J. Physiol. (1985), **359**, *pp.* 81–105
With 16 *text-figures*
Printed in Great Britain

SODIUM-DEPENDENT CONTROL OF INTRACELLULAR pH IN PURKINJE FIBRES OF SHEEP HEART

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(*Received 15 February 1984*)

SUMMARY

1. Intracellular pH (pH_i) of Purkinje fibres from sheep heart was recorded with pH-sensitive glass micro-electrodes. The cells were acidified by one of three methods: (1) exposure to and subsequent removal of NH_4Cl, (2) exposure to solutions containing 5 % CO_2 or (3) exposure to an acidic Tyrode solution. The pH_i recovery from these acidifications was studied.

2. The time constant of recovery from an acidification induced by NH_4Cl was almost twice as long as that from one induced by CO_2 or acid extracellular pH. Following an acidification induced by exposure to CO_2 the time constant of pH_i recovery was not changed when the cell was depolarized to −40 mV (by replacement of some Na^+ by K^+).

3. An intracellular acidification was produced when extracellular Na^+ was removed and replaced by quaternary ammonium ions or K^+. Such Na^+-free solutions also inhibited pH_i recovery from an acidification. A 50 % inhibition of the rate of recovery was produced by lowering the $[Na^+]_o$ to 8 mM.

4. When used as a Na^+ substitute, Li^+ could permit recovery. Tris (22 mM) changed pH_i in the alkaline direction.

5. Amiloride (1 mM) or a decrease in temperature slowed the recovery from an acidification ($Q_{10} = 2{\cdot}65$). There was no effect of SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid disodium salt; 100 μM) on the recovery.

6. Na^+-sensitive glass micro-electrodes were used to measure the intracellular Na^+ activity when $[Na^+]_o$ was lowered to levels used in our pH_i recovery experiments. From these data we have calculated the apparent Na^+ electrochemical gradient at different values of $[Na^+]_o$. If this gradient is responsible for H^+ efflux from the cell then, by applying thermodynamic considerations, it can be shown that only low concentrations (1–2 mM) of extracellular Na^+ are required.

7. Solutions containing a very low $[Ca^{2+}]_o$ ($< 10^{-8}$ M, buffered with EGTA) were used to prevent large rises of $[Ca^{2+}]_i$ which may occur on removal of external Na^+. Under these conditions pH_i recovery is still dependent upon $[Na^+]_o$, and the apparent inhibition of pH_i recovery by removal of Na^+ is not simply due to rises in $[Ca^{2+}]_i$.

8. The intracellular acidification which occurs on removal of Na^+ does not occur when $[Ca^{2+}]_o$ is very low ($< 10^{-8}$ M).

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9. A very large intracellular acidification occurs on removal of Na^+ in the presence of strophanthidin (10^{-5} M). Amiloride (1 mM) inhibits the contracture produced under these conditions and slightly decreases the magnitude of the acidification. The acidification and contracture do not occur when $[Ca^{2+}]_o$ is very low.

10. The Na^+ dependence of the pH_i regulatory system in Purkinje fibres and the close relationship of intracellular levels of Na^+, Ca^{2+} and H^+ are discussed. The results are consistent with a sarcolemmal Na^+–H^+ exchange being responsible for pH_i recovery from an acidosis, but other systems aiding pH_i recovery, e.g. an uptake of protons by mitochondria, cannot be excluded.

INTRODUCTION

Changes in intracellular pH (pH_i) have considerable effects on both the contractile and electrical properties of the heart (Cingolani, Mattiazzi, Blesa & Gonzalez, 1970; Williamson, Safer, Rich, Schaffer & Kobayashi, 1975; Fabiato & Fabiato, 1978; Fry & Poole-Wilson, 1981), but how pH_i is controlled in cardiac muscle is still poorly understood. It is clear, in common with many other cell types, that H^+ ions are not in equilibrium across the cardiac cell membrane (Ellis & Thomas, 1976; Deitmer & Ellis, 1980). The pH_i of heart muscle is almost an order of magnitude more alkaline than would be expected from a passive distribution of H^+ at equilibrium and yet the cells can rapidly recover from an imposed acidosis. The cells must therefore possess a mechanism for outward transport of H^+ (or its equivalent, i.e. inward transport of OH^- or HCO_3^-). In the light of the results of Thomas (1977) using snail neurones, Aickin & Thomas (1977) using mammalian skeletal muscle and Boron, McCormick & Roos (1981) using barnacle muscle, it seems reasonable to propose a mechanism for cardiac muscle whereby movement of Na^+ down its electrochemical gradient into the cells is coupled with the outward extrusion of H^+, that is to say an exchange of extracellular Na^+ for intracellular H^+. This mechanism was tentatively proposed by Deitmer & Ellis (1980) but the evidence was equivocal. In the present work we have used pH-sensitive glass micro-electrodes (Thomas, 1978) for continuous measurement of intracellular H^+ activity in Purkinje fibres from sheep hearts. In the first half of this paper we have extended the observations of Deitmer & Ellis (1980) and have demonstrated that removal of extracellular Na^+ causes pH_i to become more acid and also inhibits pH_i recovery from an acidosis. It is suggested that the presence of a Na^+–H^+ exchange mechanism could explain these results.

Recently, Vaughan-Jones, Lederer & Eisner (1983) presented evidence of pH_i changes in cardiac muscle following alterations of $[Ca^{2+}]_i$, which could not be ascribed to Na^+–H^+ exchange. They suggested that the mechanism for pH_i changes under these conditions was an internal sequestration of H^+, principally by the mitochondria. In addition they suggested that as $[Ca^{2+}]_i$ in cardiac muscle is also influenced by the transmembrane Na^+ gradient (e.g. Reuter & Seitz, 1968) then it is possible that the apparent Na^+ dependence of pH_i regulation is secondary to changes in $[Ca^{2+}]_i$. In the second half of this paper we have examined more closely the Na^+ concentration dependence of pH_i control and the intracellular interactions of Ca^{2+} and H^+. The results suggest that recovery from an intracellular acidosis is dependent upon the transmembrane Na^+ gradient even in the absence of large changes of $[Ca^{2+}]_i$. Some

of the results have been reported previously in preliminary form (Ellis & MacLeod, 1983*a*, *b*).

METHODS

General

Fresh sheep hearts were obtained from the local slaughterhouse. They were cut open and transported to the laboratory immersed in a bicarbonate-buffered Tyrode solution at ambient temperature and bubbled with a 95 % O_2, 5 % CO_2 gas mixture. Free-running Purkinje fibres were removed and one end pinned to the floor of the experimental chamber. A loop of fine nylon thread was tied to the other end and was connected to a force transducer constructed from piezo-resistive elements (AE801 Aksjeselskapet Mikroelektronic, Horten, Norway). The temperature of the bath was maintained at $35 \pm 1{\cdot}0$ °C. Oxygenated solutions were pre-heated to 35 °C in a water bath and carried to a multiway tap by Polythene tubing, or stainless-steel tubing when HCO_3^-/CO_2 buffered solutions were used.

The exchange rate of solution in the experimental chamber was 10 volumes/min and the solution exchange time was 10–90 % complete in 10 s (as determined by the response of a liquid ion-exchanger Na^+-sensitive micro-electrode).

Solutions

The normal Tyrode solution contained (mM): Na^+, 140; K^+, 6; Ca^{2+}, 2; Mg^{2+}, 1; Cl^-, 152; glucose, 10; HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid), 5; it was titrated with NaOH to give a pH of $7{\cdot}40 \pm 0{\cdot}05$ at 35 °C. The solution was equilibrated with 100 % O_2. When measurements were carried out at room temperature the normal Tyrode solution was titrated to give a pH of $7{\cdot}40 \pm 0{\cdot}05$ at 22 °C. For Na^+-free solutions the Na^+ was replaced by one of the following (mM): Li^+, 140; $Tris^+$ (2-amino-2-(hydroxymethyl)propane-1,3-diol(tris)), 158; BDA^+ (bis (2-hydroxyethyl)dimethylammonium), 140; TMA^+ (tetramethylammonium), 140 or K^+, 140.

Bicarbonate-buffered Tyrode solution, equilibrated with nominally 95 % O_2, 5 % CO_2, lacked HEPES but contained (mM): Na^+, 140; K^+, 6; Ca^{2+}, 2; Mg^{2+}, 1; Cl^-, 128; HCO_3^-, 24; glucose, 15; its pH was $7{\cdot}4 \pm 0{\cdot}1$ at 35 °C. Na^+-free bicarbonate-buffered solutions were produced by replacing Na^+ with one of the above substitutes (116 mM) and $KHCO_3$ (24 mM) to buffer the CO_2.

Solutions of very low $[Ca^{2+}]$ were produced by adding 1 mM-EGTA (ethylene glycolbis-(β-aminoethylether *N,N'*-tetraacetic acid) to nominally Ca^{2+}-free solutions. An extra 1 or 2 mM-Mg^{2+} was added as a Ca^{2+} substitute. Such solutions had $[Ca^{2+}] < 10^{-8}$ M. The $[Ca^{2+}]$ in these solutions was estimated by the use of Ca^{2+}-selective micro-electrodes (tip diameter *ca.* 10 μm), filled with a neutral ligand sensor (Oehme, Kessler & Simon, 1976) and calibrated by using standards as detailed by Tsien & Rink (1980).

When NH_4Cl was added an equivalent amount of NaCl was removed. Amiloride was added directly to the Tyrode solution; SITS (4-acetamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid disodium salt) was also added as solid to the Tyrode solution just before use, giving a final concentration of 100 μM.

Strophanthidin (Boehringer Mannheim) was dissolved in 50 % ethanol to produce a stock solution of 10^{-2} M. Thus, with a concentration of 10^{-5} M-strophanthidin in the Tyrode solution the amount of ethanol did not exceed 0·05 %.

Micro-electrodes

Membrane potentials were measured with conventional 3 M-KCl-filled micro-electrodes with resistances of 6–20 MΩ. pH-sensitive micro-electrodes were of the recessed-tip glass design (Thomas, 1974, 1978). Their tips were < 1 μm in diameter and they all had response times which were 90 % complete in 2 min. Their response to a unit change in pH was 52–58 mV. These electrodes were essentially free from drift. Any apparent drift on the recordings was due to either inadequate chloriding of the silver wire connecting the electrode to its amplifier or was due to a real drift of pH_i. The latter was observed following continued exposure to solutions containing NH_4Cl. Discrimination between the two types of drift was possible following removal of the micro-electrodes from the cells and observation of any change of base line. The Na^+-sensitive electrodes were also of the recessed-tip design of Thomas (1970, 1978). They all had response times which were 90 % complete within 90 s and they produced responses of 54–60 mV for a 10-fold change in $[Na^+]$. Since

Na^+-sensitive glass is slightly sensitive to K^+ and is therefore likely to produce a small voltage in response to the high intracellular K^+ activity, the electrodes were calibrated in solutions where Na^+ was replaced by K^+.

The potential measured by the Na^+-sensitive glass electrode is dependent on the pH of the solution under test (Eisenman, Rudin & Casby, 1957). The interference of H^+ becomes more apparent as the $[Na^+]$ in the test solution is decreased. We have therefore undertaken a series of measurements to quantify the effects of pH on the Na^+-sensitive electrodes at low $[Na^+]$ because our measurements of intracellular Na^+ activity (a^i_{Na}) at very low $[Na^+]_o$ were accompanied by pH_i changes. After very long periods (>1 h) in Na^+-free solution pH_i may become more acid by up to 0·7 pH units. Measurements made over a similar range of pH values reveal that the apparent a^i_{Na} would be over-estimated by $0{\cdot}057 \pm 0{\cdot}009$ mM ($n = 5$). This result yields a value of approximately 2·6 for the selectivity factor, log K^{Pot}_{NaH} (log (sensitivity to H^+/sensitivity to Na^+)); this is close to that quoted by Steiner, Oehme, Ammann & Simon (1979).

Signal recording

The output from the KCl micro-electrode with respect to an Agar–Ringer solution reference electrode was displayed on a pen recorder. pH micro-electrode potentials were measured using a varactor bridge operational amplifier (Analog Devices 311J) and filtered (time constant 0·65 s). By recording the difference in signal between the KCl and the ion-sensitive micro-electrodes the pH_i or a^i_{Na} was obtained.

RESULTS

Acidosis and pH_i recovery

The method adopted to investigate the problem of pH_i regulation was to acidify the cell and then to study the subsequent pH_i recovery. This recovery should be equivalent to an 'uphill' movement of H^+ ions against their electrochemical gradient. Acidifications were imposed by (1) the addition and subsequent removal of NH_4Cl, (2) exposure to solutions containing CO_2 or (3) changing to a more acid Tyrode solution. These three types of acid loading are illustrated in Fig. 1.

Exposure of the fibre to solutions containing NH_4Cl initially produces an intracellular alkalinization. This is followed by a slow acidification. The subsequent removal of NH_4Cl causes a rapid intracellular acidification. The transmembrane movements of NH_3 and NH_4^+ responsible for these pH_i changes have been described by Boron & De Weer (1976) but an alternative explanation for part of the changes has been given by Vaughan-Jones (1981, 1982*a*, *b*).

Exposure to solutions containing CO_2 rapidly acidifies the cell. CO_2 easily crosses the cell membrane, producing H^+ and HCO_3^- on hydration and subsequent dissociation. The rate of recovery from these acidifications is a measure of the ability of the cell to regulate its pH_i following an acidosis. The steady-state pH_i attained in CO_2/HCO_3^--buffered solutions is usually more acid than that attained in CO_2-free, HEPES-buffered Tyrode solution. This is perhaps due to the negative membrane potential driving the efflux of HCO_3^- (de Hemptinne, 1981). On returning from a CO_2/HCO_3^--buffered to a CO_2-free, HEPES-buffered Tyrode solution the pH_i rapidly becomes alkaline and overshoots its initial value as intracellular CO_2 leaves the cell. Boron & De Weer (1976) suggest that this overshoot is indicative of a previous acid extrusion and its size is a measure of the net loss of H^+ which occurred during the exposure to CO_2.

Following all three types of acidification there was a recovery (i.e. a shift of pH_i to a more alkaline value). If the values of pH_i are converted into intracellular H^+

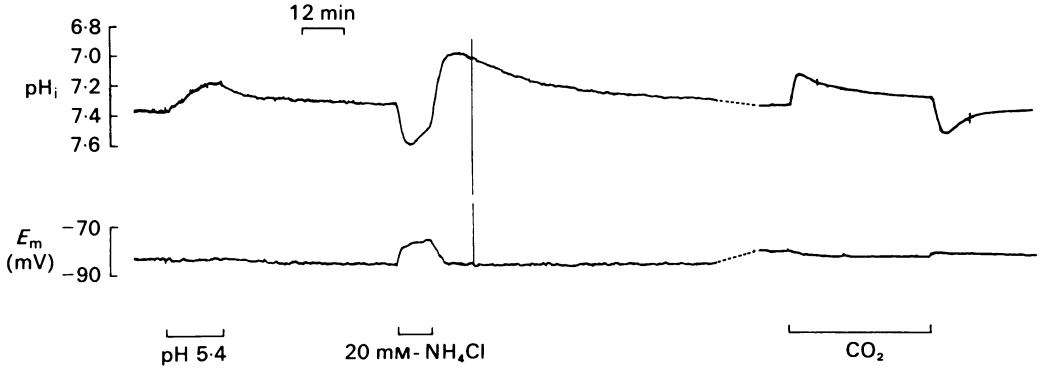

Fig. 1. The effects on intracellular pH (pH_i, top trace) and membrane potential (E_m, bottom trace) of three different methods of acid loading. The dotted line indicates a break in the recording of about 90 min, during which time the voltage electrode was removed (because it was blocked) and another cell penetrated.

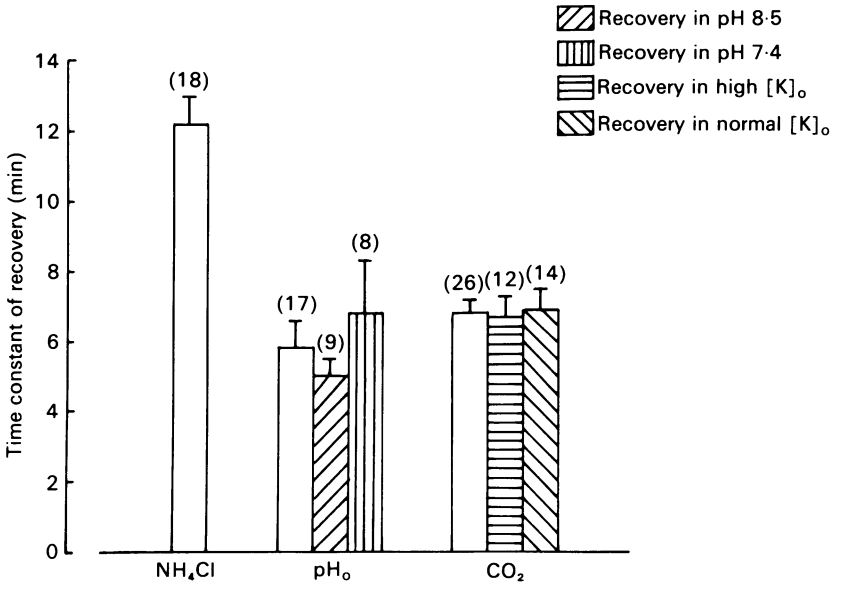

Fig. 2. The time constants of the rates of recovery of pH_i following three methods of acid loading. The unshaded columns indicate the means ± S.E. of means of the time constants of all recoveries from acidifications induced by NH_4Cl, acid Tyrode solution (pH_o 5·4) and CO_2. The number of separate experiments for each type of acid load is given above the columns. Some of the recoveries from acid pH Tyrode solution were carried out at pH_o 8·5 and some at pH_o 7·4. These results are shown separately (as diagonally and vertically hatched columns respectively). Similarly, the unshaded column for CO_2-induced acid loading represents the combined results obtained from experiments carried out under two conditions. The recoveries were in either normal $[K^+]_o$ (6 mM) or high $[K^+]_o$ (20–26 mM, the $[Na^+]_o$ being reduced by an equivalent amount). The results at normal and high $[K]_o$ are shown separately as the diagonally hatched and horizontally hatched columns respectively.

activity (a^i_H), the recovery can be shown to be approximately exponential (e.g. see Fig. 7*B*).

The time constants of recovery were different according to which type of acid loading procedure was used. Fig. 2 shows these differences in recovery time constants. The unshaded columns indicate the mean (±S.E. of mean) of the time constants from experiments where the three different types of acid loading procedures were used. The number of separate experiments for each type of acidification is given above the columns. The shaded columns illustrate the different recovery conditions used in some experiments. Following acid loading with acid extracellular pH (pH_o) the recovery was measured with pH_o at either 7·4 or 8·5. There was no significant difference in the recovery rates. Following acidification with CO_2 the recovery was measured at either a normal $[K^+]_o$ of 6 mM or at a high $[K^+]_o$ of 20–26 mM (with $[Na^+]_o$ reduced by an equivalent amount). The high $[K^+]_o$ used in these experiments produced a fall in membrane potential from about −73 to about −40 mV. There was no significant difference between the recovery rates. Taking the grouped data (unshaded columns) there was no significant difference between the time constant of recovery from acid loading produced by acid pH_o compared with CO_2 exposure ($P > 0·1$, Student's *t* test). However, Thomas (1977) showed that the buffering power of cells is higher in the presence of CO_2, which makes a direct comparison difficult. In snail neurones he found that following ionophoretic injection of H^+ there was a faster recovery of pH_i in the presence of CO_2 than in its absence. The mean time constants of recovery following acidifications induced by acid pH_o or CO_2 were both significantly faster than the time course of recovery following exposure to NH_4Cl ($P < 0·01$, Student's *t* test).

The effects of removal of extracellular Na^+ on pH_i recovery

Evidence for the existence of a sarcolemmal Na^+–H^+ exchange mechanism in heart muscle is limited. Deitmer & Ellis (1980) showed that decreasing $[Na^+]_o$ to 10% normal (i.e. 14 mM) slowed the rate of recovery from an acidification induced by removal of NH_4Cl. In order to investigate this further we have tried to inhibit the recovery from an acid load completely by removing all extracellular Na^+. In Fig. 3 acid loading was brought about by exposure to, then removal of, 20 mM-NH_4Cl. After the first exposure to NH_4Cl the superfusing Tyrode solution was changed to one where all the Na^+ had been replaced by BDA^+. The Na^+-free solution inhibited pH_i recovery from the intracellular acidification. Approximately 20 min later the Tyrode solution was changed to one which also lacked Na^+ but contained Li^+ as the Na^+ substitute. Recovery of pH_i now proceeded but at a slower rate (time constant 19 min) than in normal Tyrode solution (time constant 14·4 min). This demonstrates two important points concerning the regulation of pH_i in cardiac Purkinje fibres. First, removal of all Na^+ and the use of an appropriate Na^+ substitute can completely inhibit recovery of pH_i and, secondly, Li^+ can support recovery from an intracellular acidification. The use of TMA^+ or K^+ as the Na^+ substitute gives similar results to those using BDA^+. Tris, commonly used as a Na^+ substitute, was found to produce alkaline pH_i changes (not illustrated) of up to 0·1 pH units when added to the normal Tyrode solution to produce a concentration of only 22 mM. As much as 30% of Tris can remain undissociated at pH 7·4 (Nahas, 1962). This fraction could penetrate the cells and make the cytoplasm more alkaline (see Robin, Wilson & Bromberg, 1961). An alternative explanation is provided by the recent experiments of de Hemptinne &

Vanheel (1984). They found that a change in the buffer capacity of the superfusion solution can affect the pH at the surface of muscle cells. This can in turn alter pH_i. Thus in a solution with a high buffer capacity the intracellular pH would tend to become more alkaline.

We have therefore preferred to use either quaternary ammonium compounds (which appear to be fully dissociated in solution) or K^+ as Na^+ substitutes. However,

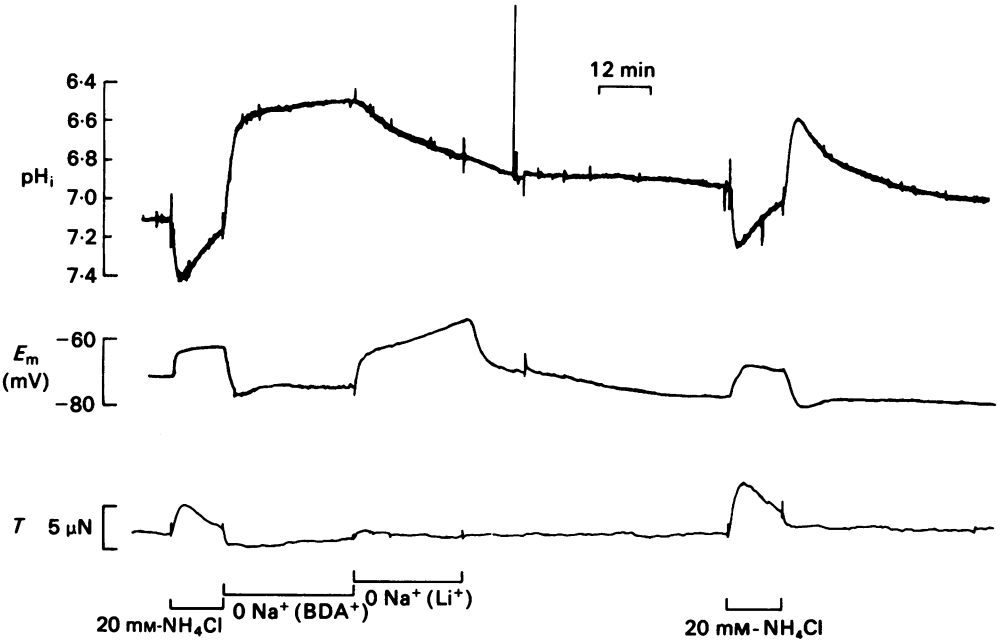

Fig. 3. The effect of removal of all extracellular Na^+ (BDA^+ substitution) on pH_i recovery from an NH_4Cl-induced acidification. Immediately after NH_4Cl exposure the Tyrode solution was changed to one lacking Na^+ and where BDA^+ was the substituted cation. After about 20 min the Na^+-free Tyrode was changed to one where Li^+ was the Na^+ substitute. The bottom trace shows the changes in force produced by the preparation.

K^+ may not be a particularly good choice; although K^+ does not suffer from the drawbacks described previously for Li^+ or Tris, solutions of high $[K^+]$ can produce small changes of pH_i. Hypertonic Tyrode solution, containing 140 mM-K^+ and 140 mM-Na^+ was found to decrease pH_i by up to 0·1 pH unit, compared with a small transient change in pH_i induced by 140 mM-TMA^+ and 140 mM-Na^+. De Hemptinne (1981) has also found that depolarization with high $[K^+]_0$ induces an acid pH_i change which he presumed was due to a release of Ca^{2+}. The effect appears to be concentration-dependent because we found that increasing $[K^+]_0$ from normal up to 70 mM (again in the presence of 140 mM-Na^+) also produced an acid pH_i change but somewhat smaller than when 140 mM-K^+ was used. The acidification could be due to the depolarization associated with high $[K]_0$ solutions.

Similar inhibitory effects of Na^+-free solutions on recovery can be demonstrated when the cell has been acidified by exposure to CO_2-containing solutions (Ellis & MacLeod, 1983*a*, *b*), or by changing pH_0 to, for example, pH 5·4. While all pH_i recoveries from acidifications induced by NH_4Cl or CO_2 showed complete inhibition

by Na^+-free solutions the degree of inhibition of recovery by Na^+-free solution from an acidification induced by an acid pH_o was variable between preparations. Following this type of acidification, the change to a Na^+-free solution at normal pH (7·4) was often accompanied by a transient recovery of pH_i. The size of this transient recovery was only $27 \pm 14\%$ (S.E. of mean, $n = 7$) of the total initial acidification. After this small recovery the pH_i became more acid with continued exposure to the Na^+-free

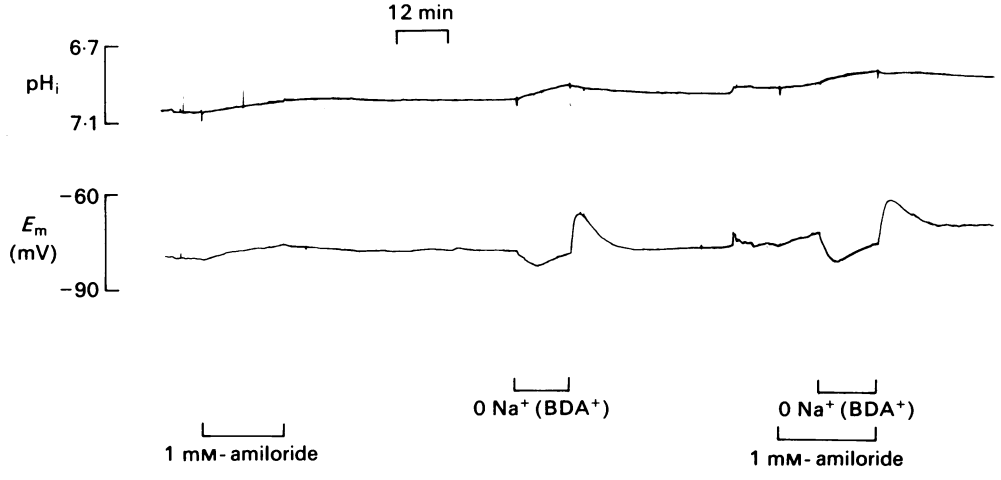

Fig. 4. A comparison of the effects of amiloride (1 mM) and Na^+-free Tyrode solution on pH_i. The Na^+ substitute was BDA^+.

solution. This transient recovery probably reflects the diffusion delay in reducing the $[Na^+]_o$ down to the very low levels (see later Results) necessary to inhibit pH_i recovery. This delay would not be apparent with the other two methods used to study extracellular Na^+-dependent pH_i recovery because in both cases any possible recovery was preceded by a period when the cell was tending to become acid (either via loss of NH_3 or entry of CO_2). Therefore by the time the acidification had developed fully, the $[Na^+]_o$ was already very low and so no recovery was apparent.

Since recovery from these types of acid loading can be almost completely blocked by removing extracellular Na^+ (with the proviso that an appropriate Na^+ substitute is used) then simple removal of extracellular Na^+ should produce an intracellular acidification because there should be a continuous 'leak' entry of H^+ into the cells in addition to metabolic production of H^+ by the cells. This is illustrated in Fig. 4 (see also Ellis & MacLeod, 1983*b*). Replacement of Na^+ by BDA^+ for 12 min decreased pH_i by 0·08 unit. Exposure to Na^+-free (K^+- or TMA^+-substituted) Tyrode solution for prolonged periods of time (i.e. >1 h) also produced slow continuous decreases in pH_i (e.g. see Fig. 12). Average initial rates of decrease of pH_i for K^+-, BDA^+- and TMA^+-substituted Tyrode solutions were similar ($0{\cdot}0062 \pm 0{\cdot}0012$, $0{\cdot}0060 \pm 0{\cdot}0007$, and $0{\cdot}0060 \pm 0{\cdot}0004$ pH units/min, mean $\pm$S.E. of mean, $n = 8, 4$ and 15 respectively). When Na^+ was replaced pH_i recovered.

These results are in contrast to the recent brief report of Coray & McGuigan (1983) who found that pH_i increased when Na^+ was replaced by TMA^+. Their experiments

were carried out on ferret ventricular muscle using electrodes containing the neutral ligand ion exchange resin of Amman, Lanter, Steiner, Schulthess, Shijo & Simon (1981) and at room temperature (22–25 °C). We have carried out similar experiments at room temperature with both glass and neutral ligand pH-sensitive micro-electrodes. In three experiments using sheep Purkinje fibres, one preparation showed a smaller acidification at 22 °C than at 35 °C in Na^+-free solution, while the other two

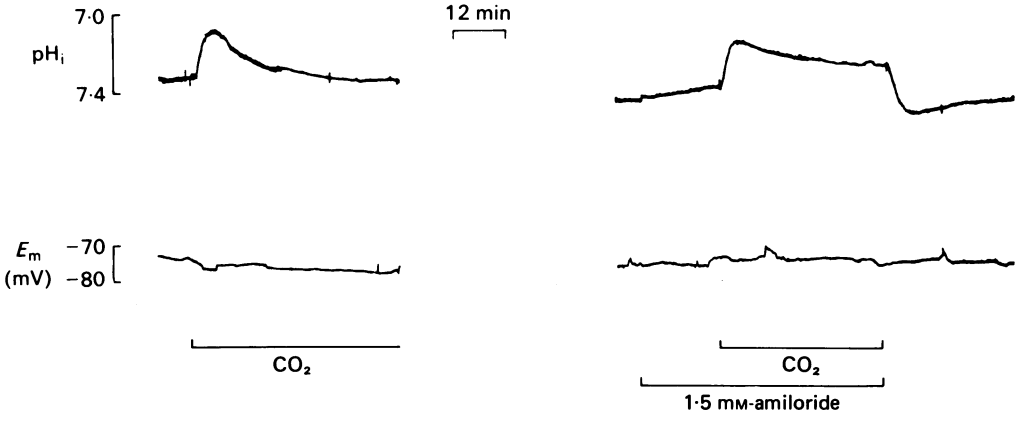

Fig. 5. The effect of amiloride (1·5 mM) on pH_i recovery from a CO_2-induced intracellular acidification. The break in the recording was for a period of about 1 h.

preparations showed little change in pH_i at 22 °C compared with the normal acidification at 35 °C. In one experiment on ferret papillary muscle at 35 °C, Na^+-free solutions produced an intracellular acidification. Thus it appears that temperature probably affects the pH_i response to Na^+-free solutions but differences between ventricular muscle and Purkinje fibres cannot be ruled out.

Effect of amiloride and SITS on pH_i recovery

Exposure of the fibre to 1 mM-amiloride (a putative Na^+–H^+ exchange inhibitor; see for example Bull & Laragh, 1968), also decreased pH_i but more slowly than removal of all extracellular Na^+ (Fig. 4). The observed rate of decrease of pH_i with 1 mM-amiloride was about 33% of that observed in Na^+-free solutions (rate of acidification in normal Tyrode solution +1 mM-amiloride = 0·0020 ± 0·0004 pH units/min, mean ± S.E. of mean, $n = 7$). No additive effect of Na^+-free (BDA$^+$- or TMA$^+$-substituted) Tyrode solution and 1 mM-amiloride could be detected. Amiloride could slow but not completely inhibit recovery from an acidification induced by exposure to CO_2 (Fig. 5) and has already been demonstrated to have similar effects on recovery from NH_4Cl-induced acidifications (Deitmer & Ellis, 1980; Vaughan-Jones, 1982*a*).

Exposure to CO_2/HCO_3^--buffered Tyrode solution would presumably allow any Cl^-–HCO_3^- transport mechanism, not operational in CO_2-free, HEPES-buffered Tyrode solution, to assist pH_i recovery (see Aickin & Thomas, 1977). We have found no effect of the stilbene derivative SITS (100 μM), an inhibitor of Cl^-–HCO_3^-

exchange (e.g. Knauf & Rothstein, 1971), on the recovery from a CO_2-induced acidification.

Effect of temperature on pH_i and pH_i recovery from acid loading

Fig. 6 shows part of an experiment illustrating the effects of temperature on pH_i recovery from an acidosis. Decreasing the temperature from 35 to 21 °C produced a decrease in the rate of recovery of pH_i by about 60 % ($n = 4$) and increased steady-state

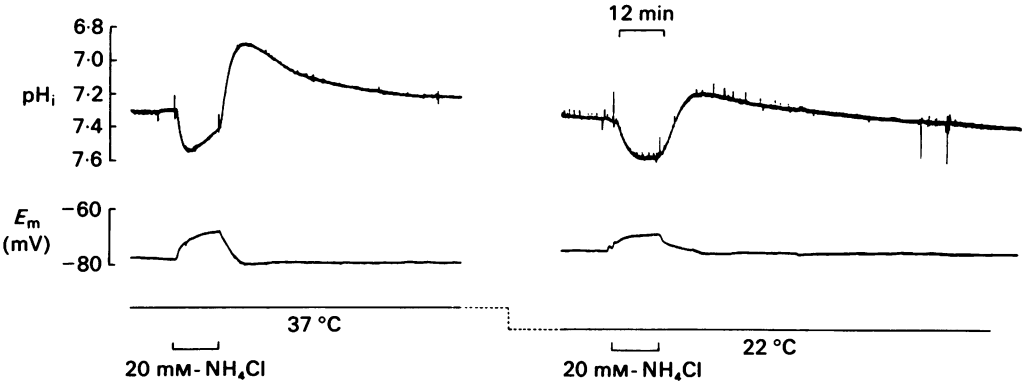

Fig. 6. The effect of temperature on pH_i during exposure to, and removal of NH_4Cl. After the recovery at 37 °C the heating unit was switched off and the Tyrode solution was changed to one which had a pH of 7·4 at 22 °C. The break in the trace was for approximately 2 h.

pH_i by 0·21 ± 0·06 pH units (mean ± S.E. of mean, $n = 4$). These results are similar to those of Aickin & Thomas (1977) in mouse skeletal muscle. From four experiments the average Q_{10} for the pH_i recovery measured over the temperature range of 21–35 °C was 2·65. This is higher than the calculated Q_{10} of 1·4 (temperature range 28–37 °C) for the Na^+–H^+ exchanger in mouse skeletal muscle (Aickin & Thomas, 1977). Notice that at low (i.e. room) temperature the acidification brought about by removal of NH_4Cl was not as great. The recovery from the *alkalinization* during exposure to NH_4Cl was also slowed. This recovery is probably due to two separate phenomena: an active extracellular Cl^-–intracellular HCO_3^- exchange mechanism (see Vaughan-Jones, 1981, 1982*a*, *b*) as well as an influx of NH_4^+ which yields H^+. Note that the depolarization, presumably associated with NH_4^+ influx, is less.

The Na^+ dependence of pH_i recovery

In order to characterize more fully the Na^+ dependence of the pH_i recovery mechanism we performed experiments of the type shown in Fig. 7*A*. The Na^+ substitute used was TMA^+ and the acidifications were induced by exposure to solutions containing CO_2.

Since TMA bicarbonate was not readily available, $KHCO_3$ was used to buffer the CO_2-containing solutions. The same quantity of KCl was therefore added to the other solutions to obtain the same degree of cell depolarization. The effect of removal of Na^+ was similar to that seen previously. Fig. 7*A* shows that when all Na^+ was removed, recovery was inhibited. With 7 mM-Na^+ present, recovery was greatly

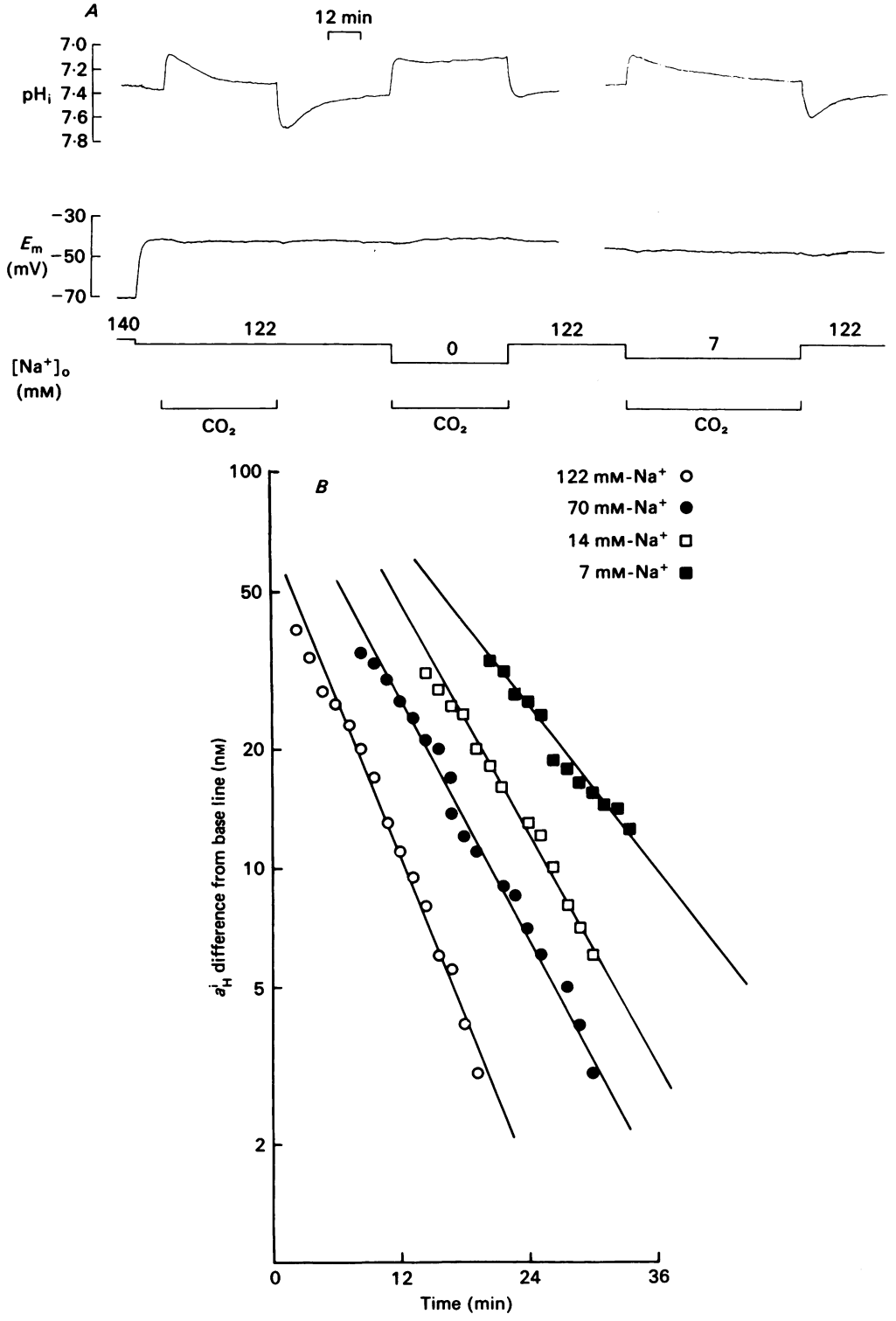

Fig. 7. For legend see following page.

slowed but pH_i returned to near normal levels. The rates of recovery of a^i_H at various $[Na^+]_o$ from this experiment are shown in Fig. 7*B*. This process appears to be exponential and it was inhibited as $[Na^+]_o$ was reduced. In this experiment the rate of recovery in 7 mM-Na^+ was 58% of that in 122 mM-Na^+. Fig. 8 shows the rate of recovery (expressed as a percentage of that in normal $[Na^+]_o$, which in these experiments was 120–124 mM) plotted against $[Na^+]_o$. The mean results from five experiments showed that $[Na^+]_o$ had to be reduced to approximately 8 mM before the rate of recovery was inhibited by 50%.

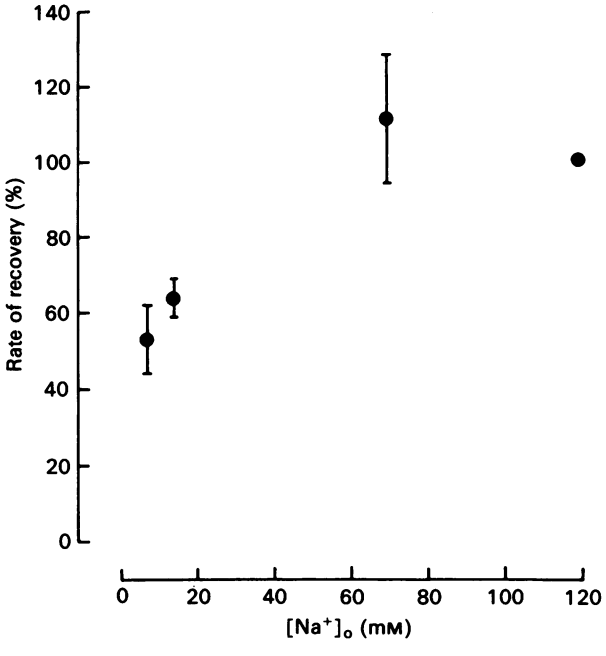

Fig. 8. The filled circles show the dependence of the rate of pH_i recovery on $[Na^+]_o$. The rate is expressed as a percentage of that in 120 mM-Na^+. The points are the means ± S.E. of means of five experiments.

It is interesting to speculate on the $[Na^+]_o$ which would be required to promote the net extrusion of H^+. Net extrusion might continue as long as the energy gradient for Na^+ entry is great enough. If it is assumed that there is a Na^+–H^+ exchange in

Fig. 7. *A*, the effect of lowering the $[Na^+]_o$ on pH_i recovery from acidifications induced by exposure to CO_2-containing solutions. When $[Na^+]_o$ was reduced it was replaced by TMA^+. Approximately 24 mM-$KHCO_3$ was used to buffer the CO_2-containing solutions. The same quantity of K^+ was added to the other solutions (HEPES-buffered) to obtain the same degree of cell depolarization (with a similar amount of Na^+ removed to maintain isotonicity). The break in the recording was for about 2·5 h, during which time a similar pH_i recovery was measured in 70 mM-Na^+. *B*, the recovery of intracellular H^+ activity (a^i_H) back to its base-line value in solutions of varying $[Na^+]_o$, following acid loading with CO_2-containing solutions. The data points were taken from the experiment in *A*. The lines drawn through the points were fitted by regression analysis. The points at different values of $[Na^+]_o$ have been offset by 6 min for clarity.

the ratio 1 : 1 (there is no evidence for an electrogenic Na^+–H^+ exchange in this tissue), then there could be net extrusion of H^+ as long as $E_{Na} > E_H$ (where E_{Na} and E_H are the equilibrium potentials for Na^+ and H^+ respectively and assuming no other ion can participate in the exchange).

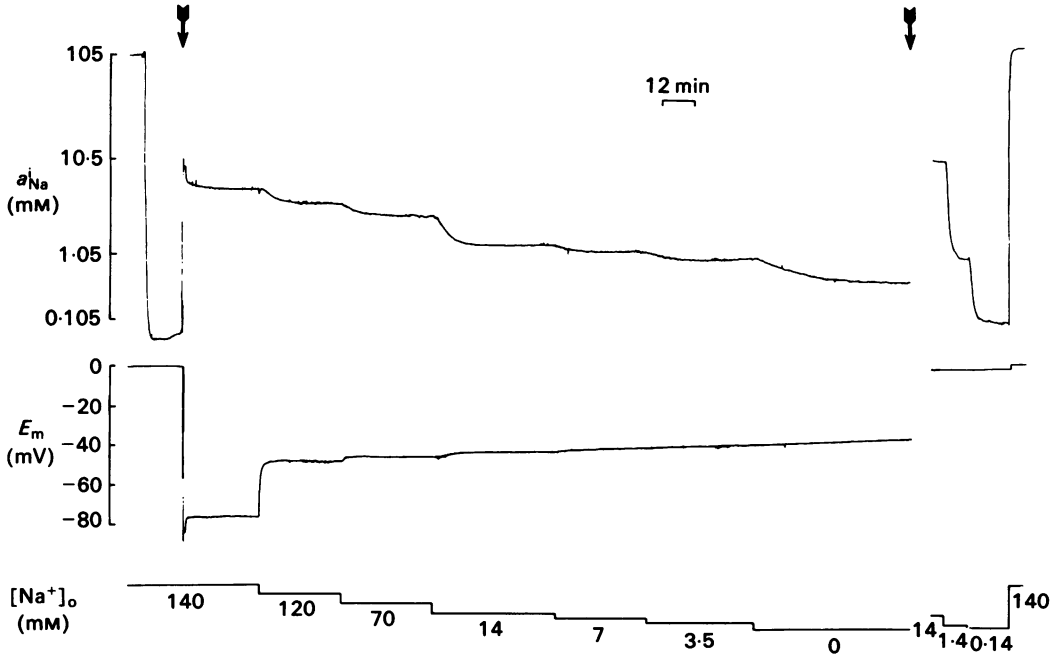

Fig. 9. The effect of changes of $[Na^+]_o$ on intracellular Na^+ activity. Top trace: intracellular Na^+ activity (a^i_{Na}); bottom trace: membrane potential (E_m). The changes in $[Na^+]_o$ made during the experiment are shown at the bottom of the illustration. At the beginning of the trace both electrodes were extracellular. Both electrodes were intracellular between the arrows. The Na^+ substitute was K^+ for the first change in $[Na^+]$ (from 140 to 120 mM). Further reductions of $[Na^+]_o$ were produced by substitution with TMA^+. A short calibration procedure for the electrode is shown at the end of the experiment. During the calibration procedure the Na^+ substitute was K^+.

Effect of $[Na]_o$ on a^i_{Na}

No thorough measurements of a^i_{Na} at very low $[Na^+]_o$ (< 14 mM) have been made previously. In order to find values of E_{Na} at low $[Na^+]_o$ we have undertaken a series of measurements of a^i_{Na} using Na^+-sensitive glass micro-electrodes. An example of such an experiment is shown in Fig. 9. For the first 25 min the preparation was superfused with normal Tyrode solution (140 mM-Na^+, 6 mM-K^+). The Tyrode solution was then changed to one with a higher $[K^+]_o$ (120 mM-Na^+, 26 mM-K^+) and the cells depolarized to about −45 mV, i.e. conditions similar to those during the CO_2 recovery experiments. From this point, when Na^+ was removed an isotonic amount of TMA^+ was substituted. The combined results of five such experiments are shown in Fig. 10. The a^i_{Na} values have been corrected as described in the Methods for the interference due to the H^+ on the Na^+-sensitive glass at very low Na^+ activities. The

a^i_{Na} activity varies linearly with $[Na^+]_o$ down to $[Na^+]_o$ values of about 14 mM. This agrees well with the results of Ellis (1977), Ellis & Deitmer (1978), Sheu & Fozzard (1982) and Chapman, Coray & McGuigan (1983*a*). The straight line drawn through the data points between 14 and 120 mM was fitted by regression analysis. At lower $[Na^+]_o$ the graph no longer remains linear but falls away steeply to yield values for

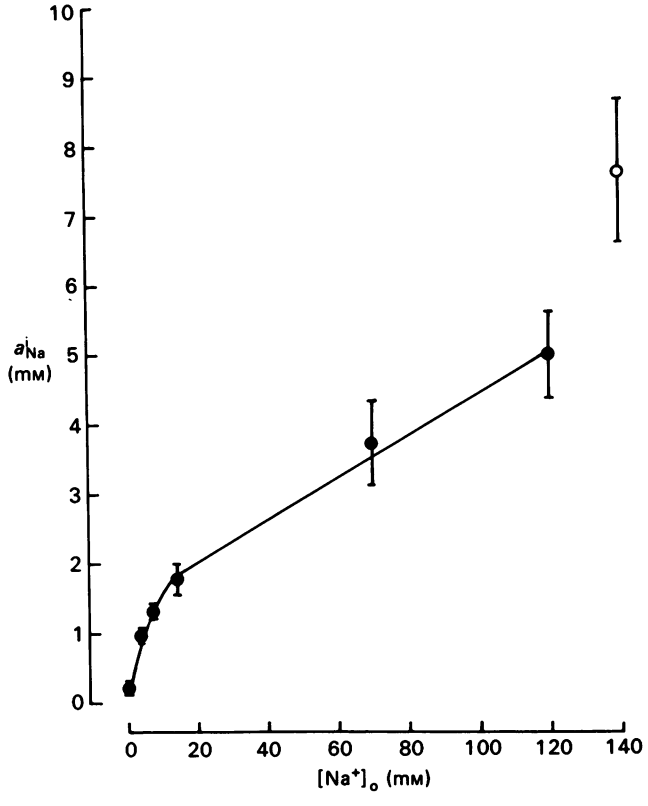

Fig. 10. The relationship between intracellular Na^+ activity (a^i_{Na}) and the $[Na^+]_o$ obtained from experiments like that shown in Fig. 9. Measurements were made in experiments carried out in 26 mM-K^+ (●) and in 6 mM-K^+ (○). The points are means ± S.E. of means of five preparations. The straight line drawn through the points between 14 and 120 mM-Na^+ was fitted by linear regression analysis and has the equation $a^i_{Na} = 0{\cdot}030\ [Na^+]_o + 1{\cdot}43$, $r = 0{\cdot}813$.

a^i_{Na} of $0{\cdot}18 \pm 0{\cdot}09$ (mean ± S.E. of mean) mM in Na^+-free Tyrode solution. (Our Na^+-free Tyrode solution was analysed for Na^+ and the concentration of contaminant Na^+ was $< 50\ \mu$M.)

There may be two components to the relationship of a^i_{Na} *versus* $[Na^+]_o$: a saturable component with an apparent half-maximal activation of approximately 3 mM and a second component that is linear up to approximately 120 mM-Na^+ (Fig. 10).

The values of a^i_{Na} from experiments of the type shown in Fig. 9 have been used to calculate E_{Na}. E_{Na} is plotted against $[Na^+]_o$ in Fig. 11. The Na^+ energy gradient only starts to fall towards E_H when $[Na^+]_o$ is less than approximately 2 mM. The $[Na^+]_o$ might therefore have to be decreased to this range before H^+ extrusion is halted.

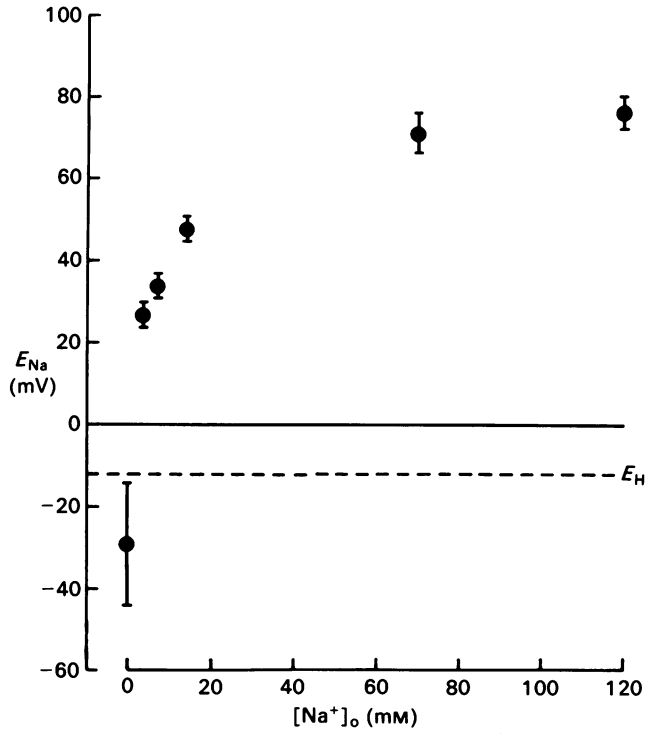

Fig. 11. The relationship of E_{Na} to $[Na^+]_o$. E_{Na} was calculated by the Nernst equation using the data from experiments of the type shown in Fig. 9. The points are the means ± S.E. of means from five experiments. The dotted line shows E_H assuming $pH_i = 7{\cdot}2$, $pH_o = 7{\cdot}4$.

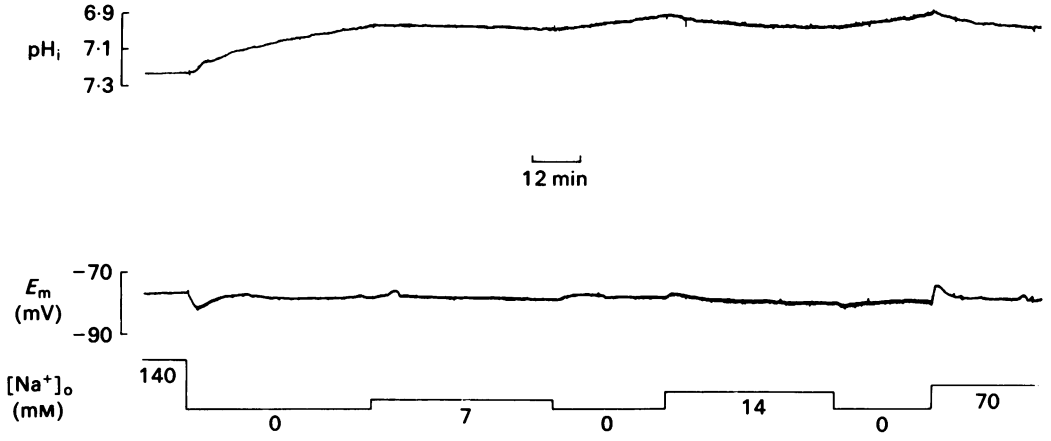

Fig. 12. The effect on pH_i of varying $[Na^+]_o$. The Na^+ substitute throughout the experiment was TMA^+. The changes in $[Na^+]_o$ are shown at the bottom of the illustration.

These thermodynamic estimates are in agreement with experiments of the type illustrated in Fig. 12. This shows the effect on pH_i of varying $[Na^+]_o$. The Na^+ substitute throughout the experiment was TMA^+. Na^+-free Tyrode solution produced an intracellular acidification which was stopped and reversed by changing to a Tyrode solution which contained 7 mM-Na^+. In other words, only a very low $[Na^+]_o$ is required to promote a recovery from an acidification induced by CO_2 or NH_4Cl or to halt the acidification that occurs in Na^+-free solutions.

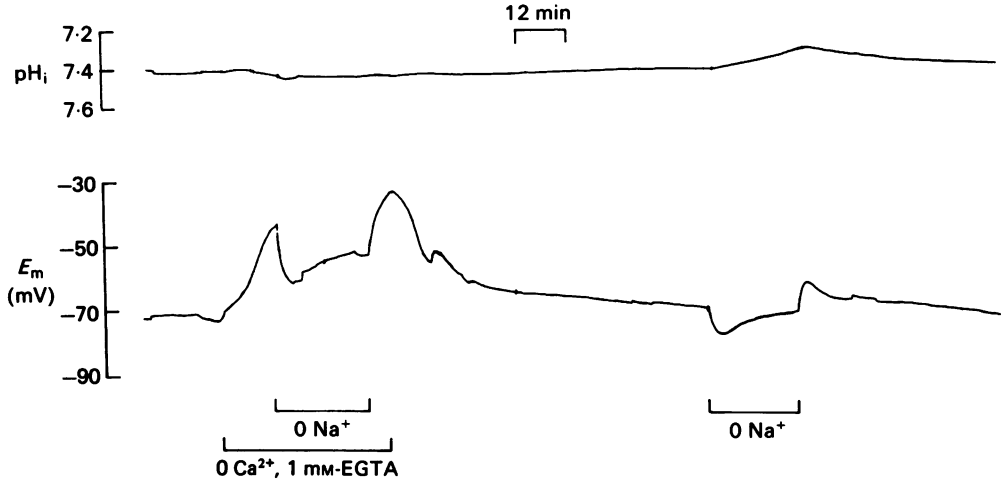

Fig. 13. The effect on pH_i of Na^+ removal in normal (2 mM) or very low ($<10^{-8}$ M) Ca^{2+} Tyrode solution. The Na^+ substitute was TMA^+.

Ca^{2+} and H^+ interaction

The experiments described above provide further evidence for involvement of Na^+ in the regulation of pH_i. However, the apparent inhibition of pH_i recovery in low $[Na^+]_o$ solutions could be mediated not only via a direct effect on a Na^+–H^+ exchange but also via an indirect process whereby a rise in $[Ca^{2+}]_i$, brought about by reducing $[Na^+]_o$, affects pH_i. The $[Ca^{2+}]_i$ is increased on reduction of $[Na^+]_o$ (Niedergerke, 1963) probably due to an altered Na^+ gradient (Reuter & Seitz, 1968; Baker, Blaustein, Hodgkin & Steinhardt, 1969; Glitsch, Reuter & Scholtz, 1970). This raised $[Ca^{2+}]_i$ can directly change pH_i, perhaps because of competition at buffering sites shared between H^+ and Ca^{2+} (Meech & Thomas, 1977; Deitmer & Ellis, 1980; Ellis, Deitmer & Bers, 1981; Vaughan-Jones *et al.* 1983).

If $[Ca^{2+}]_i$ were to rise under conditions of low $[Na^+]_o$ the observations that (i) pH_i decreases in Na^+-free Tyrode solution and (ii) pH_i recovery from acid loading is inhibited in low $[Na^+]_o$, could be explained by the rise in $[Ca^{2+}]_i$ being buffered and giving rise to an increase in the level of free H^+ within the cell. We have therefore performed a series of experiments using very low $[Ca^{2+}]_o$ solutions (buffered with EGTA) to prevent large rises of $[Ca^{2+}]_i$ when $[Na^+]_o$ was reduced. Chapman & Miller (1972) using frog atrial muscle showed clearly that Na^+-free contractures can be prevented by superfusion with solution lacking Ca^{2+} and with the addition of

1 mM-EGTA. Chapman (1974), in addition, showed that a Na^+-free contracture develops when the $[Ca^{2+}]_o$ is as low as 10^{-5} M but that the muscle relaxes in 10^{-7} M-Ca^{2+}. Miller & Moisescu (1976) reported that low-Na^+ contractures are prevented when $[Ca^{2+}]_o$ is less than 5×10^{-8} M. Fig. 13 demonstrates an experiment in which we have used low $[Ca^{2+}]_o$ solutions ($< 10^{-8}$ M). Exposing the cells to Na^+-free solution in the presence of normal $[Ca^{2+}]_o$ brings about an intracellular acidification,

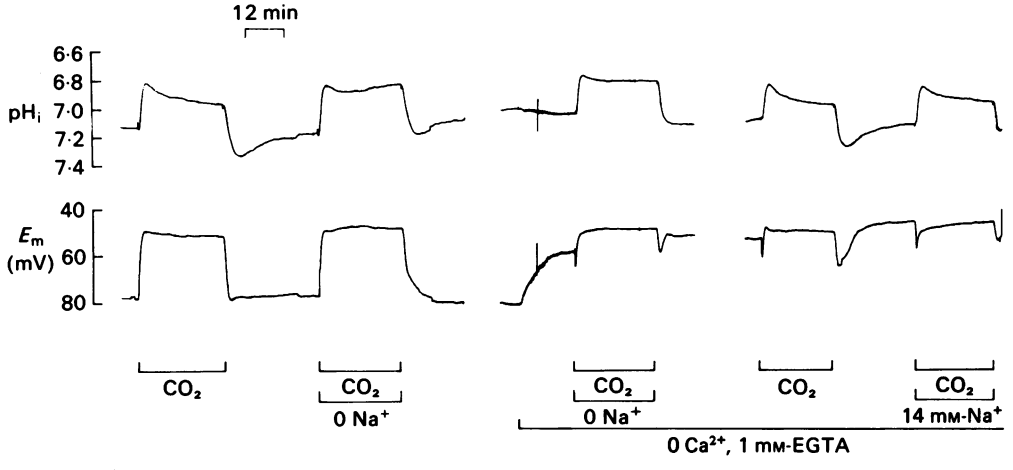

Fig. 14. The effect on pH_i recovery, from CO_2-induced acid loading, of reduction of $[Na^+]_o$ in normal (2 mM) or very low ($< 10^{-8}$ M) Ca^{2+}. When Na^+ was reduced it was replaced by TMA^+. The CO_2 was buffered by $KHCO_3$. The raised $[K^+]_o$ was responsible for the depolarization in CO_2-containing solutions.

as has been demonstrated earlier in the paper. When the fibre was superfused with a Tyrode solution containing very low $[Ca^{2+}]$ there was little change in pH_i. The depolarization of approximately 30 mV in low $[Ca^{2+}]_o$ was presumably due to an increase in cell membrane permeability. Under such conditions of very low $[Ca^{2+}]_o$, exposure of the cells to Na^+-free Tyrode solution produced no change in pH_i. The membrane potential became more negative during the Na^+-free exposure, suggesting that much of the depolarization was due to an increase in Na^+ permeability. The acidification was blocked even though the Ca^{2+}-free treatment would cause a large rise in a^i_{Na} (Deitmer & Ellis, 1978). This increase in a^i_{Na} might be expected to inhibit a Na^+–H^+ exchange system. Experiments of this type suggest that much of the intracellular acidification produced by decreasing $[Na^+]_o$ is mediated via an increase in $[Ca^{2+}]_i$, the rise in $[Ca^{2+}]_i$ being brought about by the Na^+–Ca^{2+} exchange mechanism promoting Ca^{2+} influx and inhibiting Ca^{2+} efflux when $[Na^+]_o$ is low (Vaughan-Jones *et al.* 1983). In order to check that the pH_i recovery from acid loading, which is inhibited in low $[Na^+]_o$, was not due to a change in $[Ca^{2+}]_i$, we performed experiments like that shown in Fig. 14. The depolarizations during the CO_2 exposures were due to $KHCO_3$ being used to buffer the CO_2. In Na^+-free, normal $[Ca^{2+}]_o$, the recovery was inhibited. When the same experiment was carried out with $< 10^{-8}$ M-Ca^{2+} in the superfusing Tyrode solution, complete Na^+ removal again inhibited recovery. In 120 mM-Na^+ recovery proceeded and in 14 mM recovery

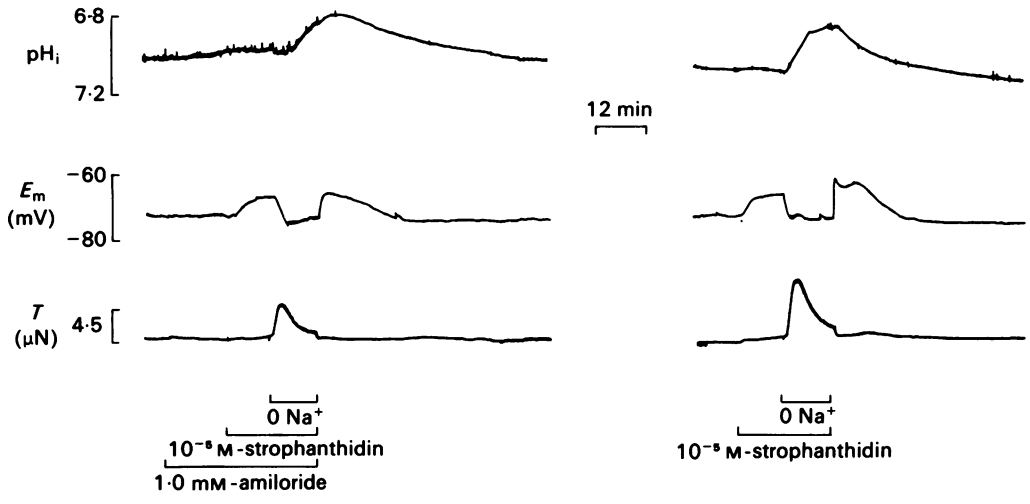

Fig. 15. The effect of amiloride (1 mM) on the contracture and the intracellular acidification produced by removal of Na^+ during exposure of the cells to 10^{-5} M-strophanthidin. Top trace: pH_i; middle trace: membrane potential (E_m); bottom trace: tension (T). When Na^+ was removed it was replaced by TMA^+. The break in the recording was for about 30 min.

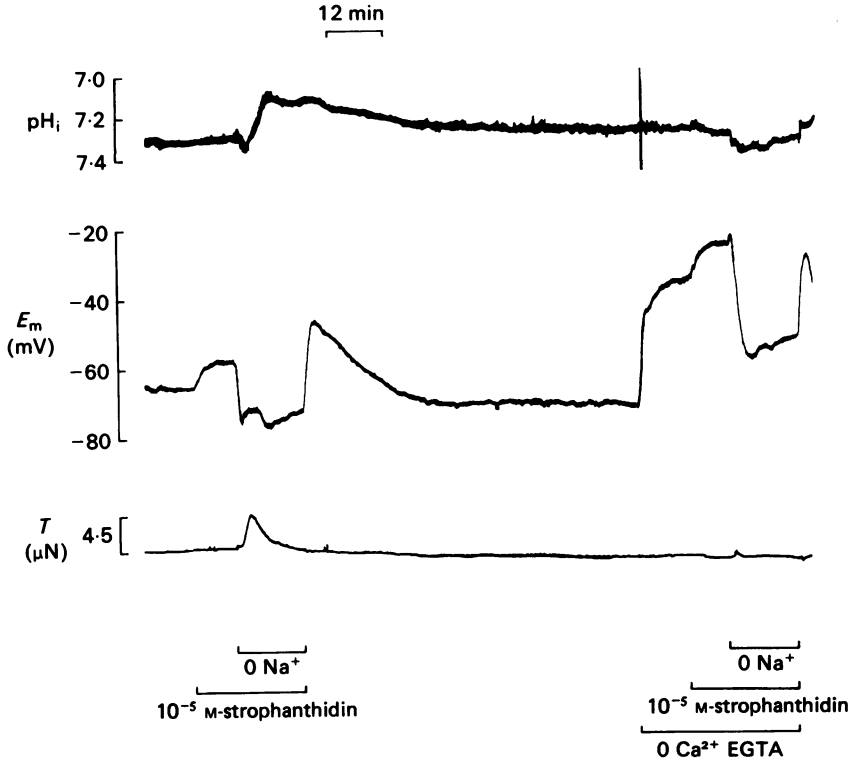

Fig. 16. The effect of very low ($< 10^{-8}$ M) Ca^{2+} on the intracellular acidification brought about by removal of extracellular Na^+ during exposure of the cells to 10^{-5} M-strophanthidin. Top trace: pH_i; middle trace: membrane potential (E_m); bottom trace: tension (T). When Na^+ was removed it was replaced by TMA^+.

proceeded but at a rate slowed by about 40 % compared with that in 120 mM-Na^+. This suggests that the inhibition of recovery in low $[Na^+]_o$ is not simply due to rises in $[Ca^{2+}]_i$.

We have further investigated the interaction of Ca^{2+} and H^+ that is apparent on removal of Na^+ during exposure of the fibre to 10^{-5} M-strophanthidin to inhibit the Na^+–K^+ pump and so increase a^i_{Na}. Under these conditions a very large acidification is produced. Deitmer & Ellis (1980) suggested that this acidification might be brought about by two mechanisms. First, the Na^+–H^+ exchange mechanism may function in reverse so that when $[Na^+]_o$ is reduced Na^+ leaves the cell in exchange for H^+, and/or, secondly, an increase in $[Ca^{2+}]_i$ may result from the reverse operation of the Na^+–Ca^{2+} exchange, i.e. reducing $[Na^+]_o$ may promote a Ca^{2+} influx coupled with a Na^+ efflux. The raised level of Ca^{2+} might then produce an increase of a^i_{Na}. It was suggested (Ellis *et al.* 1981) that the latter possibility was more likely and evidence in favour of this idea has recently been produced by Vaughan-Jones *et al.* (1983). We have used two protocols to differentiate between the two possibilities. If the acidification is brought about by the first mechanism (i.e. reversal of Na^+–H^+ exchange) we might expect that amiloride would decrease its magnitude. If the acidification is mediated via the second mechanism a very low $[Ca^{2+}]$ Tyrode solution buffered with EGTA should inhibit it. Fig. 15 shows the Na^+-free acidification in strophanthidin and its associated contracture. When this was carried out in the presence of 1 mM-amiloride, the magnitude of the acidification was largely unaffected but the contracture was substantially reduced. The inhibition of the contracture is more difficult to explain (see Discussion). It is possible that amiloride may slow the rate of rise of a^i_{Na} during strophanthidin blockade. This would result in a smaller contracture on removal of Na^+ because contracture strength is proportional to a^i_{Na} (Eisner, Lederer & Vaughan-Jones, 1981, 1983). We have measured the rate of rise of a^i_{Na} during pump inhibition with 10^{-5} M-strophanthidin in the presence and absence of amiloride. 1 mM-amiloride may slow the rate of rise of a^i_{Na} but the differences are very small. Fig. 16 shows that the intracellular acidification observed in these circumstances was indeed inhibited by lowering $[Ca^{2+}]_o$ to $< 10^{-8}$ M. This suggests that the second mechanism described above is responsible, i.e. that the intracellular acidification is mediated via Na^+–Ca^{2+} exchange.

DISCUSSION

The method used to study the pH_i regulatory system was to acidify the cells and then measure the subsequent recovery from this acidification under different conditions. Since, under normal conditions, H^+ ions are far from being in equilibrium across the cell membrane, this recovery must represent the equivalent of an active extrusion of H^+ from the cell and/or their uptake or utilization within the cell. Indeed for H^+ to be in equilibrium across the cell membrane would require pH_i to be about 6·2. If a plasma membrane Na^+–H^+ exchange mechanism uses the electrochemical gradient for Na^+ entry into the cell to supply the necessary energy for extrusion of H^+ from the cell, then removal of Na^+ from the extracellular fluid would inhibit this exchange process and the cell would lose the ability to regulate its pH_i. The present results suggest that this may be a mechanism for the pH_i recovery from acidosis in Purkinje fibres from sheep hearts.

The recovery from an intracellular acidification induced by NH_4Cl was always much slower than the recoveries from acidification produced by either CO_2 or an acid pH_o. This may indicate an additional effect of NH_4^+. On removal of the NH_4Cl most of the NH_4^+ that had entered the cell would dissociate to form NH_3 and H^+, and NH_3 would rapidly exit from the cell, leaving behind the acid load. However, if some of the NH_4^+ were slow to leave the cell (perhaps due to a slow release from an internal sequestration site) then NH_4^+ might bind to the Na^+–H^+ exchanger, competitively inhibiting the efflux of H^+ from the cell. It has recently been shown that the Na^+–H^+ exchanger in renal microvillus membrane has affinity for NH_4^+ and can mediate a Na^+–NH_4^+ exchange (Kinsella & Aronson, 1981).

In Tyrode solutions where Na^+ was totally replaced by BDA^+, TMA^+ or K^+, pH_i recovery was inhibited. In addition, in Na^+-free Tyrode solution (Na^+ replaced by TMA^+, BDA^+ or K^+) the pH_i decreased. It was shown that Li^+ appears to be able to substitute, at least partially, in the process that allows pH_i recovery (Fig. 3). Normally, if all the extracellular Na^+ was substituted by Li^+ then the recovery from an acid load was slowed but the effect of Li^+ substitution was variable and sometimes recovery proceeded in Li^+-substituted Tyrode solution after a delay at a rate comparable with that in normal Tyrode solution.

Boron *et al.* (1981), reported that the rate of recovery from acidification in barnacle muscle was reduced by 33% when Li^+ was the Na^+ substitute and recovery was completely abolished when Na^+ was substituted by choline or *N*-methylglucamine. Moody (1981) has reported that, in crayfish neurones, Na^+-free (Li^+) Ringer solution was about 80–95% *as effective* in blocking pH_i recovery as Na^+-free (BDA^+) Ringer solution. Kinsella & Aronson (1981) found that the Na^+–H^+ exchanger in renal microvillus membrane vesicles had an affinity for Li^+ but their technique was not sufficiently sensitive for them to be able to determine whether the rate of Li^+–H^+ exchange was significantly slower than that of Na^+–H^+ exchange. However, Li^+ appeared to be unable to substitute for Na^+ in mouse skeletal muscle (Aickin & Thomas, 1977). There is a progressive depolarization of the cells in Na^+-free solution substituted by Li^+, in contrast to the small hyperpolarizing effect of Na^+-free solutions in which Na^+ is replaced by BDA^+ or TMA^+. This is presumably due to the permeability of Li^+ being greater than that of the quaternary ammonium ions. It has been shown that Li^+ can accumulate inside cardiac cells under low $[Na^+]_o$ conditions (Carmeliet, 1964).

The results indicate the need for an appropriate choice of Na^+ substitute in such experiments. We conclude that TMA^+ or BDA^+ are good substitutes because they do not appear to support pH_i recovery from acidosis, they are fully dissociated in solution and do not cause substantial depolarization. Tris causes alkaline pH_i changes (perhaps due to its entry into cells or perhaps because of its buffering capacity (de Hemptinne & Vanheel, 1984)), and K^+ induces small acid pH_i changes (see also de Hemptinne, 1981, who interpreted the acidification as being mediated by a release of Ca^{2+} from internal stores).

Na^+ dependence of pH_i regulation

The results suggest that $[Na^+]_o$ needs to be reduced to about 8 mM for the rate of pH_i recovery from an acidification to be inhibited by 50%. This would imply that

the Na^+–H^+ exchange in this tissue has a relatively high affinity for extracellular Na^+, of which it requires comparatively low concentrations for activation, and that it is essentially saturated with respect to extracellular Na^+ under physiological conditions. This is in contrast to the Na^+–H^+ exchange examined in skeletal muscle (Aickin & Thomas, 1977) where only small reductions in $[Na^+]_o$ (11 %) were required to inhibit the recovery from acid loading by 57 %. Boron *et al.* (1981) found a K_m of 59 mM for barnacle muscle, i.e. reductions of $[Na^+]_o$ of 87 % were required to inhibit the acid extrusion rate by 50 %. Boron & Boulpaep (1983) have recently found a K_m of 5–10 mM-Na^+ in renal proximal tubule of the salamander. Kinsella & Aronson (1980) report a K_m for the Na^+–H^+ exchange in renal microvillus vesicles of 5 mM-Na^+. In similar experiments where K^+ was used as the Na^+ substitute, the range of $[Na^+]_o$ required to activate pH_i recovery was very similar to that when TMA^+ was used as the Na^+ substitute. This suggests that the large depolarizations in high $[K^+]$ have only a small effect on pH_i recovery.

Interactions between pH_i and $[Ca^{2+}]_i$

Although these results provide further evidence that pH_i regulation in sheep heart Purkinje fibres is dependent upon $[Na^+]_o$, care must be taken in interpreting these effects as necessarily implicating a Na^+–H^+ exchange mechanism. One alternative possibility is that on reduction of the $[Na^+]_o$ (to try to inhibit the postulated Na^+–H^+ exchange system) Ca^{2+} levels would rise and this could produce a decrease of pH_i. When $[Na^+]_o$ is decreased the rise in $[Ca^{2+}]_i$ (Niedergerke, 1963) probably occurs via Na^+–Ca^{2+} exchange (Reuter & Seitz, 1968; Glitsch *et al.* 1970). Increases in intracellular Ca^{2+} activity, while decreasing $[Na^+]_o$, have been observed using Ca^{2+}-sensitive micro-electrodes in ventricle (e.g. Lee, Uhm & Dresdner, 1980; Marban, Rink, Tsien & Tsien, 1980) and in Purkinje fibres from sheep hearts (Bers & Ellis, 1982).

Evidence suggests that much of the Ca^{2+} which enters the cells when $[Na^+]_o$ is reduced is sequestered by intracellular organelles, i.e. the sarcoplasmic reticulum and also the mitochondria (see Chapman, Coray & McGuigan, 1983*b* for further discussion). The uptake of Ca^{2+} by the mitochondria may also be associated with the release of H^+ (e.g. Williams & Fry, 1979). Vaughan-Jones *et al.* (1983) have shown that changes in $[Ca^{2+}]_i$ can alter pH_i, at least when the Na^+–K^+ pump is inhibited. Bers & Ellis (1982) found that changes in pH_i could alter intracellular Ca^{2+} activity (a^i_{Ca}). Both these phenomena were suggested to be due to Ca^{2+} and H^+ ions sharing common intracellular buffering sites. Meech & Thomas (1977) have suggested such an interaction in snail neurones where mitochondria are thought to provide the main site for this interaction. As total cell $[Ca^{2+}]$ rises under low $[Na^+]_o$ conditions the observations that (1) pH_i decreases in Na^+-free Tyrode solution and (2) pH_i recovery from an acidosis is inhibited in low $[Na^+]_o$ could be explained by the rise in a^i_{Ca} being buffered with a consequent intracellular release of H^+. It is difficult to differentiate between the two possibilities (viz. a low $[Na^+]_o$-induced inhibition of Na^+–H^+ exchange or an increase of a^i_H as a result of a low $[Na^+]_o$-induced rise in total cell $[Ca^{2+}]$ (i.e. bound and free Ca^{2+}). We have tried to overcome this problem by using superfusing Tyrode solutions of very low $[Ca^{2+}]$ ($< 10^{-8}$ M). In this way large rises in $[Ca^{2+}]_i$ can be prevented when $[Na^+]_o$ is reduced. The results confirm the suggestion that extracellular Na^+ is important for pH_i recovery in cardiac muscle (Fig. 14). As

$[Na^+]_o$ is reduced the ability of the cell to recover from CO_2-induced acid loading declines even when large rises of $[Ca^{2+}]_i$ have been prevented. However, it seems that the acidification brought about by Na^+ removal alone (e.g. Figs. 4 and 12) may be a secondary effect of an initial rise in total cell $[Ca^{2+}]$ because the low $[Na^+]_o$ acidification can be prevented by using very low $[Ca^{2+}]_o$ solutions (Fig. 13).

From the observation that the acidification produced by removal of extracellular Na^+ is prevented if the $[Ca^{2+}]_o$ is very low, it could be suggested that Na^+–H^+ exchange plays no part in the regulation of the 'resting level' of pH_i. This need not necessarily be the case, however, if the intracellular systems buffering Ca^{2+} and H^+ are 'unloaded' in the very low $[Ca^{2+}]_o$ medium. Thus, the tendency for a^i_H to rise due to the inhibition of Na^+–H^+ exchange could be balanced by an increased intracellular buffering capacity for Ca^{2+} and H^+. The Na^+–H^+ exchange may only be activated if the cell is acidified. There is evidence that pH_i recovery from an *alkalosis* can be initiated by Cl^-–HCO_3^- exchange but that this might have little influence on the normal steady-state level of pH_i, i.e. Cl^-–HCO_3^- exchange appears to be activated by an alkalosis (Vaughan-Jones, 1982*b*).

As in previous work (Deitmer & Ellis, 1980; Vaughan-Jones, 1982*a*, *b*; Vanheel & de Hemptinne, 1982) we have been unable to find evidence of a Cl^-–HCO_3^- contribution to pH_i recovery from an acidosis. There was no effect of the stilbene derivative SITS on pH_i recovery from an intracellular acidification brought about by any of the three methods used in these experiments.

Further evidence of Ca^{2+} involvement in changing pH_i comes from experiments using strophanthidin. The intracellular acidification seen on removal of Na^+ during exposure to strophanthidin can be prevented if the $[Ca^{2+}]_o$ is very low (i.e. $< 10^{-8}$ M) (Fig. 16). This suggests that this acidification is mediated via a Na^+–Ca^{2+} exchange mechanism increasing total cell $[Ca^{2+}]$ which further mediates an increase in a^i_H. If this is the case then the actions of amiloride (Fig. 15) are particularly interesting. Amiloride appeared to have only small effects on the acidifications produced by reduction of $[Na^+]_o$ in the presence of strophanthidin. This would be consistent with most of these pH_i changes being due to perturbations of Na^+–Ca^{2+} exchange rather than effects on Na^+–H^+ exchange. Amiloride, however, substantially reduced the contracture produced on reduction of $[Na^+]_o$. This unusual result might be explained if, as suggested in brief reports by Siegl, Kaczorowski, Trumble & Cragoe (1983) and Cragoe, Kaczorowski, Reeves & Slaughter (1984), amiloride and its analogues can inhibit Na^+–Ca^{2+} exchange. Such an effect could explain, at least partially, the inhibition produced by amiloride on recovery from induced acidosis. If this were the case, we would predict an increase in total cell $[Ca^{2+}]$. This increase could acidify the cell or appear to inhibit the recovery from an induced acidification. It may also explain the small depolarization seen in the presence of amiloride (Fig. 4). This depolarization could, however, be due to a non-specific effect of amiloride on membrane permeability (the effect on K^+ permeability being apparent in this instance).

To conclude, it is difficult to investigate the putative Na^+–H^+ exchange mechanism in cardiac muscle due to the close interactions of Ca^{2+} and H^+ levels within the cells and the presence of a powerful Na^+–Ca^{2+} exchanger. Some evidence is presented against the hypothesis that a Na^+–H^+ exchange mechanism controls the resting level

of pH_i. A Na^+–H^+ exchange may only be important in cardiac muscle following conditions producing acidosis. Many of the pH_i changes observed following alteration of $[Na^+]_o$ can be explained by secondary effects as a result of changes of $[Ca^{2+}]_i$. The only substantial evidence in favour of a sarcolemmal Na^+–H^+ mechanism is provided in the experiments utilizing low $[Ca^{2+}]_o$ solutions where effects on the Na^+–Ca^{2+} exchange, when reducing $[Na^+]_o$, are minimized.

Our experiments do not necessarily implicate or exclude intracellular mechanisms for uptake of H^+, for example an uptake of H^+ by the mitochondria or the sarcoplasmic reticulum. Until they are quantified precisely it is impossible to ascertain the importance of such mechanisms in the regulation of pH_i in cardiac muscle.

We wish to thank Drs R. D. Vaughan-Jones and D. A. Eisner for helpful discussions during the course of this work and valuable criticism of an early version of this manuscript. We would also like to thank the M.R.C. and the British Heart Foundation for financial support. K.T.M. received an M.R.C. Scholarship during part of this work.

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