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SODIUM–HYDROGEN EXCHANGE IN GUINEA-PIG VENTRICULAR MUSCLE DURING EXPOSURE TO HYPEROSMOLAR SOLUTIONS

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SUMMARY

1. The effect on intracellular pH (pH_i) and intracellular Na^+ activity (a^i_{Na}) of exposure to hyperosmolar solutions was investigated in guinea-pig ventricular muscle using ion-sensitive microelectrodes.

2. Exposure of tissue to solution made hyperosmolar by the addition of 100 mM-sucrose produced an intracellular alkalinization of 0·10 pH units and hyperpolarization of the membrane potential.

3. When extracellular Na^+ was reduced to 15 mM by substitution of NaCl with choline chloride, exposure to hyperosmolar solutions caused a decrease in pH_i. Identical experiments using LiCl as the sodium substitute resulted in an increase in pH_i of a magnitude similar to that seen at physiological Na^+ levels.

4. In the presence of 50 μM-5-(*N*,*N*-dimethyl)amiloride (DMA), an inhibitor of Na^+–H^+ exchange, pH_i decreased upon exposure to hyperosmolar solution.

5. The recovery of pH_i from an intracellular acidosis (induced by brief exposure to NH_4Cl) was enhanced in hyperosmolar solution when compared to recovery in isosmolar solution. This enhancement was observed even when a^i_{Na} was markedly elevated (> 25 mM) by inhibition of the Na^+–K^+ pump.

6. There was an increase in a^i_{Na} during exposure to hyperosmolar solutions. When the Na^+–K^+ pump was inhibited with dihydro-ouabain a component of this increase in a^i_{Na} was sensitive to DMA.

7. We conclude that exposure of cardiac tissue to hyperosmolar solutions results in an intracellular alkalosis due to activation of the sarcolemmal Na^+–H^+ exchanger. Such changes should be considered when exposure to hyperosmolar solutions is used in the study of excitation–contraction coupling and cardiac muscle mechanics.

INTRODUCTION

Studies on red blood cells (Cala, 1980), thymocytes (Grinstein, Cohen, Goetz & Rothstein, 1985; Grinstein & Cohen, 1987), glial cells (Jean, Frelin, Vigne & Lazdunski, 1986) and osteoblasts (Green, Yamaguchi, Kleeman & Muallem, 1988*a*) have demonstrated that an intracellular alkalosis develops during exposure to an hyperosmolar milieu due to activation of the plasmalemmal Na^+–H^+ exchanger. This activation is thought to play a role in cellular volume regulation (see Grinstein *et al.*

1985 for discussion of mechanism). In contrast, however, the intracellular pH (pH_i) of cardiac Purkinje fibres has been reported to decrease during exposure to hyperosmolar solutions (Ellis & Thomas, 1976).

The apparent discrepancy in the response of pH_i to an hyperosmolar milieu between cardiac and non-cardiac cells is of interest for two reasons. Firstly, it suggests the possibility that the mechanism for volume regulation in cardiac cells may be different from that of other cells. One might speculate that such a difference could somehow be related to the existence of an extensive collagen network which binds myocytes together (Caulfield & Borg, 1979) and which can restrict myocyte volume changes in responses to an anisosmolar milieu (Pine, Brooks, Nosta & Abelmann, 1981). Secondly, the effect of an anisosmolar milieu on pH_i is of interest because exposure of cardiac tissue to solutions made hyperosmolar with solutes impermeable to the sarcolemmal membrane has been used in many studies of cardiac contractility and excitation–contraction coupling (see for example Koch-Weser, 1963; Little & Sleator, 1969; Goethals, Adele & Brutsaert, 1975; Chapman, 1978; Lado, Sheu & Fozzard, 1984; Beyer, Jepsen, Lullmann & Ravens, 1986; Allen & Smith, 1987). Exposure to hyperosmolar solutions causes shrinkage of myocytes and loss of osmotically obliged intracellular water. As a consequence, intracellular K^+, Na^+ and Ca^{2+} activities increase (Fozzard & Lee, 1976; Lado *et al.* 1984; Allen & Smith, 1987). These easily reversible ionic changes are important determinants of the contractile properties of the tissue. Intracellular pH is, however, also expected to markedly influence contractility (see Orchard & Kentish, 1990, for a review), and any change in pH_i is expected to be of importance for interpretation of data.

Working myocardium rather than cardiac Purkinje fibres have been used in most of the studies on contractility in hyperosmolar solutions. The decrease of pH_i reported for Purkinje fibres (Ellis & Thomas, 1976) in response to such exposure should not, however, be assumed to be representative of working myocardial tissue. We have therefore studied pH_i of guinea-pig ventricular muscle exposed to hyperosmolar superfusates. We have found that an intracellular alkalosis develops, and we provide evidence indicating that this is due to activation of the sarcolemmal Na^+–H^+ exchanger. Preliminary results have been published (Whalley, Hemsworth & Rasmussen, 1990).

METHODS

Guinea-pigs of either sex, weighing 300–500 g, were killed by cervical dislocation and exsanguination. The heart was rapidly removed and right ventricular papillary muscles or free-running trabeculae, less than 750 μm diameter, were isolated. Specimens were pinned to the Sylgard bottom of a 150 μl Perspex tissue bath. The flow rate of superfusates was maintained at 3 ml min^{-1}. Recordings with ion-sensitive electrodes demonstrated complete exchange of solutions within 10 s. The design of the tissue bath allowed the temperature to be maintained at 35 ± 0.5 °C during change of solutions.

Solutions

The standard modified Tyrode solution contained (in mM): NaCl, 140; KCl, 5·6; $CaCl_2$, 2·16; NaH_2PO_4, 0·44; glucose, 10; $MgCl_2$, 1·0; *N*-2-hydroxyethyl piperazine-*N*-2-ethanesulphonic acid (HEPES), 10 and was titrated with $\simeq$ 5·80 ml of 1 M-NaOH to pH 7.40 ± 0.01 at 35 °C. This solution was bubbled with 100% O_2. Unless otherwise stated, experiments were performed in this nominally HCO_3^--free solution to eliminate the possible contribution of HCO_3^--dependent pH-regulating mechanisms.

In one series of experiments solutions were buffered with bicarbonate. We reduced NaCl to 116 mM and added 24 mM-$NaHCO_3$. These solutions were bubbled with 5% CO_2–95% O_2 for at least 1 h to achieve a pH of $7{\cdot}40 \pm 0{\cdot}05$ units at 35 °C. Loss of CO_2 was minimized by using thick-walled Tygon R-3603 tubing (Norton Co., Akron, OH, USA) to connect the reservoir and tissue bath. Samples of solution were taken from the bath for measurement of pH and pCO_2 on a temperature controlled pH meter (Radiometer ABL-30; Copenhagen Denmark).

Sucrose was added to solutions to increase their osmolarity. The osmolarity of samples of solutions used, measured by freezing-point depression, (Advanced Microosmometer, Model 3NO; Advanced Instruments, MA, USA) increased from $310 \pm 1{\cdot}6$ mosM (mean ± S.E.M.; $n = 16$) in control solutions to $357 \pm 4{\cdot}0$ mosM ($n = 6$), $418 \pm 2{\cdot}0$ mosM ($n = 8$) and $460 \pm 5{\cdot}1$ mosM ($n = 5$) after the addition of 50, 100 and 150 mM-sucrose respectively.

Superfusates containing 5-(*N*,*N*-dimethyl)amiloride (DMA), purchased from Dr E. J. Cragoe, 2211 Oak Terrace Drive, Lansdale, PA, USA, or dihydro-ouabain (DHO), purchased from Sigma Chemical Co., St Louis, MO, USA, were made by dissolving the compounds directly into the solution immediately prior to use.

Microelectrodes

Microelectrodes for measuring membrane potential were made from filamented borosilicate glass tubing (TW100F-4; World Precision Instruments (WPI), New Haven, CT, USA). When filled with 3 M-KCl they had resistance of 10–20 MΩ.

Ion-selective microelectrodes were made from unfilamented borosilicate glass tubing (1B100-4 WPI) and were pulled on a Flaming-Brown type horizontal puller (Sutter P-87; San Rafael, CA, USA). Electrodes were placed in a Petri dish and baked in an oven at 200 °C for 30 min to drive off surface moisture. Ten microlitres of hexamethyldisilazane was added to the dish which was then covered, and the microelectrodes were baked for another hour to achieve silanization. After cooling, the pipettes were back-filled with a solution containing (in mM): NaCl, 100 and HEPES, 20. The solution was titrated to a pH of 7·60 with NaOH. Filling was aided with a cat's whisker and a microforge.

Microelectrodes were then dry bevelled on Imperial Film Lapping Paper (0·3 μm Al_2O_3 grain size; 3M Company, MN, USA) glued to the optically flat disc of a WPI 1300M Micropipette Beveller. The tip of the micropipette was placed in H^+-sensitive (Ammann, Lanter, Steiner, Schulthess, Shijo & Simon, 1981) or Na^+-sensitive (Steiner, Oehme, Ammann & Simon, 1979) resin, and a short (< 100 μm) column was drawn into the tip under microscopic visualization by applying suction to the back of the micropipette.

H^+-sensitive microelectrodes were calibrated in solutions containing (in mM): KCl, 150; NaCl, 10; HEPES, 10. Solutions were titrated to a pH of 6·5 or 7·5 with NaOH. The slope was measured immediately after and usually before experiments. The slope for forty-nine electrodes was $54{\cdot}2 \pm 0{\cdot}6$ mV (pH unit)$^{-1}$. We ascertained that electrodes were not sensitive to K^+ or Na^+ in the concentrations expected to be encountered during experiments.

Na^+-sensitive microelectrodes were calibrated using the reciprocal dilutions method (Vaughan-Jones & Aickin, 1987). The sum of KCl and NaCl concentrations in calibration solutions was constant at 150 mM for the determination of a^i_{Na} in isosmolar solutions. The calibration solutions were nominally Ca^{2+}-free and contained 0·1 mM-EDTA.

The intracellular K^+ concentration is expected to increase in hyperosmolar solutions (Lado *et al.* 1984). We corrected for the expected increased interference from K^+ by also calibrating electrodes in solutions in which the sum of KCl and NaCl was larger. The numerical values for these sums were adopted from Lado *et al.* (1984). Figure 1 shows an example of calibration curves used to determine a^i_{Na} in isosmolar superfusate and in superfusate made hyperosmolar with 100 mM-sucrose. It can be seen that use of the appropriate calibration curve is necessary for accurate measurements of a^i_{Na} in the physiological range. At higher levels of Na^+ activities (> ≃ 30 mM) the calibration curves are almost superimposable.

Na^+-sensitive microelectrodes are also sensitive to Ca^{2+}, and a part of the voltage deflection recorded on entry into cells is due to the large transmembrane Ca^{2+} gradient. To account for this, electrode potentials in the calibration solutions (see Fig. 1) were recorded relative to potentials in normal Tyrode solution containing 2·16 mM-Ca^{2+}. Because interference from changes in intracellular Ca^{2+} during experimentation should be minimal (Kaila & Vaughan-Jones, 1987) this was not corrected for.

Recording system

The tissue bath was coupled to ground via a Ag–AgCl pellet. Microelectrodes were connected to the recording system by means of Ag–AgCl junctions. Signals from the voltage-sensitive electrodes were fed into an electrometer (Intra 767, WPI) and signals from ion-sensitive electrodes were fed

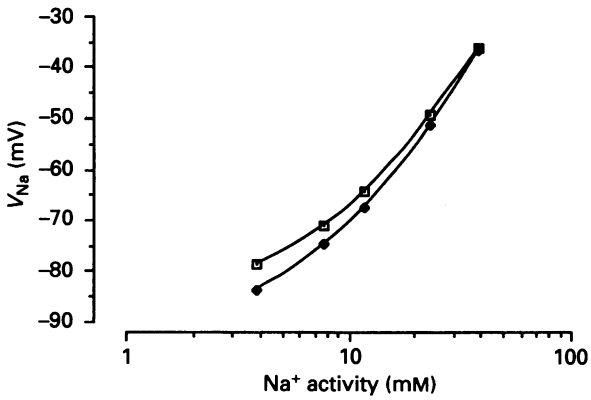

Fig. 1. Calibration curve for sodium-sensitive microelectrodes. The electrode potential, V_{Na}, is plotted against Na^+ activity. The composition of calibration solutions was varied to emulate the expected intracellular milieu in isosmolar solutions (◆) and solutions made hyperosmolar by the addition of 100 mM-sucrose (⊡). NaCl and KCl were altered in a reciprocal manner such that the sum of their concentrations was 150 mM (isosmolar) or 200 mM (hyperosmolar).

into a high-impedance electrometer (FD 223, WPI). The output from both electrometers was connected to a computerized data acquisition system with 12-bit resolution (R.C. Electronics Inc. Santa Barbara, CA, USA). Permanent records were made on an NEC P5200 printer.

Statistical analysis

Results are expressed as means ± S.E.M. Comparisons were made with paired and unpaired *t* test and analysis of variance.

RESULTS

Effects of hyperosmolar solution on pH_i

Figure 2 shows a recording of membrane potential (E_m) and pH_i in a guinea-pig papillary muscle superfused with HEPES-buffered isosmolar modified Tyrode solution. Exposure to solution made hyperosmolar with 100 mM-sucrose caused E_m to hyperpolarize by 4 mV to a new steady-state level within 90 s of a change in superfusate. This hyperpolarization is expected from the resultant increase in intracellular K^+ (Fozzard & Lee, 1976). A slowly developing intracellular alkalinization of $\simeq$ 0·1 pH units relative to control occurred during exposure to the hyperosmolar solution. Upon re-exposure to isosmolar superfusate E_m and pH_i returned to their original levels. As shown in Fig. 2, the effects of hyperosmolar solution on pH_i and E_m were reversible and reproducible. In experiments on six separate tissue specimens pH_i increased from 7·09 ± 0·02 in isosmolar solution to 7·19 ± 0·02 in Tyrode solution containing 100 mM-sucrose. This difference was

statistically significant ($P < 0.01$, paired t test). During exposure to hyperosmolar solution E_m hyperpolarized from 83.7 ± 1.7 to 86.4 ± 0.6 mV. An intracellular alkalinization of 0.03 ± 0.005 units could be detected in eight experiments when specimens were exposed to solution containing 50 mM-sucrose. Solutions containing

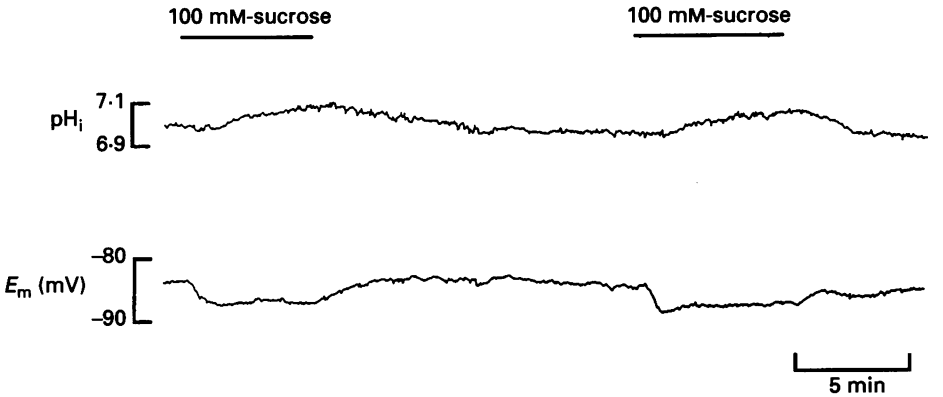

Fig. 2. The effect of hyperosmolar solutions on intracellular pH (pH_i), and membrane potential (E_m). Horizontal bars indicate the timing of exposures to solution made hyperosmolar by the addition of 100 mM-sucrose. The trace showing pH_i is derived from the difference between the membrane potential (conventional microelectrode) and ion-sensitive electrode recordings.

more than 100 mM-sucrose caused no further alkalinization beyond that observed with 100 mM.

Effect of Na^+–H^+ exchange inhibition

Effect of low extracellular Na^+

The effect on pH_i of inhibition of the Na^+–H^+ exchanger during exposure of specimens to hyperosmolar solutions was examined. In one series of experiments we utilized the dependence of H^+ extrusion on extracellular Na^+. To completely abolish Na^+–H^+ exchange Na^+ must be omitted from the superfusate. Microelectrode impalements, however, could not be maintained in hyperosmolar superfusates when Na^+ was omitted. We therefore reduced the Na^+ concentration to 15 mM to decrease rather than completely abolish Na^+–H^+ exchange activity. The Na^+ concentration was reduced by isosmolar substitution of NaCl with choline chloride. Atropine (1 μM) was added to solutions to prevent any possible effects of choline mediated through a muscarinic receptor mechanism. Control experiments indicated that choline in the concentration used had no effect on the H^+-sensitive microelectrode potential.

Figure 3 shows the effect of hyperosmolar solutions at low extracellular Na^+. Exposure to isosmolar solution containing 15 mM-Na^+ caused a depolarization of E_m by 3 mV and a barely detectable decrease in pH_i. Upon switching to a similar solution made hyperosmolar by the addition of 100 mM-sucrose E_m rapidly hyperpolarized by 3 mV while pH_i decreased by 0·06 units. The intracellular acidosis partially recovered upon returning to low-Na^+ isosmolar solution. Complete recovery

of pH_i was observed after switching to superfusate with physiological levels of Na^+. In five identical experiments pH_i was $7{\cdot}01 \pm 0{\cdot}02$ units at 140 mM-extracellular Na^+ and $7{\cdot}00 \pm 0{\cdot}02$ units after 5 min exposure to superfusate containing 15 mM-Na^+. There was a decrease to $6{\cdot}94 \pm 0{\cdot}02$ units with a switch to low-Na^+ hyperosmolar

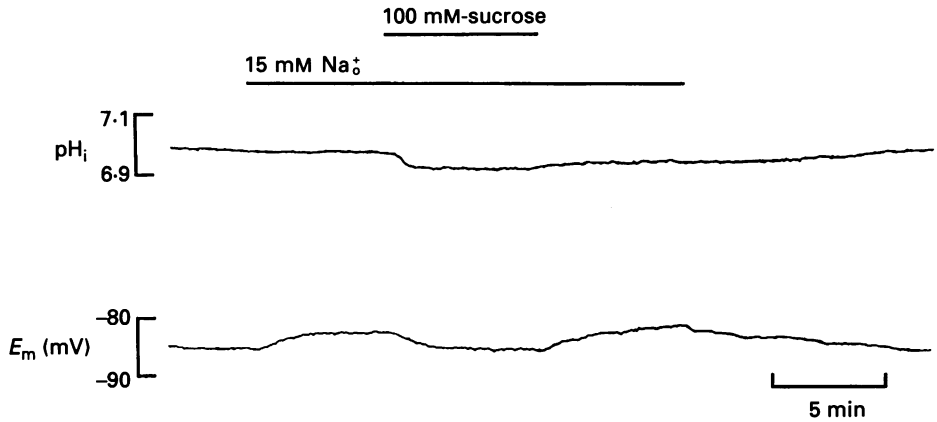

Fig. 3. The effect of low extracellular sodium (Na_o^+) on the response of intracellular pH (upper trace) and membrane potential (lower trace) to hyperosmolar solutions. NaCl was replaced by choline chloride to maintain osmolarity. Tissue was exposed to 15 mM-Na_o^+ for 5 min before switching to an identical solution made hyperosmolar by the addition of 100 mM-sucrose.

superfusate. This difference between pH_i in isosmolar and hyperosmolar low-Na^+ superfusate was statistically significant ($P < 0{\cdot}05$, paired t test).

Thus, when H^+ extrusion via Na^+–H^+ exchange was decreased by reducing extracellular Na^+ the intracellular alkalosis observed during exposure to a hyperosmolar superfusate was converted to an acidosis. This suggests that any stimulation of Na^+–H^+ exchange by hyperosmolar superfusate which may occur at 15 mM-extracellular Na^+ is insufficient to counterbalance presumed acidifying effects of increases in intracellular Ca^{2+} activity (a^i_{Ca}). Acidifying effects of an increase in a^i_{Ca} should be larger at low than at physiological levels of extracellular Na^+ because Ca^{2+} extrusion via Na^+–Ca^{2+} exchange should be reduced. This might account for the conversion of an increase in pH_i (Fig. 2) to a decrease during exposure to hyperosmolar superfusates with low extracellular Na^+ (Fig. 3). To examine this possibility we reduced the Na^+ concentration in the superfusate to 15 mM by isosmolar substitution with LiCl. Li^+ in the concentration used had no effect on the potential of the H^+-sensitive microelectrode. After superfusing specimens with isosmolar Tyrode solution containing a physiological level of Na^+ we switched to Li^+-substituted solution made hyperosmolar with 100 mM-sucrose. This was accompanied by an initial brief decrease in pH_i followed by the development of an intracellular alkalosis. Although pH_i increased more slowly than during exposure to hyperosmolar superfusates containing physiological levels of Na^+ the increase was of a similar magnitude (0·07 and 0·11 units above the level in control Tyrode solution in two experiments). Complete recovery of pH_i occurred after return to control Tyrode

solution. Since extracellular Li^+ can support H^+ extrusion via the Na^+–H^+ exchanger (Mahnensmith & Aronson, 1985) but not Ca^{2+} extrusion via the Na^+–Ca^{2+} exchanger (Reuter & Seitz, 1968) these findings suggest that the conversion of an increase to a decrease in pH_i during exposure to hyperosmolar superfusates by low extracellular

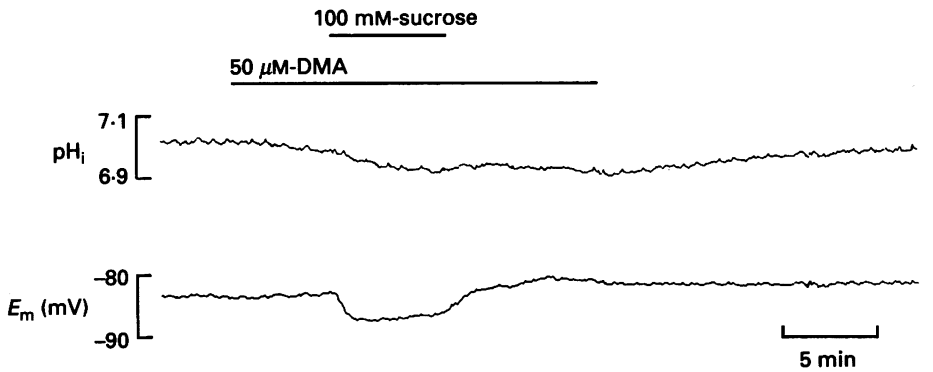

Fig. 4. Effect of 5-(*N*,*N*-dimethyl)amiloride (DMA) on the response of pH_i to hyperosmolar solutions. Tissue was exposed to a modified Tyrode solution containing 50 μM-DMA for 5 min before exposure to an identical solution containing 100 mM-sucrose. Traces show pH_i (upper) and membrane potential (lower).

Na^+ is not related to differences in a^i_{Ca}. The findings therefore indicate a direct dependence of the alkalinization upon extracellular Na^+. The initial acidification and the slower rate of the subsequent alkalinization than that observed during exposure to hyperosmolar superfusates with physiological levels of Na^+ probably reflects the lower affinity of Li^+ for extracellular transport sites of the Na^+–H^+ exchanger compared with that of Na^+ (Mahnensmith & Aronson, 1985).

Effect of DMA

We also examined the effect of 50 μM-DMA on pH_i in isosmolar and hyperosmolar superfusates. In this concentration DMA had no effect on the H^+-sensitive microelectrode potential. Figure 4 shows a representative experiment. Exposure to isosmolar solution containing DMA caused no change in E_m while there was a small decrease in pH_i. Upon switching to a similar solution made hyperosmolar by the addition of 100 mM-sucrose E_m rapidly hyperpolarized by $\simeq$ 5 mV and pH_i decreased by an additional 0·05 units. The intracellular acidosis persisted upon return to DMA-containing isosmolar solution. Slow recovery of pH_i to control levels occurred after switching to DMA-free isosmolar solution. In six experiments pH_i decreased from $7{\cdot}04 \pm 0{\cdot}03$ in DMA-free control solution to $6{\cdot}98 \pm 0{\cdot}03$ units in solution containing 50 μM-DMA. On switching to an hyperosmolar superfusate containing DMA pH_i decreased by an additional $0{\cdot}06 \pm 0{\cdot}01$ units to $6{\cdot}92 \pm 0{\cdot}03$. The difference between pH_i in isosmolar and hyperosmolar DMA-containing solutions was significant ($P < 0{\cdot}05$, paired t test).

Effect of hyperosmolar solution on a^i_{Na}

Exposure of cardiac tissue to hyperosmolar solution is expected to cause shrinkage of myocytes and hence an increase in a^i_{Na}. To confirm this experimentally we measured a^i_{Na} during exposure to hyperosmolar solutions. The concentration of

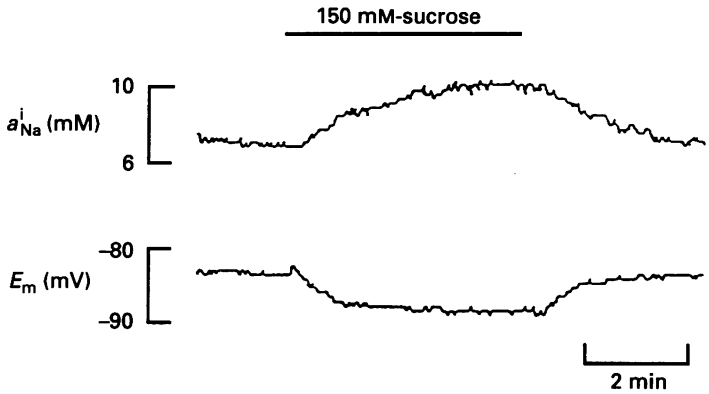

Fig. 5. Measurement of a^i_{Na} (upper trace) and membrane potential (lower trace) during exposure to Tyrode solution containing 150 mM-sucrose.

sucrose used to render solutions hyperosmolar in these experiments was 150 mM to allow comparison with a previous study (Lado *et al.* 1984). Figure 5 shows recordings from a representative experiment. After an initial increase in a^i_{Na} a new steady state was reached within 5 min. In eight experiments a^i_{Na} increased from $7{\cdot}6 \pm 0{\cdot}4$ mM in isosmolar solution to $10{\cdot}9 \pm 0{\cdot}6$ mM at steady state in hyperosmolar solution; an increase of $43 \pm 5\%$. This increase is not statistically significantly different from the 37 and 27% increases reported for sheep Purkinje fibres and sheep ventricular muscle respectively exposed to similar hyperosmolar solutions (Lado *et al.* 1984).

If the Na^+–H^+ exchanger is activated by exposure of specimens to hyperosmolar solutions it should be possible to demonstrate that a part of the increase in a^i_{Na} during such exposure is sensitive to DMA. We examined the effect of DMA on a a^i_{Na} during exposure to solution made hyperosmolar with 100 mM-sucrose. Since extrusion by the Na^+–K^+ pump of Na^+ entering myocytes through Na^+–H^+ exchange might obscure any DMA-sensitive increases in a^i_{Na} we inhibited the Na^+–K^+ pump with dihydro-ouabain (DHO). This drug has a very rapid onset of action and is rapidly washed off guinea-pig cardiac tissue (Daut, 1983).

In initial experiments we recorded a^i_{Na} during superfusion with isosmolar DHO-free solution and then switched to solution which was made hyperosmolar with 100 mM-sucrose and which contained 0·2 mM-DHO. The time course for the mean increase in a^i_{Na} summarized for experiments on four specimens is plotted in Fig. 6. After DHO wash-off and return of a^i_{Na} to control levels we superfused the specimens with isosmolar solution containing 50 μM-DMA. This caused a decrease in a^i_{Na} by $0{\cdot}5 \pm 0{\cdot}01$ mM to a new steady state within 5 min. After an additional 10 min we switched to a solution which was made hyperosmolar with 100 mM-sucrose and which

contained both DMA and DHO. The time course for the mean increase in a^i_{Na} during exposure of the four specimens to this superfusate is also plotted in Fig. 6. Analysis of variance indicated that the rate of rise of a^i_{Na} was slower in DMA-containing than in DMA-free superfusate. We conclude that a part of the increase of a^i_{Na} during exposure to hyperosmolar solutions is sensitive to DMA.

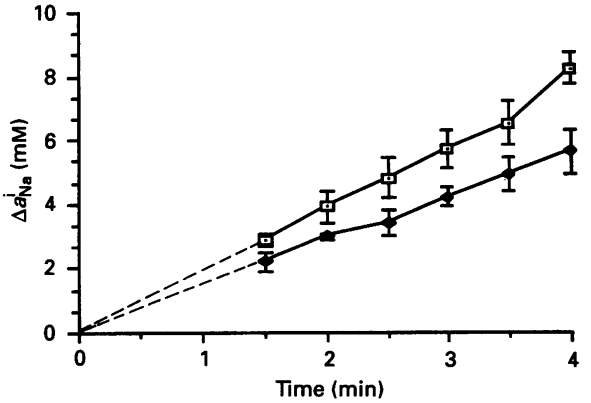

Fig. 6. Effect of DMA on the increase in a^i_{Na} with exposure to hyperosmolar solutions. In these experiments the Na^+–K^+ pump was inhibited with 0·2 mM-dihydro-ouabain (DHO). Change in a^i_{Na} (Δa^i_{Na}) is plotted against time after exposure to hyperosmolar (100 mM-sucrose) DHO-containing solution (⊡) and hyperosmolar solution containing both DHO and 50 μM-DMA (◆). Results are expressed as the mean ± S.E.M. of paired experiments in four different preparations.

To reflect the shrinkage of cells and increase in intracellular K^+ during exposure to hyperosmolar superfusates (Lado *et al.* 1984) the data in Fig. 6 were obtained using calibration curves for which the sum of Na^+ and K^+ was either 150 or 200 mM. The former curve was used to determine a^i_{Na} in isosmolar solution. The latter curve was used to determine a^i_{Na} during exposure to hyperosmolar superfusate. Since shrinkage of cells in the multicellular preparation is not instantaneous, neither curve provides an accurate determination of a^i_{Na} during the initial period after the change. Therefore a^i_{Na} was not determined until 90 s after the change in superfusate. This delay was chosen because the hyperpolarization of E_m, reflecting an increase in intracellular K^+ (Fozzard & Lee, 1976), was 90% complete within 90 s. Any residual error in the determination of a^i_{Na} which might remain with this approach should be small and identical in experiments with and without DMA.

Acid loading

Effect of hyperosmolar solution on recovery from acidosis

The effect of DMA and low-Na^+ superfusate on pH_i, and the effect of DMA on uptake of Na^+ suggests that the Na^+–H^+ exchanger is activated in response to exposure to hyperosmolar solutions. To further support this hypothesis we studied pH_i during recovery from an experimentally induced acidosis in both isosmolar and hyperosmolar solutions. Such recovery in cardiac tissue in the nominal absence of HCO_3^- is mediated by the Na^+–H^+ exchanger (Ellis & MacLeod, 1985).

Specimens were superfused for 3 min with solution containing 20 mM-NH_4Cl (made by isosmolar substitution for NaCl). Upon re-exposure to NH_4Cl-free solution an intracellular acidosis developed (see Thomas, 1984, for discussion of mechanism) followed by recovery towards more alkaline levels. Following removal of NH_4Cl in six

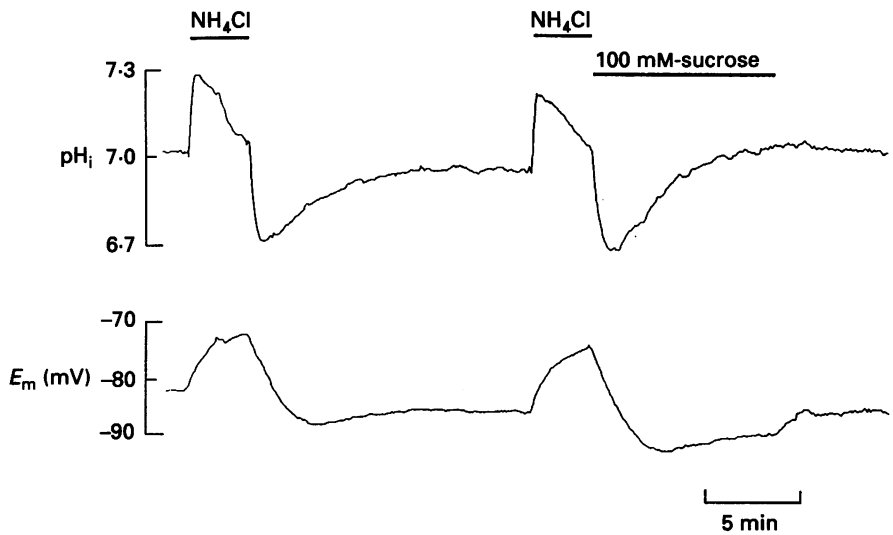

Fig. 7. Recovery from intracellular acidosis. Traces show intracellular pH (upper) and membrane potential (lower). An intracellular acidosis was induced by exposure to and subsequent withdrawal of 20 mM-NH_4Cl as indicated by horizontal bars. The rate of pH recovery in isosmolar and hyperosmolar (100 mM-sucrose) solution is compared.

experiments pH_i fell by $0{\cdot}28 \pm 0{\cdot}03$ units in isosmolar solution and $0{\cdot}25 \pm 0{\cdot}03$ units in hyperosmolar solution. The pH_i nadirs were $6{\cdot}78 \pm 0{\cdot}04$ and $6{\cdot}79 \pm 0{\cdot}05$ units respectively. As illustrated in Fig. 7 the rate of pH_i recovery after the NH_4Cl prepulse was faster in hyperosmolar than in isosmolar solution. In six paired experiments the maximal pH_i recovery rate increased significantly from $0{\cdot}041 \pm 0{\cdot}006$ units min^{-1} in isosmolar solution to $0{\cdot}064 \pm 0{\cdot}009$ units min^{-1} in hyperosmolar solution ($P < 0{\cdot}01$, paired t test). In hyperosmolar solution pH_i recovered to a new steady state $\simeq 0{\cdot}07$ units more alkaline than the initial pH_i in isosmolar solution. The time courses of pH_i recovery in isosmolar and hyperosmolar solutions are summarized in Fig. 8*A* and *B*.

Intracellular Na^+ during recovery from acidosis

Exposure of specimens to hyperosmolar solutions causes an increase in a^i_{Na} (Fig. 5). This is expected to reduce H^+ extrusion via the Na^+–H^+ exchanger (Grinstein, Cohen & Rothstein, 1984) yet, as shown in Fig. 8, the rate of pH_i recovery was actually faster in hyperosmolar than in isosmolar solution. It is therefore of interest to measure a^i_{Na} during recovery from acidosis in both isosmolar and hyperosmolar solutions. Figure 8*C* and *D* summarizes the time course of a^i_{Na} during such recovery in five experiments. It is notable that a^i_{Na} at the time of maximal pH_i recovery rate was higher in hyperosmolar ($8{\cdot}8 \pm 0{\cdot}5$ mM) than in isosmolar solution ($6{\cdot}5 \pm 0{\cdot}8$ mM).

The data shown in Fig. 8 demonstrate that the recovery rate of pH_i from acidosis is enhanced in hyperosmolar solutions despite an increase in a^i_{Na} relative to control by $\simeq$ 2 mM. We also examined if enhanced pH_i recovery in hyperosmolar solutions could be demonstrated at even higher levels of a^i_{Na}. To cause an increase in a^i_{Na} we

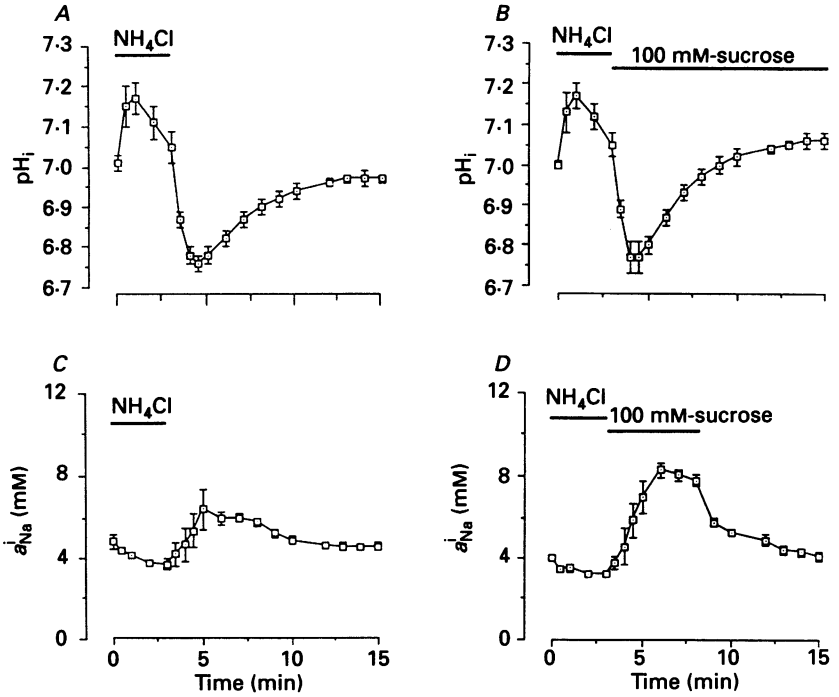

Fig. 8. Effect of hyperosmolar solutions on pH_i and a^i_{Na} during recovery from intracellular acidosis. The upper traces summarize the time course of pH_i recovery after an NH_4Cl-induced acidosis. Recovery in isosmolar (*A*) and hyperosmolar (*B*) solutions is compared. Results are from six paired experiments. The lower traces summarize the changes in a^i_{Na} during an identical experimental protocol in isosmolar (*C*) and hyperosmolar (*D*) solutions. Results are from five paired experiments. The time scale is identical for both the pH_i and a^i_{Na} traces.

inhibited the Na^+–K^+ pump by using K^+-free solutions after the NH_4Cl pre-pulse. The time course of pH_i recovery in six paired experiments is summarized in Fig. 9*A* and *B*. As expected, the rate of recovery of pH_i was slower in K^+-free isosmolar solutions ($0{\cdot}031 \pm 0{\cdot}003$ units min^{-1}) than in K^+-containing solutions ($0{\cdot}041 \pm 0{\cdot}006$ units min^{-1}) the difference being statistically significant ($P < 0{\cdot}05$, unpaired *t* test). However, even in K^+-free solution the rate of pH_i recovery after the pre-pulse was faster in hyperosmolar than in isosmolar solutions. The maximal pH_i recovery rate increased from $0{\cdot}031 \pm 0{\cdot}003$ units min^{-1} in isosmolar solution to $0{\cdot}055 \pm 0{\cdot}006$ units min^{-1} in hyperosmolar solution. This increase is statistically significant ($P < 0{\cdot}01$, paired *t* test). Figure 9*C* and *D* summarizes the time course of a^i_{Na} during and after NH_4Cl pre-pulses in three experiments. It is notable that a^i_{Na} was $\simeq$ 15–20 mM at the time of maximal pH_i recovery in isosmolar K^+-free solution

and $\simeq$ 25–30 mM at the time of maximal pH_i recovery in hyperosmolar K^+-free solution. Thus, hyperosmolarity enhances recovery from acidosis, even when a^i_{Na} is markedly elevated.

Are differences in pH_i recovery due to different rates of acid loading?

Bountra, Powell & Vaughan-Jones (1990) have shown that the rate of pH_i recovery after an NH_4Cl pre-pulse in guinea-pig papillary muscle is reduced due to

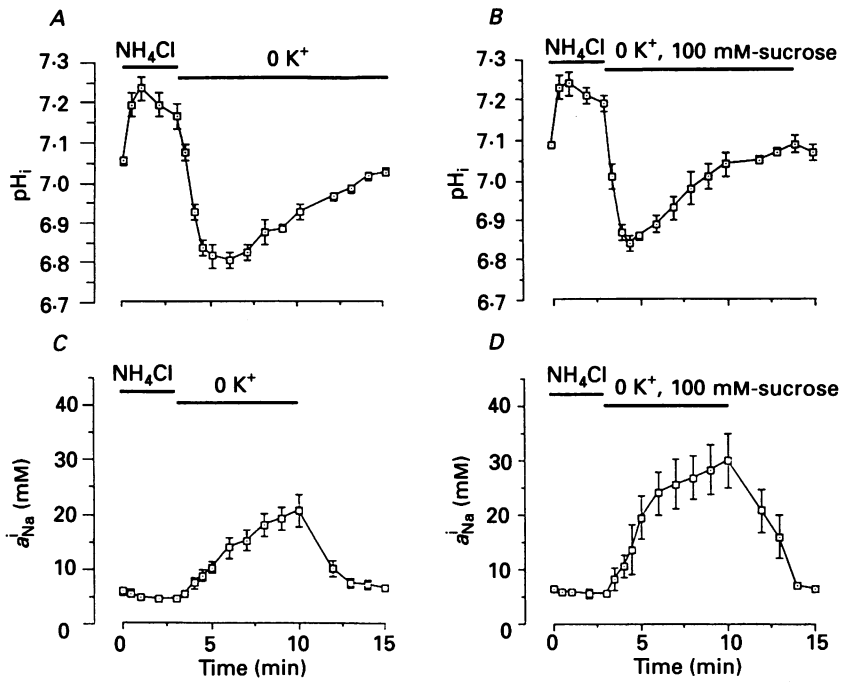

Fig. 9. Effect of elevated intracellular Na^+ during recovery from acidosis. Following a 20 mM-NH_4Cl pre-pulse, tissue was superfused with K^+-free solution to inhibit the Na^+ pump and elevate a^i_{Na}. The upper traces show pH_i during recovery in isosmolar (*A*) and hyperosmolar (*B*) K^+-free solutions ($n = 6$). Lower traces show a^i_{Na} during recovery from intracellular acidosis in isosmolar (*C*) and hyperosmolar (*D*) K^+-free solution ($n = 3$). Note that pH_i recovery is faster in hyperosmolar than in isosmolar solution even with marked elevation of a^i_{Na}. The time scale is identical for pH_i and a^i_{Na} traces.

effects of relatively slow acid loading. Since acid loading depends upon simple diffusion of NH_3 out of the cell it should be facilitated by the increased surface area: volume ratio of cells shrunken in hyperosmolar solution. This in turn might increase the rate of pH_i recovery in hyperosmolar solution independent of any direct effects on the Na^+–H^+ exchanger. To determine the extent to which such effects of cell shrinkage may contribute to faster pH_i recovery in hyperosmolar solution, we performed experiments designed to achieve complete acid loading before pH_i recovery in isosmolar and hyperosmolar solution was studied. In these experiments we utilized the effect of extracellular acidosis to inhibit cellular acid extrusion by the Na^+–H^+ exchanger (Vaughan-Jones & Wu, 1990).

Specimens were exposed for 2 min to a superfusate which contained 20 mM-NH_4Cl and which had a pH of 7·4. They were then exposed to an NH_4Cl-free superfusate which had a pH of 6·2. This resulted in an intracellular acidosis from which no significant recovery occurred over $\simeq$ 8 min. After a stable pH_i was maintained for

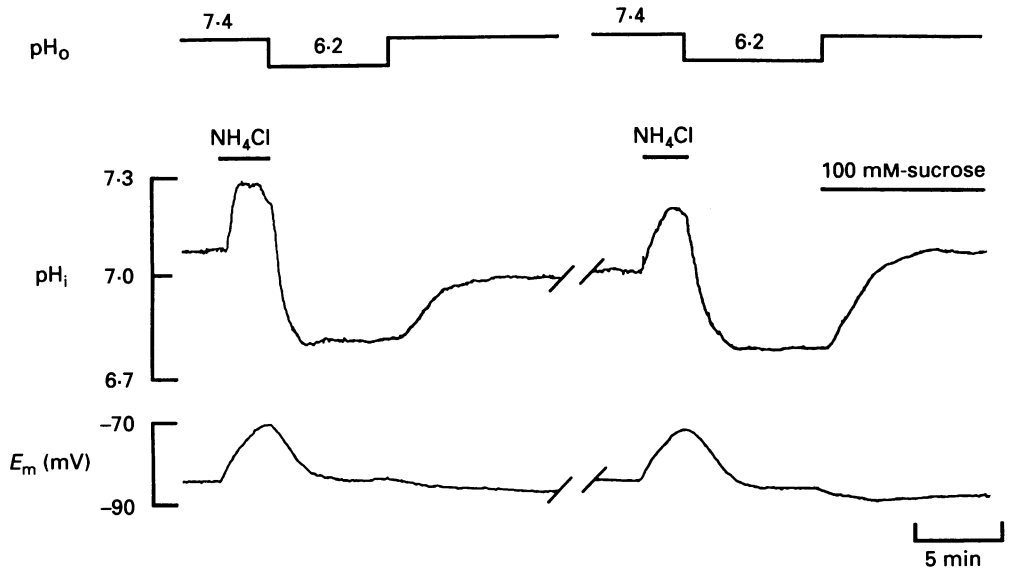

Fig. 10. Recovery from intracellular acidosis after inhibition of Na^+–H^+ exchange by low extracellular pH. Traces show intracellular pH (upper) and membrane potential (lower). Recovery from intracellular acidosis after a 20 mM-NH_4Cl pre-pulse was prevented by superfusing tissue with solution with a pH of 6·2. After a stable pH_i was maintained for 5 min recovery from the acidosis was achieved by switching to isosmolar or hyperosmolar superfusate with a pH of 7·4.

5 min recovery was allowed to occur in isosmolar or hyperosmolar superfusate with a pH of 7·4. A representative experiment is shown in Fig. 10. In five such experiments maximal pH_i recovery rate was $0{\cdot}045 \pm 0{\cdot}005$ units min^{-1} in isosmolar solution and $0{\cdot}071 \pm 0{\cdot}006$ units min^{-1} in hyperosmolar solution. The difference between these rates was statistically significant ($P < 0{\cdot}001$, paired t test). In hyperosmolar solution pH_i consistently recovered to a level more alkaline than at steady state in isosmolar solution. Thus, when the potentially confounding influence of different rates of acid loading is removed, recovery from acidosis remains faster in hyperosmolar than isosmolar solution.

Buffer capacity in isosmolar and hyperosmolar solutions

When recovery from acidosis was studied in hyperosmolar superfusates the tissue was exposed to NH_4Cl in the pre-shrunken state. The total amount of NH_4^+ entering the cell, and hence the subsequent acid load, should therefore be identical to that in control experiments. However, cell volume after the NH_4Cl pre-pulse should be less in hyperosmolar than in isosmolar superfusate. It follows that pH_i should fall to a lower nadir in hyperosmolar than in isosmolar superfusate. In addition to this, the

expected increase in a^{i}_{Ca} may further lower pH_i in hyperosmolar solution. Contrary to these expectations the nadirs were similar (see Figs 7 and 8). In an effort to account for this we examined whether a significant increase in buffer capacity occurs with exposure to hyperosmolar solutions.

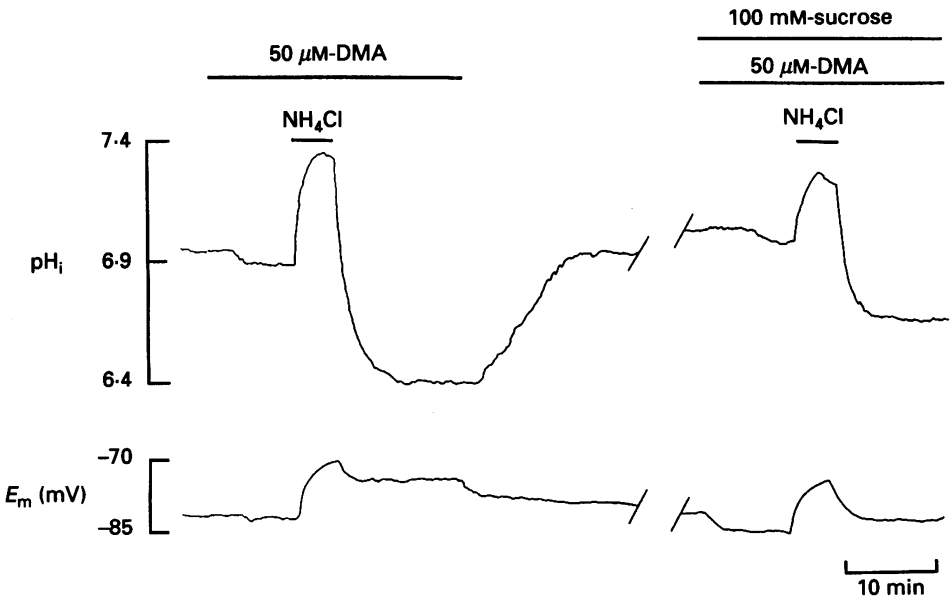

Fig. 11. Estimation of buffer capacity in isosmolar and hyperosmolar solutions. Traces show intracellular pH (upper) and membrane potential (lower) during acid loading with a brief 20 mM-NH_4Cl pre-pulse in isosmolar and hyperosmolar solution. Tissue was superfused with DMA, as indicated by the horizontal bars, to inhibit acid extrusion. Buffer capacity was estimated using the fall in pH_i after NH_4Cl withdrawal. The break in the traces indicates a period of 20 min. The apparent buffer capacity was 24·3 mmol l^{-1} in isosmolar and 44·2 mmol l^{-1} in hyperosmolar solution.

For estimation of the apparent buffer capacity in isosmolar solution we exposed specimens for 10 min to 50 μM-DMA. They were then superfused for 5 min with solution which also contained 20 mM-NH_4Cl before being re-exposed to NH_4Cl-free solution. For estimation of buffer capacity in hyperosmolar solution we used the same experimental protocol except that all superfusates were hyperosmolar (100 mM-sucrose added). The timing of exposure to DMA-, NH_4Cl- and sucrose-containing superfusates is shown in a representative experiment depicted in Fig. 11. After the NH_4Cl pre-pulse pH_i rapidly decreased to a new stable level from which recovery did not occur until DMA was washed off. The time to achieve the nadir was $5·1 \pm 0·6$ min in six experiments performed in isosmolar solution and $4·0 \pm 0·4$ min in five experiments in hyperosmolar solution. This difference was not statistically significant ($P = 0·3$, unpaired t test). DMA was used to prevent acid extrusion via Na^+–H^+ exchange during acid loading. Such extrusion has been shown to contribute to a marked over-estimation of buffer capacity in guinea-pig papillary muscles (Bountra *et al.* 1990). Using a method identical to that described by Bountra *et al.* (1990), we

estimated the apparent buffer capacity from the decrease in pH_i which occurred upon withdrawal of NH_4Cl. In isosmolar solution the estimated buffer capacity was $29{\cdot}9 \pm 2{\cdot}2$ mmol l^{-1}. In hyperosmolar solution the estimated buffer capacity was $43{\cdot}3 \pm 2{\cdot}8$ mmol l^{-1}. This increase in buffer capacity was statistically significant ($P < 0{\cdot}01$, unpaired t test).

Effect of HCO_3^-–CO_2 buffering in the superfusate

In contrast to the present findings a study on sheep Purkinje fibres has demonstrated a decrease in pH_i by $\simeq 0{\cdot}1$ unit during exposure to 100 mM-sucrose (Ellis & Thomas, 1976). In that study CO_2–HCO_3^- rather than HEPES was used to buffer solutions. To examine if the difference in buffer could somehow account for the discrepancy we performed additional experiments using solutions containing 24 mM-$NaHCO_3$ instead of 10 mM-HEPES. NaCl in the solution was reduced from 140 to 116 mM to maintain Na^+ concentration and osmolarity constant. In six experiments pH_i increased from $7{\cdot}11 \pm 0{\cdot}02$ to $7{\cdot}17 \pm 0{\cdot}02$ units when tissue was exposed to solution containing 100 mM-sucrose. The difference between pH_i in isosmolar and hyperosmolar solution was statistically significant ($P < 0{\cdot}05$, paired t test). We conclude that use of different buffers does not account for the discrepancy between our study and results from studies on sheep Purkinje fibres.

DISCUSSION

During exposure of cardiac tissue to hyperosmolar solutions the a^i_{Ca} increases (Lado *et al.* 1984; Allen & Smith, 1987). This, in turn, should cause a decrease in pH_i due to the sharing of intracellular binding and uptake sites by H^+ and Ca^{2+} (see Orchard & Kentish, 1990, for review) and due to a Ca^{2+}-induced generation of intracellular acid equivalents (Allen, Eisner, Morris, Pirolo & Smith, 1986). An intracellular acidification is therefore expected. Contrary to this expectation the present study shows that an alkalosis develops in hyperosmolar superfusates.

Evidence for Na^+–H^+ exchange activation

The Na^+ dependence of the intracellular alkalinization during exposure to hyperosmolar superfusates suggests that the Na^+–H^+ exchanger is activated. Further support for this was obtained when the alkalosis induced by hyperosmolar solutions was converted to an acidosis by DMA. However, an indirect effect of DMA on pH_i mediated through changes in intracellular Na^+ and Ca^{2+} should also be considered since amiloride and its derivatives may inhibit Na^+–Ca^{2+} exchange and Na^+ channels. Blockade of Na^+ channels should decrease a^i_{Na} and should therefore not have an acidifying effect. Inhibition of Na^+–Ca^{2+} exchange might increase intracellular Ca^{2+} at the levels of E_m recorded in this study. Because of an effect of Ca^{2+} on the binding and generation of intracellular H^+, this in turn could cause an acidosis. However, available evidence indicates that amiloride in concentrations 20–40 times higher than that of DMA used in this study has no effect on Na^+–Ca^{2+} exchange (Kaila & Vaughan-Jones, 1987) and since the potency of DMA for Na^+–Ca^{2+} exchange inhibition is only twofold higher than that of amiloride (Kleyman & Cragoe, 1988) it seems highly unlikely that an inhibition of Na^+–Ca^{2+}

exchange accounts for the effects of DMA on pH_i during exposure to hyperosmolar superfusates. Rather, the effects of DMA on pH_i during such exposure supports the conclusion that an hyperosmolar milieu stimulates the Na^+–H^+ exchanger.

Determinants of the time course of pH_i after an NH_4Cl pre-pulse

The maximal rate of recovery of pH_i was 55% (95% confidence interval 41–70%) faster in hyperosmolar than in isosmolar solutions. This should not, however, be taken to indicate a 55% increase in activity of the Na^+–H^+ exchanger in hyperosmolar solutions because determinants of the time course of recovery of pH_i also include the cell surface area:volume ratio and the intrinsic buffer capacity as well as the rate of H^+ efflux via the exchanger (Bountra et al. 1990).

The increase in the surface area:volume ratio during shrinkage of myocytes exposed to hyperosmolar solutions should increase the rate of pH_i recovery. If the myocyte alters its volume as a perfect osmometer whilst its surface area remains constant the surface area:volume ratio is predicted to increase by 35% for the increase in extracellular osmolarity used in this study. However, there is evidence suggesting that cardiac myocytes shrink less in hyperosmolar solutions than predicted. Extrapolating from the data of Drewnowska & Baumgarten (1991) one would expect only an $\simeq 18\%$ increase in the surface area:volume ratio. Any acceleration in the rate of pH_i recovery resulting from this increase should be opposed by the effect of an increased intrinsic buffer capacity which is expected to slow pH_i recovery during exposure to hyperosmolar solutions. We found that the buffer capacity increased by 45% (95% confidence interval 20–67%). It follows that if the activity of the Na^+–H^+ exchanger was similar in isosmolar and hyperosmolar solution the net result of these changes in surface area:volume ratio and buffer capacity should be to maintain the pH_i recovery rate at similar levels in isosmolar and hyperosmolar solutions. Therefore the enhanced pH_i recovery rate in hyperosmolar solutions actually observed in this study supports the conclusion that the rate of H^+ efflux via the Na^+–H^+ exchanger is stimulated.

In agreement with a previous study (Kaila & Vaughan-Jones, 1987) we found that recovery from acidosis could occur even when a^i_{Na} was markedly elevated during inhibition of the Na^+–K^+ pump. The high a^i_{Na} might have inhibited Na^+–H^+ exchange-mediated pH_i recovery because Na^+ competes with H^+ at the intracellular transport site of the exchanger (Grinstein *et al.* 1984; Green, Yamaguchi, Kleeman & Muallem, 1988*b*). In addition, a high a^i_{Na} should enhance cellular acid generation and thus cause a decrease in the apparent rate of Na^+–H^+ exchange-mediated pH_i recovery (Kaila & Vaughan-Jones, 1987). In agreement with these expectations the rate of recovery of pH_i was slower during Na^+–K^+ pump inhibition with K^+-free superfusate than in K^+-containing superfusate. However, when a^i_{Na} was increased further by shrinkage of cells in hyperosmolar superfusates the rate of recovery of pH_i from acidosis was enhanced relative to that in isosmolar superfusates. This finding is consistent with the observation made on an osteosarcoma cell line indicating that exposure to hyperosmolar solutions increases the affinity of intracellular transport sites of the Na^+–H^+ exchanger for H^+ while the affinity for Na^+ decreases (Green *et al.* 1988*a*). However, because of the well-documented limitations in estimating kinetic parameters in multicellular cardiac preparations (see Bountra *et al.* 1990) the

intracellular affinities of the transported ionic species and possible changes in these affinities were not determined in this study.

Comparison with previous studies of media osmolarity and pH_i

Two previous studies have reported the effects of hyperosmolar solution on pH_i of cardiac tissue. In one of these (Ellis & MacLeod, 1985), a decrease in pH_i of $\simeq 0{\cdot}1$ unit occurred during exposure to Tyrode solution made hyperosmolar with 140 mM-KCl. It was proposed that the acidification was due to the depolarization associated with the high extracellular K^+ concentration. In support of this idea, when solutions were made hyperosmolar with 140 mM-tetramethylammonium chloride there was only a small transient change in pH_i. In the other study (Ellis & Thomas, 1976) pH_i decreased by $\simeq 0{\cdot}1$ unit when superfusates were made hyperosmolar with 100 mM-sucrose. Sheep Purkinje fibres were used in both studies and one might speculate that tissue and/or species differences account for the discrepancy with the present study. It should be noted, however, that the absence of intracellular alkalinization during exposure to hyperosmolar superfusates does not necessarily indicate an absence of Na^+–H^+ exchange activation since it is possible that any increased activity of the exchanger is outweighed by an increase in intracellular acid generation and/or displacement from binding and uptake sites. Thus, differences between sheep Purkinje fibres and guinea-pig ventricular muscle in the response of pH_i homeostasis to an hyperosmolar milieu could be of a quantitative rather than a qualitative nature.

Implications for studies on contractility

Exposure of cardiac tissue to hyperosmolar solutions has been used in many studies of cardiac contractility. Determinants of contractility during such exposure include an increase in intracellular Ca^{2+}, K^+ and ionic strength and an increase in viscosity of the tissue. The increase in ionic strength is expected to directly inhibit developed tension through an effect on cross-bridge formation between actin and myosin (Kentish, 1984). The increase in intracellular K^+ should decrease the sensitivity of myofibrils to Ca^{2+}, and should, therefore, also decrease cardiac contractility (Kentish, 1984). Finally, contractility should be decreased by the increase in tissue viscosity (Allen & Smith, 1987). The inhibitory influence of these changes in viscosity, intracellular ionic strength and K^+ on contractility should be counteracted by the positive inotropic effect of an increase in intracellular Ca^{2+} (Lado *et al.* 1984; Allen & Smith, 1987). All these determinants have been considered in previous studies addressing the effect of hyperosmolar solutions on cardiac contractility. However, to our knowledge, the influence of pH_i has not been taken into account in any published quantitative analysis. This may introduce a significant error in the analysis since small changes in pH_i ($\simeq 0{\cdot}1$ unit) can have a major effect on cardiac contractility (Vaughan-Jones, Eisner & Lederer, 1987; Bountra & Vaughan-Jones, 1989; Orchard & Kentish, 1990). Comparisons of experimental evidence from sheep and guinea-pig cardiac tissue provides some indirect support for the importance of considering changes in pH_i during studies involving exposure to hyperosmolar solutions. Ellis & Thomas (1976) showed that pH_i decreases by $\simeq 0{\cdot}1$ unit during exposure of sheep Purkinje fibres to solutions made hyperosmolar

with 100 mM-sucrose. In contrast our study shows that pH_i increases by $\simeq 0{\cdot}1$ unit during exposure of guinea-pig working myocardial tissue to similar solutions. These differences may account for the findings that contractility of sheep cardiac Purkinje fibres decreases relative to control during exposure to solution made hyperosmolar with 100 mM-sucrose (Lado *et al.* 1984) while the contractility of guinea-pig atrial tissue increases during exposure to similar solutions (Little & Sleator, 1969).

Possible implications for in vivo effects of hyperosmolarity

In the clinical setting elevations of extracellular osmolarity similar to that used in this study may occur. In diabetic hyperosmolar coma, for example, plasma osmolarities can be ~ 410 mosM (Gerich, Martin & Recant, 1971), and with intravenous infusion of mannitol values up to ~ 450 mosM have been reported (Aviram, Pfau, Czackes & Ullmann, 1967). Stimulation of Na^+–H^+ exchange and an increase in pH_i might enhance cardiac contractility in these clinical settings. We are not aware of published clinical data to support such speculation. However, animal studies indicate that cardiac contractility *in vivo* can be enhanced by similar increases in plasma osmolarities induced by infusion of sucrose (Wildenthal, Mierzwiak & Mitchell, 1969) or mannitol (Atkins, Wildenthal & Horowitz, 1973). This enhancement of contractility is sustained for at least 30 min after the increase in osmolarity.

The increase in plasma osmolarity which occurs with elevation of plasma urea in renal failure is of particular interest. One might speculate that because urea permeates cell membranes it should not cause changes in steady-state cell volume and hence perhaps not activation of Na^+–H^+ exchange. To examine this possibility we superfused three specimens with solution made hyperosmolar with 100 mM-urea. A transient intracellular alkalosis did develop; however, pH_i returned to control levels within 5 min despite continued exposure to urea (data not shown). In this regard it is interesting to note that the positive inotropic effect on cat papillary muscle of solution made hyperosmolar with urea is also transient (Koch-Weser, 1963). These findings may be explained in part by changes in pH_i.

Conclusion

Despite the physical constraints of the extracellular matrix characteristic of cardiac tissue, the Na^+–H^+ exchanger in cardiac myocytes responds to an hyperosmotic milieu in a manner similar to that reported for the plasmalemmal Na^+–H^+ exchanger of non-cardiac cells free of such constraints. Stimulation of the Na^+–H^+ exchanger by hyperosmolar solutions causes an increase in pH_i. This increase is expected to be an important determinant of cardiac contractility and should be taken into account in any quantitative analysis when exposure to hyperosmolar solutions is used in the study of excitation–contraction coupling and cardiac muscle mechanics.

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