^{3+} (1 mM) and Mn^{2+} (20 mM) were capable of totally blocking the Ca^{2+} uptake induced by exposure to low $[Na^+]_o$ (as reported, for La^{3+}, by Barry & Smith, 1982) but they could only block 55–70% of the countergradient movement of Na^+. The disparity in the effects of La^{3+} and Mn^{2+} on Na^+ and Ca^{2+} movements may indicate the presence of a La^{3+}- and Mn^{2+}-insensitive Na^+-coupled transport system which enables Na^+ to leave the cell against its electrochemical gradient even when Na^+–Ca^{2+} exchange is blocked. Since E_m fails to hyperpolarize negative to E_K in the presence of La^{3+} and Mn^{2+}, it is the Na^+–Ca^{2+} exchange which is electrogenic rather than any other putative Na^+-coupled transport system.

Inhibition of Na^+–Ca^{2+} exchange in cardiac sarcolemmal vesicles by divalent or trivalent cations has been uniformally observed and is independent of the direction of the exchange (Reeves & Sutko, 1979; Trosper & Philipson, 1983). The actions of multivalent cations on Na^+–Ca^{2+} exchange in whole-cell preparations is less consistent. In frog atrial tissue, 3 mM-La^{3+} or 15 mM-Mn^{2+} blocked Na^+-free contractures (Horackova & Vassort, 1979) and 2 mM-La^{3+} blocked a current associated with Na^+–Ca^{2+} exchange (Mentrard *et al.* 1984). Similarly Hume *et al.* (1985), using frog atrial myocytes, found that 10 μM-La^{3+} blocked a current which they associated with Na^+–Ca^{2+} exchange, irrespective of the polarity of the current. On the other hand, 0·2 mM-La^{3+} did not block $[Na^+]_o$-dependent Ca^{2+} efflux from pig or sheep ventricular trabeculae (Katzung, Reuter & Porzig, 1973) nor did 1–5 mM-La^{3+} block low-$[Na^+]_o$ hyperpolarizations in sheep cardiac Purkinje fibres (Coraboeuf, Gautier & Guiraudou, 1981). In our preparation, neither La^{3+} nor Mn^{2+} had any effect on the movements of Na^+ and Ca^{2+} stimulated by re-establishing $[Na^+]_o$. This could be due to the efficacy of the inhibiting cation being determined by a competition between Na^+, Ca^{2+} and the inhibitor at an external site on the membrane. Na^+ and Ca^{2+} compete for the Na^+–Ca^{2+} exchanger (e.g. Reeves & Sutko, 1983) and Ca^{2+} and La^{3+} compete

(Rahamimoff & Spanier, 1984), implying a competition between all three ions. Thus the ability of La^{3+} to bind to the exchanger could depend on $[Na^+]_o$, as appears to be the case in cardiac sarcolemmal vesicles (Trosper & Philipson, 1983). This could explain our results since Na^+–Ca^{2+} exchange was measured in the Ca^{2+} uptake mode with $[Na^+]_o = 27$ mM and in the Ca^{2+} release mode with $[Na^+]_o = 125$ mM. The discrepancy between results obtained with different preparations may be related to different experimental external Na^+ and Ca^{2+} concentrations: alternatively, the relative affinities for Na^+ and Ca^{2+} may vary according to the type of preparation (see, for example, Wakabayashi & Goshima, 1981).

In conclusion, stimulation of Na^+–Ca^{2+} exchange by manipulations of $[Na^+]_o$ was confirmed by the observation of countergradient movements of both Na^+ and Ca^{2+} that were coupled to a downhill movement of the counter-ion. The hyperpolarization induced by exposure to low $[Na^+]_o$ confirmed that the exchange is electrogenic because E_m could be hyperpolarized negative to the reversal potentials for Na^+, K^+ and Cl^-. Apart from La^{3+} and Mn^{2+}, none of the putative blockers consistently and significantly inhibited both Na^+ and Ca^{2+} movements. The dependence of the effects of La^{3+} and Mn^{2+} on the direction of the exchange could be due to competition between Na^+, Ca^{2+} and the inhibiting cation.

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