THE CONTROL OF INTRACELLULAR pH IN CARDIAC MUSCLE

BY

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I, Kenneth T. MacLeod, hereby declare that this thesis, entitled "The Control of Intracellular pH in Cardiac Muscle", submitted for the degree of Doctor of Philosophy at the University of Edinburgh, has been composed by myself and is a result of work done entirely by myself.

Kenneth MacLeod
October, 1983

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Some of this work has already been published in the Journal of Physiology. (Ellis, D. & MacLeod, K. T. (1983), J. Physiol. 336, 69-70P and Ellis, D. & MacLeod, K. T. (1983), J. Physiol. 342, 54-55P). These papers are inserted as an Appendix.

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LIST OF ABBREVIATIONS

All abbreviations used in the text have been specifically defined when initially brought into use. However, a list of abbreviations was thought useful.

a^i_{Ca} - intracellular Ca^{2+} activity
a^i_{Cl} - intracellular Cl^- activity
a^i_H - intracellular H^+ activity
a^i_{Na} - intracellular Na^+ activity
BDA - Bis (2-hydroxy-ethyl) dimethylammonium
$[Ca]_i$ - intracellular Ca^{2+} concentration
$[Ca]_o$ - extracellular Ca^{2+} concentration
$[Na]_i$ - intracellular Na^+ concentration
$[Na]_o$ - extracellular Na^+ concentration
DIDS - 4,4'diisothiocyanostilbene-2,2'-disulphonic acid
DMO - 5,5-dimethyl-2,4-oxazo-lidinedione
EGTA - ethyleneglycol-bis-(β-aminoethylether N,N'-tetraacetic acid)
HEPES - N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
pH_i - intracellular pH
pH_o - extracellular pH
pH_i^∞ - steady-state intracellular pH
ΔpH_i^∞ - change in steady-state intracellular pH
SITS - 4-acetamido-4'-iso-thiocyanatostilbene-2,2'-disulphonic acid disodium salt
TMA - tetramethyl ammonium
Tris - 2-amino 2-(hydroxymethyl) propane-1,3 diol (tris)

The small letters '$_i$' and '$_o$' refer to intracellular and and extracellular respectively and if not preceded by activity notation (a) or concentration notation ([ ]) they denote an ionic species to which no reference to activity or concentration is intended.

CONTENTS

SUMMARY

SUMMARY

(1) The intracellular pH (pH_i) of sheep heart Purkinje fibres and ferret and guinea-pig ventricular muscle was recorded using recessed-tip pH-sensitive glass microelectrodes.

(2) The cells were acidified by one of three methods - (i) exposure to and subsequent removal of NH_4Cl, (ii) exposure to 5% CO_2 containing solutions or (iii) exposure to an acidic Tyrode solutions. The pH_i recovery from these acid loads was studied. The time constant of recovery from an NH_4Cl induced acid load was almost twice as long as from a CO_2 or acidic Tyrode-induced load.

(3) Following a CO_2-induced acid load the time constant of pH_i recovery was not changed when the cell was depolarized to -40mV (by adding more K and removing Na).

(4) Decreasing the temperature caused an increase of pH_i and considerably slowed recovery from an acid load. Results over the range of 22 - 35°C yield an average Q_{10} of 2.65 for the pH_i recovery process.

(5) When extracellular Na was removed and replaced by quaternary ammonium ions or K an intracellular acidification was produced. Such Na-free solutions also inhibited pH_i recovery from acid loading. Li could permit recovery from acid loading but not as well as Na. Tris changed pH_i in the alkali direction when used as a Na-substitute.

(6) Removal of Na_o could also slow the recovery from an alkalinization. This recovery was also temperature sensitive.

(7) Amiloride (1.0 - 1.5mM) produced an intracellular acidification but to a lesser extent than Na-free solutions. Amiloride (at this concentration) could slow but not completely inhibit recovery from an acid load.

(8) No effect of SITS (a Cl^-/HCO_3^- exchange inhibitor) was found on pH_i recovery from an acid load.

(9) The steady-state pH_i became more acid upon depolarization of the cell with K.

(10) The rate of pH_i recovery from a CO_2-induced acid load was found to decrease as $[Na]_o$ was lowered. A 50% inhibition of the rate of recovery was produced by lowering $[Na]_o$ to about

7mM. The rate of recovery (r) (expressed as a percentage of that in normal Tyrode) was related to $[Na]_o$ by the equation:-

$$r = \frac{1}{\frac{a}{[Na]_o} + b}$$

where a and b had values of 0.073mM/ and 0.0091 respectively.

(11) Using Na-sensitive glass microelectrodes intracellular Na activity (a^i_{Na}) was measured as the $[Na]_o$ was lowered to levels used in the pH_i recovery experiments. From this data the apparent Na electrochemical gradient at different values of $[Na]_o$ was calculated. Thermodynamic considerations were applied and it was shown that the efflux of H^+ ions from the cell could be maintained by small quantities (<3.5mM) of Na .

(12) Very low $[Ca]_o$ solutions ($<10^{-8}$M, buffered with EGTA) were used to prevent large rises of Ca_i which may occur on removal of Na_o. These experiments indicated that the pH_i recovery is dependent upon $[Na]_o$ and that the apparent inhibition of pH_i recovery, by removal of Na_o, is not simply due to rises in Ca_i.

(13) The intracellular acidification, which occurs on removal of Na_o does not occur when $[Ca]_o$ is very low.

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

(15) The Na dependence of the pH_i regulatory system in heart muscle and the close interrelationship of intracellular levels of Ca, H and Na are discussed.

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INTRODUCTION

Section 1 - WHAT IS pH?

Arrhenius developed the classical ionisation theory in 1887 and from this time it became clear that the degree of acidity of a solution was related to the number of hydrogen ions in it.

A working definition of the degree of acidity of a solution was first produced by Sorensen in 1909. He defined "wassertoffionenexponent" or pH to be the negative logarithm of the hydrogen ion concentration i.e.

$$pH = -\log [H]$$

The Arrhenius Theory applies fairly satisfactorily to the dissociation of weak electrolytes but cannot explain or model the dissociation of strong electrolytes particularly in more concentrated solutions. In solutions of strong electrolyte the force of attraction between oppositely charged ions can be so great that a proportion of the ions are drawn together and remain, in effect, undissociated. This will be discussed in more detail in the methods section (see page 54) but the effect forms the basis for the Lewis Activity Theory of 1921. This theory relates the apparent concentration (activity) to the actual concentration by an activity coefficient so that:-

$$\text{activity coefficient } (f) = \frac{\text{activity } (a)}{\text{concentration } (c)}$$

If the concentration is expressed as a molarity i.e. mol/l then:-

$$f = \frac{a}{c}$$

and so

$$a = f \times c$$

Thus Sorensens original formula has been re-defined to be pH = negative logarithm of the hydrogen ion activity.

$$\text{i.e.} \quad pH = -\log a_H$$

The task of defining and measuring hydrogen ion activity presents complex problems (see Bates, 1954) the greatest of which is obtaining a value for the activity coefficient of Cl^-. Due to the need to maintain electroneutrality it is impossible to change the amount of H^+ in a solution without also changing the amount of some anion or exchanging the H^+ with some cation. Measuring the chemical potential of the H^+ ion with a hydrogen sensitive electrode is only possible by using a reference electrode e.g. Ag/AgCl so that one measures the sum of the chemical potentials of the hydrogen ions and the chloride ions. Thus, for example a cell of the type:-

$$Pt/H_2/\text{buffer solution (including } Cl^- \text{ ions)/AgCl/Ag}$$

gives emf values

$$E = E_{Ag,AgCl} - \frac{(2.303\ RT)}{F} \log c_H f_H c_{Cl} f_{Cl}$$

Thus to calculate the chloride activity in the system we need to know the Cl activity coefficient. This is impossible to calculate absolutely and assumptions have to be made. Such assumptions are explained by Bates (1954), Britton (1955) and by Mattock & Band (1976). Using these assumptions, the U. S. Bureau of Standards provides a working definition of the activity coefficient of Cl^- (based on the Debye-Huckel Theory) in six solutions of simple electrolytes. These six solutions are the primary pH standards and the pH of these solutions is defined as the pa_H.

$$pa_H = -\log a_H$$

Operational Definition

From the Handbook of Chemistry & Physics 63rd ed. (1982 - 1983) CRC press inc., Florida.

"In all existing national standards the definition of pH is

an operated one. The electromotive force E_X of the cell:

reference electrode/conc. KCl solution : solution X/H /Pt

is measured and likewise the electromotive force E of the cell:

reference electrode/conc. KCl solution : solution S/H /Pt

both cells being at the same temperature throughout and the reference electrodes and bridge electrodes being identical in the two cells. The pH of solution X, denoted by pH(X), is then related to the pH of the solution S, denoted by pH(S), by the definition:

$$pH(X) = pH(S) + \frac{(E_s - E_x)\,F}{RT \ln 10}$$

where R = gas constant, T = thermodynamic temperature and F = Faraday constant. Thus defined the quantity pH is a number"..... "To a good approximation, the hydrogen electrodes in both cells may be replaced by other hydrogen-ion responsive electrodes e.g. glass.

The difference between the pH of two solutions having been defined as above, the definition of pH can be completed by assigning a value of pH at each temperature to one or more chosen solutions designated as standards."

A series of standards are available for which pH(S) has been carefully determined so that

$$pH(S) = pa_H(S) = -\log a_H(S)$$

where a_H is the hydrogen ion activity (defined on a molarity basis). Thus if the composition of the test solution is the same as that of the standards then the measured pH will approximate to pa_H. However, this is often not the case for normal pH measurements and certainly not intracellular pH (pH_i). The composition of the cytoplasm of cell is completely different from the calibrating standard.

To summarize the measured pH of any solution is thus a

measurement made relative to a standard solution. If the measured solution is composed of similar ionic constituents to that of the standard the value of pH will be very near to the true pH i.e. $-\log a_H$. However, if the test solution is widely different from the standard, then the pH value found can only be a relative measure of the acidity of the solution. Thus a value of (say) $pH_i = 7.2$ is taken to mean that this value has been measured in relation to an extracellular pH of 7.4 which has been measured in relation to NBS standard buffers. It is thus not necessary to know the hydrogen ion activity coefficient.

The true value of the activity coefficient of H^+ ions may not be one. It is probably about 0.8 in human plasma (Siggard-Anderson, 1974).

Section 2 - METHODS USED TO STUDY AND MEASURE pH_i

The aim of this Section is to give the reader some appreciation and historical perspective of the various techniques that have been used to measure pH_i. I do not intend to fully review the methods and their drawbacks (except perhaps the section on pH microelectrodes). For a more detailed review see Poole-Wilson (1978) and also Roos & Boron (1981).

There are five main methods which have been used to measure pH_i on a variety of cell types. They are: (1) Homogenization of the preparation (2) Distribution of weak acids and bases (3) Colorimetric and Fluorimetric methods (4) Nuclear Magnetic Resonance (5) Microelectrode methods.

(1) pH Measurements on homogenized samples

This method basically involves homogenizing or lysing the preparation so breaking up the cells and measuring the pH of the homogenate or lysate. Significant problems are associated with this method. Firstly, lactic acid and CO_2 production continue after the cell destruction and this can lead to a fall in pH_i. Secondly mixing of the intra and extracellular fluids may lead to erroneous results because their respective pH's and buffering systems differ and

thirdly, a disruption of intracellular organelles makes it impossible to calculate the pH of the cytoplasmic fluid.

The first problem can be overcome by freezing the preparation to halt metabolism and this was first successfully done in 1927 by Furusawa & Kerridge. These authors obtained a pH_i value for cardiac muscle measured at just above 0°C of 7.07 (which, using their temperature correction factor, is equivalent to 6.92 at 37°C). This is a commendable result but still some 0.2 - 0.3 units less than that measured by other techniques.

Good agreement between lysate pH and pH_i measured by the DMO method (see next Section) has been obtained on red blood cells (Bromberg et al, 1965). It seems that careful use of this method can yield acceptable pH_i measurements in this preparation. However, for other types of preparation the method is very unreliable essentially for reasons two and three mentioned above.

(2) Utilising the distribution of weak acids and bases

This method relies on the assumption that the cell membrane is highly permeable to the uncharged member of an acid-base pair. Thus at equilibrium the concentration of the uncharged species will be the same on both sides of the membrane. For a weak acid (HA) with a dissociation constant K (which is also assumed to be the same on both sides of the membrane) then pH_o can be expressed by the Henderson-Hasselbalch equation:-

$$pH_o = pK_o + \log \frac{[A^-]_o}{[HA]_o}$$

similarly,

$$pH_i = pK_i + \log \frac{[A^-]_i}{[HA]_i}$$

pH_i can thus be calculated from the expression below which combines the above two equations:-

$$pH_i = pK_i + \log \left[\frac{[A^-]_i + [HA]_i}{[A^-]_o + [HA]_o} \quad (10^{pH_o - pK_o} + 1) - 1 \right]$$

In practice it is the total acid concentration in the entire tissue which is measured and so a correction is necessary for acid contained in the extracellular space. One of the disadvantages in using the distribution technique is that the pH_i indicator (i.e. the weak acid or base) may be metabolised by the cell. This is a significant problem with the weak base, nicotine (Adler 1970, 1972). Waddell & Butler (1959) introduced DMO (5,5-dimethyl-2,4-oxazo-lidinedione) for measuring pH_i. The advantages of this compound are that it does not appear to be significantly bound or metabolised by cells. DMO distribution has since been used to measure pH_i in a variety of tissues e.g. rat superior cervical ganglia (Brown & Garthwaite, 1979). pH_i = 7.37; barnacle muscle (Boron & Roos, 1976), pH_i = 7.29; frog skeletal muscle (Izutsu, 1972), pH_i = 7.16; mammalian skeletal muscle (Adler et al, 1965; Roos, 1975) pH_i = 6.89 and 7.10 respectively; rabbit erythrocytes (Calvey, 1970) pH = 7.27 and in heart muscle by, amongst others, Schloerb & Grantham (1965) pH_i = 6.86; Clancy & Brown (1966) pH_i = 7.01; Poole-Wilson & Cameron (1975a,b) pH_i = 6.80. The advantage of the weak acid/base technique is that it is simple to carry out and can be used on very small cells or even cell organelles.

Some of the disadvantages of the technique are: (1) that it involves assuming the dissociation constant is the same intracellularly as extracellularly and that $[HA]_o = [HA]_i$ i.e. we assume that the charged form is impermeable (2) that it requires knowing the volume of the extracellular space - calculation of such volumes are often difficult (3) it yields an average pH_i of the cell which includes the pH of the compartments inside organelles (4) the indicators can take quite a long time (15 min.) to equilibrate so this limits the use of the technique to steady-state conditions or to very slowly occurring pH_i transients and (5) only one measurement can be made and in addition to (4) this makes it impossible to follow pH_i transients accurately.

(3) Colourimetric and fluorimetric methods

According to Roos & Boron (1981) "the application of foreign

dyes dated back to 1893 when Metchinkoff observed that particulate litmus injected into protozoa changes in colour from blue to red." The basis of the method involves injecting or in some way incorporating a pH-sensitive dye into the cells and measuring any subsequent colour changes which take place and converting these absorbance changes into pH from a calibration curve. Compounds which are fluorescent at particular wavelengths can also be used to measure pH. The relative intensity of the fluorescence at two different wavelengths is dependent upon pH. pH-sensitive absorbance changes have been measured in frog skeletal muscle (MacDonald & Jobsis, 1976) during twitch contractions. A colourimetric or fluorimetric measuring system presents several advantages. The response of the dyes to pH changes is very rapid and this allows one to study rapidly changing pH transients. Secondly, it can be used with very small cells although limitations can be imposed by the procedure of incorporating the dye into the cell. Recently, however, this obstacle has been surmounted by Gerson & Burton (1977) and Ohkuma & Poole (1978) who have exposed slime mould cells and macrophages to fluorescent probes which phagocytose the probe material. Thirdly, it can be used to study the pH of the cell compartments. The main problem in interpreting the optical signal as a function of pH_i seems to be that of calibrating for absolute pH_i. The calibration of the probes is done in control conditions and these have to be interpolated to in vivo conditions. The calibration curve in control conditions may not be applicable to intracellular conditions.

(4) Nuclear magnetic resonance (NMR) technique

The basis of this technique is as follows:- certain atomic nuclei (e.g. ^{31}P) possess magnetic properties and when these nuclei are placed in a strong magnetic field they interact with the field. The interaction can be studied more closely by observing the characteristic frequencies at which the nuclei absorb and emit radiation of radiowave frequency. At a certain frequency, unique to each nuclei, they absorb energy, resonate, and then "jump" to a higher energy state. From this state they emit signals which can be transformed into a spectrum of peaks for each chemically distinct ^{31}P nucleus. The NMR technique for measuring pH_i relies on inorganic phosphate (present inside cells) generating a ^{31}P-NMR signal whose frequencies are sensitive to

pH variations in the normal physiological range. Inorganic phosphate exists, at pH 7.00, primarily as HPO_4^{2-} and $H_2PO_4^-$ which , in solution produce an NMR spectrum determined by the relative amounts of each species. The proportion of these species varies with pH since their respective pK values are 12.7 and 7.2. It is thus possible to determine pH_i by measuring the chemical shift of the inorganic phosphate signal and determining, from a standard curve, the pH to which this chemical shift corresponds.

Moon & Richards (1973) were the first to apply the technique of ^{31}P NMR to measure the intracellular pH of erythrocytes. Since then it has been used to measure pH_i of other tissues including heart muscle (Jacobus et al, 1977; Garlick et al, 1979). Accurately calibrated, the results from this technique are well in agreement with those measured by other methods.

(5) pH sensitive microelectrodes

Caldwell (1954) was the first to succeed in using miniaturised microelectrodes to measure intracellular pH. The pH-sensitive part of his microelectrode was about 5mm long and the rest was insulated with shellac. The disadvantage in using such a long pH-sensitive piece of glass was that in order to measure pH_i correctly the whole of the exposed glass had to be inside the cell. It was only possible to measure pH_i of large cells, (Caldwell's preparation was crab muscle fibres) and even then the electrodes had to be inserted longitudinally. Caldwell measured pH_i values of about 6.9.

Spyropoulous (1960) used a miniaturised form of pH electrode to measure the cytoplasmic pH of squid axon. He obtained an average value of 7.35.

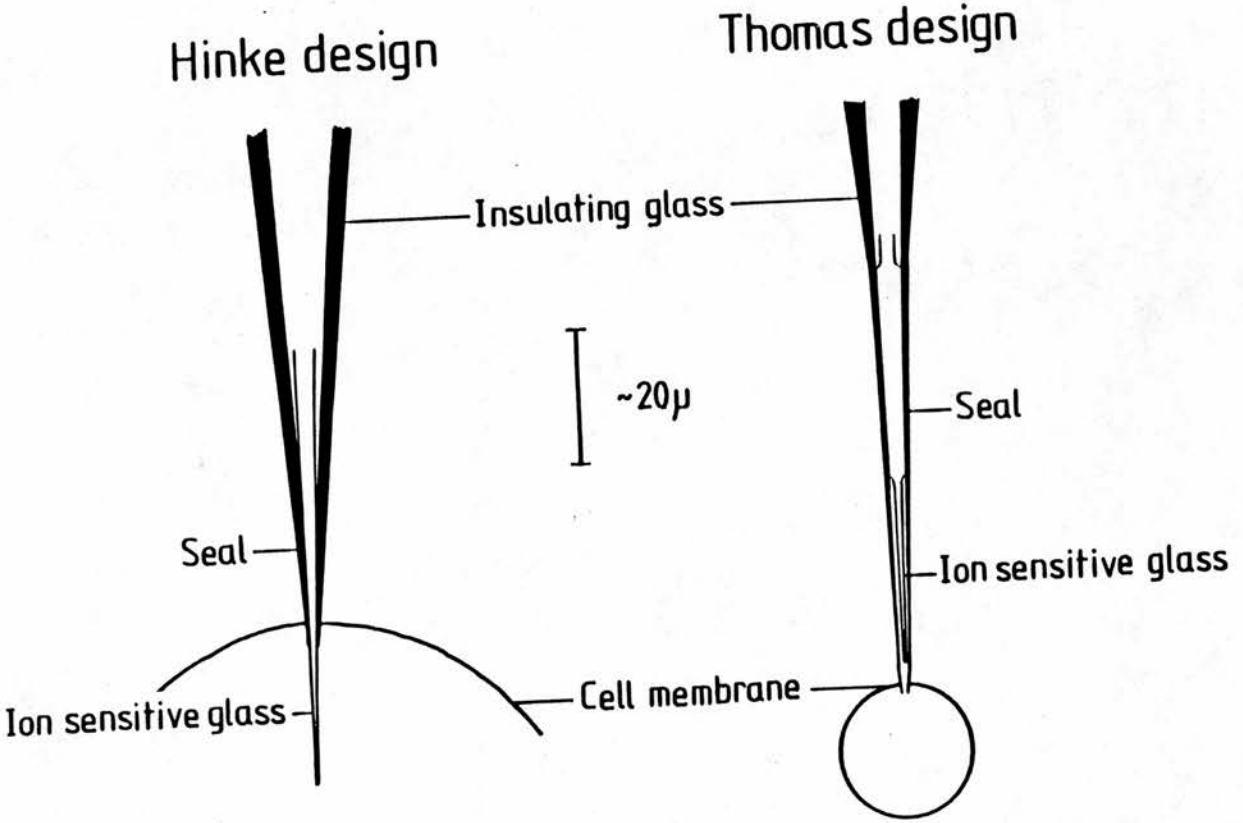
The next major advance in the manufacture of ion-sensitive microelectrodes was made by Hinke in 1959. The development of Na^+ and K^+ sensitive glass by Eisenman et al, (1957) (for review see Eisenman (1967)) allowed Hinke to design Na and K sensitive microelectrodes. These had ion-sensitive tips protruding through an insulating lead-glass micropipette (see Figure 1). Hinke (1967) produced a

FIGURE 1

A comparison of the tip configurations of the Hinke and Thomas-style microelectrodes.

The Hinke-style of microelectrode (left) has the ion-sensitive glass protruding through the end of the insulating glass. All the ion-sensitive portion requires to be inside the cell for correct measurement.

The Thomas-style of microelectrode (right) has the ion-sensitive glass recessed within the insulating glass. Only the tip of the outer insulating glass need penetrate the cell.


Hinke design
Thomas design
Insulating glass
~20μ
Seal
Seal
Ion sensitive glass
Cell membrane
Ion sensitive glass

pH-sensitive microelectrode to the same design and succeeded in measuring pH_i in the giant fibres of barnacle muscle (Hinke & Menard, 1976).

The advantages of Hinke's style of microelectrode are that it responds to pH changes very rapidly (~1 sec.) and is fairly robust. It has been used for many pH_i studies on giant barnacle muscle fibres (Boron, 1977; Boron et al, 1979, 1981; Boron & Roos, 1976; Boron et al, 1978) and in squid giant axon (Russell & Boron, 1976; Boron & de Weer, 1976). The main disadvantages of the Hinke type of microelectrode is that it cannot be used to measure pH_i in small cells because the protruding ion-sensitive glass tips ($\leqslant 100\mu m$) have to be completely inside the cell.

The problem of the protruding tip of ion-sensitive glass was solved initially by Thomas (1970, 1974 & 1978) and separately by Walker (1971). Walker incorporated newly synthesized liquid ion-exchange materials inside the micropipette tip. These liquid ion-exchange resins are usually neutral ligands dissolved in a hydrophobic organic solvent. The resin could be sucked, or pushed from the back, into the tip of the micropipette. The difficulty, solved by Walker, was that the hydrophobic glass surface usually allowed water to displace the hydrophobic ligand into the tip. Walker treated the glass, prior to incorporation of the ligand into the tip, with a silicon compound. This compound prevented the formation of water at the tip. Liquid ion-exchange microelectrodes can have very small tips and have very fast response times (of the order of milliseconds). They are comparatively easy to make but they suffer from the significant problem of interference (or binding) from other ions. If any other ion binds to a significant degree then it is likely that it will produce an additional voltage which, if not corrected, would lead to inaccurate measurements of activity. This is discussed by Lanter et al, (1982) and Meier, (1982). Harman & Poole-Wilson (1981) have succeeded in incorporating a pH-sensitive resin into microelectrodes. Recently Ammann et al (1981) have produced a better pH ligand and this has been used by Corey & McGuigan (1983). The sensitivity and selectivity of this resin in intracellular use has not yet been well described.

Thomas (1970) originally designed his recessed-tip microelectrode (see Figure 1) around Na-sensitive glass. In this electrode the ion-sensitive portion of the glass was recessed inside the tip of an insulating "outer" glass micropipette. Later the Na-sensitive microelectrode design was modified to allow recessed-tip pH sensitive microelectrods to be made (1974). A fuller description of the manufacture of these microelectrodes can be found in Thomas' monograph (1978). The design has the advantage that since only the open tip of the outer insulating glass need penetrate the cell, very small cells can be impaled as long as the tip is fine enough.

The recessed-tip design has been further modified to a double-barrelled pH-sensitive microelectrode, one barrel is the KCl reference electrode and the other the recessed-tip pH-sensitive electrode. (de Hemptinne & Marranes, 1980)

A disadvantage of the recessed-tip microelectrode is that its response time is slow compared with the other types of pH microelectrodes. It depends on the geometry of the recess and the tip diameter.

Other types of pH-sensitive electrodes have been produced (Lavallee, 1964; Bicher & Ohki, 1972; Pucacco & Carter, 1976; Yamaguchi & Stephens, 1977) but these, for varying reasons, have not been popular.

There are several advantages in using pH-sensitive microelectrodes. Firstly, they give a direct measurement of activity of H^+ ions and as discussed on pages 6, 7 and 54 it is the ion activity which is, in many cases, more important than concentration. Secondly, continuous measurements of pH often over many hours are feasible. Thirdly, the microelectrodes sample the cytoplasmic H^+ ion activity and not an "overall" pH_i of the cell inclusive of compartments or organelles. Fourthly, the microelectrodes give an almost instant measure of pH_i. Other methods rely on time consuming calculations and chemical analysis.

There are also several disadvantages of employing pH-sensitive

microelectrodes. The recessed-tip microelectrodes are difficult to make. Secondly, the microelectrodes have a very high electrical resistance and are very prone to electrical interference. Thirdly, the nature of the technique requires that two microelectrodes be inserted into the cell. This is discussed at greater length in the Methods Section but essentially is because the ion-sensitive microelectrode measures, in addition to the resting membrane potential, the changes in potential due to any changes in ion activity. Thus a conventional microelectrode has to be inserted into the cell to independently measure the membrane potential which is then subtracted from the combined signal measured by the ion-sensitive microelectrode. This double impalement can only be carried out with robust preparations.

In preparations in which the cells are electrically coupled both microelectrodes need not necessarily be in the same cell (although they should be in cells which lie close to each other i.e. less than one space constant). This point is discussed in greater detail in the Methods Section.

Section - 3 THE IMPORTANCE OF pH TO CARDIAC CELL FUNCTION

Gaskell, W. H. (1880)

"When, by means of an acid solution, the beats of the ventricle have been very much lowered in force, then the alkaline solution brings back the force of the beat to its original height, and then produces its own characteristic effect, and upon sending through the acid solution the action of the alkali is gradually overpowered and the ventricle slowly falls from a position of full contraction to one of extreme dilation."

In his conclusions.....

"One can then say that the presence of an alkaline fluid must tend to keep the cardiac and arterial muscles in a state of tonicity, and that upon the extent of the alkalinity the amount of tonicity will, in part, at all events depend."

Ringer, S. (1883)

"The solution composed of saline solution, with calcium chloride and potassium chloride is neutral, hence the ventricle will beat perfectly when supplied with a neutral fluid, and therefore alkalinity of circulating fluid is not necessary for contractility.

The alkaline reaction of the blood is no doubt necessary indirectly, for contractility, for muscular contractions develop acidity and this must give an acid reaction to a neutral fluid, and the heart cannot contract when supplied with an acid circulating fluid."

The physiological principles underlying these observations made by Gaskell and Ringer in the 1800's are still under critical experimentation to-day.

Changes in pH have many and varied effects on tissues and in this next section I am primarily concerned with the effects pH changes may have on cardiac muscle. I intend, in this section, to review some of the experiments which indicate the effects alteration in pH may have on the function of cardiac muscle. In this way, it may become apparent to the reader that it is of great importance to the cardiac cell that it is able to control the pH of its internal milieu about which this study is concerned.

Many experiments utilise changes in extracellular pH (pH_o). These can bring about intracellular pH (pH_i) changes and thus it is difficult to interpret whether the effects produced are mediated purely extracellularly or purely intracellularly. Similarly, experiments which change pH_i may change the pH at the immediate extracellular surface of the cell.

The dependence of fixed membrane charges upon pH

Frankenhaeuser & Hodgkin (1957) described the influence of Ca^{2+} ions on the conductance mechanisms of the squid giant axon. They suggested that Ca might act by binding to negative sites

on the membrane and so exert a stabilising influence. Since these observations, other workers have suggested that part of the electrical field across the membrane which controls the gating of ionic channels, depends on the presence of a negative surface charge. The magnitude of this surface charge would therefore be dependent on the number of ions which are adjacent to or are in contact with the surface. Ca may not be the only ion which affects the membrane surface charge.

Once fixed negative charges at the surface of the axon have been introduced into the Hodgkin-Huxley model (Hodgkin & Huxley, 1952), it becomes essential also to consider the effects of pH upon the surface density of charge. Bass & Moore (1973) suggest that a decrease in pH below a value of about 7.3 would neutralize an appreciable fraction of the postulated surface charges, and thus increase the transmembrane field strength.

In 1965 Tasaki et al briefly reported that if the pH of the perfusion medium in a squid axon was decreased below 6.4, conduction was blocked, whereas an increase in pH above 7.5 caused spontaneous repetitive firings. These data were the first indication that the electrical properties of perfused axons are extremely sensitive to the pH_i.

Hille (1968) observed g_{Na} (Na conductance) at the node of Ranvier of a frog nerve as he decreased pH_o from 7.3 to 4.0. The g_{Na} decreased with pH in accordance with the binding of protons to a single site with pK = 5.2. Dronin & The (1969) observed the current-voltage (I-V) characteristics of both Na and K as functions of pH_o in frog nerve. The peak value of the Na current was decreased by decreasing pH and the I-V curves were shifted in the depolarizing direction. Thus shifting of the external pH in these experiments appeared to indicate some effects due to surface charges.

A more recent study of pH_i action on the ionic channels of the squid axon (Carbone et al, 1981) suggests that the steady-state Na conductance might be controlled by the degree of protonation of two titratable groups, one with a pK_o of about 5.6. According to this scheme, high pH_i would increase the average number of channels in a

conducting state by blocking or affecting the fast-inactivation process. Conversely, although less straightforwardly, low pH_i may increase the average number of channels in the inactivated state.

In addition to the Na channel Moody & Hagiwara (1982) find that decreased pH_i can block anomalous rectification in starfish oocytes.

Changes in pH_i or pH_o may alter the fluidity of the lipid bilayer. (Trauble & Eibl, 1974; Verkleij et al, 1974) H^+ ions may change the order $\leftrightarrow$ disorder transition temperature of lipid bilayers such that when the phospholipid head groups are mostly uncharged (protonated) the fluidity of the membrane is lower because of the decreased intramolecular separation caused by the lower electrostatic repulsion. This would produce effects similar to lowering the temperature (slowing down of rate constants).

The action of pH on cardiac muscle electrophysiology is not as well described as in other tissues (but see Marranes et al, 1981; Van Bogaert & Carmeliet, 1972).

Brown & Noble (1978) found that acid solutions shift the Na threshold (i.e. the amount of depolarization from holding potential required to activate the large inward Na current) substantially in a positive direction while alkali solutions produce small but probably not significant shifts in a negative direction. They found that the effects of changes of solution pH in the range 5.5 to 9.0 were fully reversible but a change to pH 4.0 was accompanied by a rapid deterioration of the preparation. They also found that acid solutions reduce iK_2 whereas alkali solutions have no effect. (Note - iK_2 represents the pacemaker current in Purkinje fibres; the diastolic depolarizations were thought, by Noble & Tsien (1968), to be deactivations of a K current. Recent work by Di Francesco (1981) and by Callewaert et al, (1982) suggests that instead of a K current activated on depolarization, the pacemaker can better be described as a Na current activated on hyperpolarization. The reasons underlying this interpretation are summarised by Carmeliet (1982)).

Brown & Noble suggested that many of the changes connected with the Na channel could be due to H^+ titrating external surface charges near the channel. The pH effects on the iK_2 activation parameter (S_∞) was not in the direction consistent with an external surface charge titration and they considered there were two possibilities. The first was that H^+ ions influenced internal as well as external surface charges. This idea was also suggested as a reason for the effects on Purkinje iK_2 of raised pH_i (by NH_4Cl exposure) found by van Bogaert et al, (1978). The second idea was that the postulated internal charge titration was not necessarily by H^+ ions. They proposed that H^+ may depress the Na/K pump which in turn altered the activity of the Na/Ca exchange so as to increase $[Ca]_i$. The increase of $[Ca]_i$ might result in titration of internal surfaces charges near the iK_2 channels thereby producing a hyperpolarising shift in the S_∞ curve. This second alternative has been supported by a further finding (Brown, Cohen & Noble, 1978) that the effects of acidosis on iK_2 are prevented by increasing $[K]_o$ from the normal range (in their experiments 2.7 -5.4mM) to 10.8mM.

Slow inward current (Isi)

There is great debate about the nature and origin of the delayed inward currents observed in cardiac muscle under voltage clamp much of which has centred around the techniques used and the quality of spatial and temporal control of membrane potential achieved by them. I do not intend to review the mass of literature describing this current but only wish to point out that Isi can be changed by pH and this may have pertinent effects on cardiac muscle contraction. For a description of the properties of Isi see e.g. Kass & Tsien, 1975; Noble, 1979; McDonald, 1982. Correlation of the Isi with contraction showed that both have a common threshold and both are increased and decreased in amplitude on increasing and decreasing $[Ca]_o$ respectively (Becker & Reuter, 1970; Ochi & Trautwein, 1971). Good evidence that Isi is an essential step in the initiation of tension comes from experiments like that of Thynum (1974). Using partially depolarized muscles (the fast inward current thus inactivated) the upstrokes of the slow rate of rise action potentials are carried largely by Ca ions through the slow inward pathway. There is a good correlation between the change

in maximum rate of rise of these action potentials and the strength of the accompanying contraction. Increased $[Ca]_o$, or isoproterenol increased the contraction, while verapamil or decreased $[Ca]_o$ decreased contraction. Normally the Isi carries only enough Ca to activate up to 10% of the contractile proteins (see Fozzard & Beeler, 1975; New & Trautwein, 1972; Chapman, 1979) and because evidence suggests that it may be involved in the initiation of contraction then some means must exist of augmenting Ca release into the sarcoplasm which is coupled to the Isi in some way. For explanations of possible mechanisms for E-C coupling see the reviews of Chapman, (1979) and Fabiato & Fabiato , (1979).

Since Isi is obviously important in initiating contraction its alteration by pH provides yet another method by which acidosis or alkalosis can alter tension.

Chesnais et al (1975) observed a shortening of action potentials at pH_o 5-6 which could primarily be attributed to the suppression of the slow inward current.

Kohlhardt et al (1976) show that decreased extracellular pH decreases Isi. Considering the pH sensitivity of other ionic currents their results are not surprising. Kohlhardt et al point out that both Isi and isometric contractile force decreased at a pH_o of 5.5. However, decreased Ca influx seems not to be <u>exclusively</u> responsible for the development of a negative inotropic effect because generally the decrease of contraction at low pH_o was always more pronounced than the decrease in Ca current.

<u>Changing sensitivity of contractile proteins</u>

In experiments involving mechanically skinning (removing) the sarcolemma Fabiato & Fabiato (1978) observed that decreased pH from 7.40 to 6.20 produced an increase, by a factor of about 5, in the [free Ca] required for the myofilaments to develop 50% of the maximum tension. That is to say, acidosis shifted the tension against pCa curve to the right so that a higher [Ca] was required to obtain the

same tension when the pH was lower. In addition they found that acidosis decreased the maximum tension developed in the presence of a saturating [free Ca].

They found, however, that there was a non-proportional shift in the tension/pCa curve on varying pH. On decreasing pH, both the [free Ca] threshold for contraction and the submaximal tension developed at [free Ca] lower than pCa 6.00 were more affected than when the pH was increased by the same fraction of a pH unit. This result argues against a simple competition between H^+ and Ca^{2+} for a single class of binding site on troponin as proposed by Fuchs, Reddy & Briggs (1970). However, Fuchs (1974) later questioned his previous data and in a more precise study found little systematic effect of pH on Ca binding at any [free Ca].

It was Katz & Hecht (1969) who first suggested that the profound decrease in contractility in myocardial ischaemia commonly related to intracellular acidosis could be accounted for by a direct competition between Ca and H for receptor sites on troponin.

Godt (1981), however, proposes a useful hypothesis to explain the shift of the relative force-P_{Ca} curve with pH. It is known that the myofilaments bear a net negative surface charge at near neutral pH and that the resultant electrostatic field surrounding the myofilaments tends to attract cations and to repel anions. Changes in solution pH will alter the surface charge and hence the field, and will thus alter the concentration of ions immediately around each myofilament. Since the Ca binding sites on the thin filament are exposed to the Ca concentration near the filament surface, changes in the electrostatic field from titration of the ionised surface charges by protons can indirectly change the Ca binding at the sites and can thus influence force production. In this model, H^+ does not compete directly with Ca^{2+} at a binding site, rather alters the electrostatic field surrounding the thin filament and thus indirectly affects the effective Ca concentration at or near the Ca binding sites.

Ashley (1978) also finds that the tension vs pCa curve of barnacle muscle is shifted to the right by decreasing pH_i, i.e. the

relationship becomes less sensitive to [Ca] as [H] is increased. However, there is no change in the steepness of the curves. Eisner et al (1983) find increased twitch tension with increased pH_i in sheep heart Purkinje fibres.

pH_i effects on the SR

The second main finding of in the elegant experiments of Fabiato & Fabiato (1978) was that decreased pH depressed the Ca loading of the SR, the rate of Ca accumulation (expressed by the frequency of the cyclic contractions) and also the Ca-induced release of Ca. Nakamaru & Schwartz (1972) also find an increased affinity and decreased release of Ca by the SR in acid conditions (pH 6.46). Thus, depression by acidosis of the functions of the SR could be at least part of the mechanism which leads to the decrease in contractility of the myocardium during an acidotic event.

Fabiato & Fabiato (1978) find that the action of acidosis on the SR always accentuates its depressive effects on the myofilaments in cardiac muscle. Firstly, the amount of Ca released from the SR of cardiac muscle is reduced by even moderate acidosis. Secondly, the myofilaments require a greater [free Ca] for the same amount of contraction in acidosis. Consequently, the Ca released from the SR will produce a lower level of activation, for which the effects of acidosis are much more pronounced than they would be for maximal activation.

Contrastingly, a small acidosis <u>increases</u> the Ca^{2+} content of the SR of <u>skeletal</u> muscle and the large amount of Ca^{2+} released will compensate for any decrease in the sensitivity of the myofilaments to the Ca^{2+}. This may be one reason why skeletal muscle does not show such a dramatic decrease in tension production in acidotic conditions. Lea & Ashley (1981) propose that an intracellular acidification may acidify the lumen of the SR and modify either the Ca^{2+}-ATPase or the Ca^{2+}-induced release process.

Tate et al (1981) find that alkaline pH inhibits the rate of ATP dependent Ca uptake. The optimal pH is in fact somewhat more

acid (pH 6.6) and Mandel et al (1982) find that pH 6.0 markedly decreased the rates of formation of phosphorylated intermediate by Ca Mg ATPase from cardiac SR while between 7.6 and 6.8 the rates were unchanged.

Inhibition of Na/Ca exchange mechanism

There is growing evidence the Na/Ca exchange across the sarcolemmal membrane is a mechanism responsible for the control of myocardial contractility by extracellular Ca. (see Chapman, 1979; Mullins, 1981; Langer, 1982 for reviews). The Na/ Ca exchange may contribute indirectly to pH_i changes in heart muscle and this is discussed in greater detail in the Discussion. At present, it is not possible to accurately quantitate and compare the amount of Ca which contributes to excitation/contraction coupling through the slow channel, to that which contributes through the Na/Ca exchanger and that derived directly via regenerative release from the S.R. Thus the relative importance of the Na/Ca exchanger in the control of beat-to-beat contraction is unknown.

It is possible that the positive inotropic action of the cardiac glycosides is due to the action of the Na/Ca exchange mechanism although disagreement exists as to the myocardial response to low doses of these drugs (see Noble, 1980 for review). At higher doses it is proposed that since the drug inhibits the Na/K pump then Na_i will increase. This increase in Na_i will augment Ca influx due to the carrier taking Ca into the cell in exchange for intracellular Na (Neidergerke, 1963; Reuter & Seitz, 1968; Baker et at, 1969; Glitsch et al, 1970).

At present there have been few investigations of the effects of pH on this Na/Ca exchange mechanism. Considering the possible important role of the exchange mechanism in cardiac E-C coupling, the regulation of this system by pH could be of major significance to tension production. In the squid axon the Na/Ca exchange (Ca dependent Na efflux) can be inhibited at pH_i of 6.8 and at pH_i of 6.5 Na/Ca exchange is almost 100% inhibited (Baker & McNaughton, 1977). Na dependent Ca efflux is inhibited at pH_i below 7.3 (50% at 6.3) (Di Polo

& Beaugé, 1982).

Coraboeuf et al (1981) find that pH_o decreased to 6.0 also inhibits Na/Ca exchange in dog Purkinje fibres (as judged by Na-free contractures). They suggest the effects are mediated through pH_i changing but also find a possible effect of external pH on this mechanism.

Recently, Philipson et al (1982) have studied the Na/Ca exchange in isolated canine cardiac sarcolemmal vesicles. They found that the exchange is extremely sensitive to pH and that reductions in pH_i (but not apparently pH_o) will considerably decrease Ca transport mediated by the Na/Ca exchange system.

Decreased Ca binding to the sarcolemma

The model for the Na/Ca exchange system in heart muscle as proposed by Langer (see 1983 for review) places the control of Ca movement at sites which are influenced directly by changes in the interstitial space. Large amounts of Ca are bound to negatively charged sites located on the glycoproteins which constitute the external lamina. This Ca is in rapid equilibrium with that in the interstitial space and is the immediate supply of Ca used by the Na/Ca exchange carrier. Thus increasing or decreasing $[Na]_o$ decreases or increases Ca binding respectively at the cell surface and this competition affects the amount of Ca that, in some way, feeds the carrier system. Bound Ca may also enter the cell via the slow channel. Work using cation uncouplers (La^{3+}, Mn^{2+}) (e.g. Bers & Langer, 1979; Chapman & Ellis, 1977) and pharmacological uncouplers (D-600, verapamil) (e.g. Kohlhardt et al, 1972) also provides evidence to support the conclusion that a major store of coupling Ca is bound to the sarcolemma. Indeed tension depends on extracellular Ca concentration in very much the same way as sarcolemmal Ca binding (Bers & Langer, 1979). Thus if protons decrease Ca binding to the sarcolemma they may decrease tension production. Some evidence that this is a possibility was produced by Williamson et al (1975). The inhibition of contractile force induced by verapamil is reversed by an increase of extracellular Ca and this is very similar to the inhibition of contraction induced by decreasing

pH which is also reversed by increasing $[Ca]_o$.

Cell/Cell communication

The hypothesis that activity in cardiac muscle spreads by local circuit flow has been extensively investigated. The injection of a subthreshold current pulse into the cytosol of cardiac cells changes the membrane potential of neighbouring cells (Weidman, 1952, 1970). In cardiac Purkinje fibres it was shown that the distribution of the electronic potential follows closely the predicted results for a uniform cable (Weidman, 1952). The core resistivity of these fibres was low (105ohms /m) and the space constant (1.9mm) high in comparison with the length of a single cell (~125μm) (Weidman, 1952) which indicates that the electrical resistance of the intercellular junctions is very low.

Loewenstein & Rose (1978) show that there may be a role for calcium in the regulation of permeability of the cell-cell membrane channel (their experiments were carried out on Chironomus salivary gland cells). Although the precise mechanism by which Ca controls junctional resistance is unknown, they suggested that the calcium ion causes a change in the channel's fixed charge configuration or molecular conformation that decreases its effective size.

The finding that intracellular [free Ca] modulates cell-to-cell communication does not preclude the possibility that other intracellular factors influence the junctional permeability. Meech & Thomas (1977) showed a fall in pH_i of snail neurones when Ca was injected into the cytoplasm. The suggestion has been made that the effect of high [free Ca] on cell coupling is due to a fall in pH_i. Turin & Warner (1977) demonstrated that when embryonic cells of Xenopus were exposed to 100% CO_2 the pH_i decreased from 7.7 to 6.4. This pH_i change was accompanied by a decrease in E_m and the electrical coupling was abolished.

Recently Reber & Weingart (1982) have found that changes in pH_i produced by application and removal of NH_4Cl, brought about accompanying biphasic and transient shifts in internal longitudinal

resistance (r_i) of similar time course. Increased pH_i decreased r_i by up to 16.4% while decreased pH_i increased r_i by up to 30.4%. It is known that the velocity of impulse conduction depends, amongst other things, on r_i and is closely related to fibre diameter. Therefore, an increment in r_i can impair impulse conduction.

However, intracellular acidosis can bring about a release of Ca_i. Lea & Ashley (1978), for instance, found an elevation of [free Ca] in barnacle muscle exposed to 100% CO_2 although Baker & Honerjager, (1978) studied Ca_i when varying pH_i and found that decreases in pH_i decreased Ca_i. It is therefore not easy to decide which effects are due to Ca and which are due to H. This inter-relationship between H and Ca is discussed in the next section. Suffice to say that it is as yet not known if the pH_i regulates the junctional resistance under physiological conditions but the electrical synchronisation of heart cells is dependent upon the maintenance of a high junctional conductance and it is therefore easy to visualise cardiac arrhythmias generated by hypoxia, ischaemia or digitalis toxicity that can in part be explained by cell decoupling.

Changes in mitochondrial function

It should now be apparent that H and Ca have a close inter-relationship as cations affecting intracellular processes. They both screen negative surface charge in a similar way, their relative concentrations are important for developing tension, Ca uptake and release from the SR is dependent upon sarcoplasmic [H^+] and cell:cell communication depends on Ca and H. It is now important to discuss another method by which the cell can regulate its intracellular [Ca], namely by mitochondria and how this regulation can be changed by pH_i.

The influx of Ca into mitochondria is probably mediated by a carrier that is inhibited by low concentrations of La^{3+} and ruthenium red and which transfers Ca^{2+} passively (i.e. through a uniport system), in response to a negative potential generated inside mitochondria by active extrusion of H^+ (Åkerman & Nicholls, 1983). The efflux of Ca is necessary to prevent build up of an unreasonably high Ca gradient across the inner mitochondria membrane and also to provide

additional control, other than by altering the potential across the mitochondria which would in turn upset oxidative phosphorylation and metabolic transport. The observation that Na can specifically promote the extrusion of Ca from heart mitochondria (Crompton et al, 1976; Carafoli & Crompton, 1978) led to the proposal of a specific carrier that exchanges cytoplasmic Na for mitochondrial Ca. This seems to be the main way of Ca efflux from mitochondria. This carrier system is inhibited by La^{3+} and can also perform Ca/Ca exchange.

At least in liver mitochondria there is some evidence of a Ca/H antiport. Low pH induces an efflux of Ca from mitochondria (Åkerman, 1978). There are many reports, summarised by Borle (1981), which are consistent with the idea that H^+ is a competitive inhibitor of Ca uptake by mitochondria. A high pH increases the affinity of the transport system for Ca and increases Ca transport while a low pH does the reverse. This seems reasonable since Ca uptake by the mitochondria is dependent upon the difference in pH across the mitochondrial membrane. There are reports of Ca/H exchange in heart muscle mitochondria (e.g. Williams & Fry, 1979). Fry & Poole-Wilson (1981) found an efflux of Ca from rabbit cardiac mitochondria on abruptly changing pH from 7.2 to 6.8. Fry et al (1981, 1983) find that optimal Ca accumulation by the mitochondria occurs at alkaline sarcoplasmic pH. At acid pH's the Ca uptake decreased.

Other evidence for Ca/H interaction at the mitochondria comes from experiments of Meech & Thomas (1977). They found that injection of small volumes of $CaCl_2$ into snail neurones caused immediate decreases in pH_i which appeared to be directly proportional to the amount of Ca injected. They also found that injection of $CaCl_2$ and La^{3+} produced only a small decrease in pH_i compared with the decrease apparent with $CaCl_2$ injected alone. This is good evidence of the Ca uptake by mitochondria in exchange for protons. As has already been discussed, La can inhibit Ca uptake by mitochondria with consequently less proton 'dumping'.

Gerstenblith et al (1983) find that lowering Ca_o decreased contractility and increased pH_i in rat heart. Conversely they found that increased Ca_i decreased pH_i. Ahmed & Connor (1980) also find

that entry of Ca into the cell can acidify the cytoplasm. Zucker (1981) finds that increased pH_i decreases Ca buffering in Aplysia neurones.

It is apparent that a number of cardiac cell processes are changed by pH and many by pH_i. It is pertinent that a brief review be made of how other cell types regulate their pH_i so that a comparison of these mechanisms, with those possessed by heart muscle cells - the subject of this study - will be more straightforward.

Section 4 - INTRACELLULAR pH REGULATION IN OTHER CELL TYPES

Many recent reviews exist on intracellular pH (pH_i),(Waddell & Bates, 1969; Poole-Wilson, 1978; Boron, 1980; Roos & Boron, 1981 and Boron, 1983). Other works which contain sections on pH_i which are of interest are Walker & Brown, (1977); Woodbury, (1974); Lee, (1981).

Measured pH_i in most healthy cells is always found to be far more alkaline, often by almost an order of magnitude, than that predicted by an equilibrium distribution of H^+ ions. The exceptions are oocytes. In order to maintain pH_i at this level the cells must effectively extrude H^+ against a sizeable (at least 45mV) driving force. That pH_i is not in equilibrium need not imply that there is an extrusion of H^+ ions. The inward transport of OH^- would have the same effect and, as yet it is not certain whether there is transport of OH^- into the cells. For the purposes of the discussion the extrusion of H^+ ions is usually taken to mean that the process may well involve OH^- ions. Almost all of the experiments which are referred to in this section have been carried out using pH-sensitive microelectrodes of the Hinke type or the Thomas type.

Cells which regulate pH_i by an anion exchange

Squid giant axon

In 1958, Caldwell found that pH_i in giant axons of the squid was not in equilibrium, although his measured pH_i was low compared with that of Spyropoulos (1960) and Boron & de Weer (1976a). More

direct evidence of an acid extrusion mechanism comes from experiments like those of Boron & de Weer (1976) where the axon was exposed to 5% CO_2 containing sea water at constant pH_o. This caused a sharp fall in pH_i as CO_2 entered the cells and hydrated to form H_2CO_3 which subsequently dissociated to form H^+ and HCO_3^- ions. The effects of CO_2 on pH_i are examined more thoroughly in the Results Section 1 and the Discussion Section 2. pH_i slowly recovers from the CO_2-induced acidification. The gradual return of pH_i near to original baseline levels cannot be accounted for by passive movements of H^+ or HCO_3^- as these would have to move against their electrochemical gradient. Boron & de Weer (1976b) demonstrated that HCO_3^- was important in allowing pH_i to recover from a NH_4Cl^- induced acid load (Acid loading with NH_4Cl is also discussed in depth in Results Section 1 and Discussion). In nominally HCO_3^- free sea water the pH_i recovery from a NH_4Cl acid load was very slow. When HCO_3^- was added to the sea water (+ CO_2 to ensure constant pH_o) then pH_i recovered very rapidly.

Russell & Boron (1976) further showed that not only was external HCO_3^- required but also internal Cl^-. They found that when the axon was dialyzed with Cl^--free solution pH_i recovery was halted. In addition they found that SITS (4-acetamido-4'-isothiocyanostilbene-2,-2'-disulphonic acid) an inhibitor of Cl^-/HCO_3^- exchange in red blood cells (Cabantchik & Rothstein, 1972) also inhibits pH_i recovery in axons with normal $[Cl^-]_i$ and with $HCO_3^-{}_o$ present. The data suggest that the pH_i regulating system in squid axon is Cl^-/HCO_3^- exchange which functions to exchange intracellular Cl^- for extracellular HCO_3^-. Boron & de Weer (1976b) also found pH_i regulation to be affected by the presence of cyanide and 2,4-dinitrophenol which pointed to the pH_i regulating system requiring ATP though, as yet, it is not clear what role ATP plays. Russell & Boron (1976) also demonstrated that the pH_i regulatory mechanism required ATP.

Cells which regulate pH_i by an anion exchange tightly linked with the Na electrochemical gradient.

Barnacle muscle

Barnacle muscle pH_i also is far more alkaline than is

expected if H^+ ions are passively distributed (Boron, 1977). The barnacle muscle fibre also recovers from NH_4Cl-induced or CO_2-induced acid loads and these pH_i recoveries depend upon the presence of extracellular HCO_3^- and are inhibited by SITS (Boron, 1977). Again this points to a Cl^-/HCO_3^- exchange system which regulates pH_i.

Boron et al (1979) found that at constant $[HCO_3^-]_o$ the rate of pH_i recovery became greater as pH_i was decreased. They also found that the higher the pH_o then the faster the rate of recovery at a particular pH_i. In 1981 Boron et al further described the pH_i regulation and its dependence on $[HCO_3]_o$. At a particular pH_i the rate of pH_i recovery was greater as the $[HCO_3]$ was increased. They concluded from both sets of results that pH_o and HCO_3 altered the pH_i recovery process. Boron et al (1978) found that if pH_i was low then an increase of $[HCO_3^-]_o$ at constant pH_o produced a fall in Cl^- influx which was coincident with an increase in the acid extrusion rate. Boron et al (1981) also described the Na dependency of pH_i regulation. The pH_i recovery from an acid load could be completely inhibited when Na was removed from the bathing solution and replaced by choline or n-methyl-D-glucamine even when HCO_3^- was present. The dependence of the acid extrusion rate on the $[Na]_o$ could be described by simple Michaelis-Menten kinetics. When $[HCO_3]_o$ was decreased the Vmax for the process also decreased but the K_m was not significantly altered. The K_m of the Na dependent pH_i recovery system increased about 3 times when pH_o was decreased from 8.0 to 7.4. Vmax changed inconsistently though often remained unaffected. Thus the system of pH_i regulation in barnacle muscle seems to involve a Cl^-/HCO_3^- exchange which is Na dependent though the dynamics of such a carrier remain to be defined. This mechanism appears also to require cAMP (Boron et al, 1978; Russell & Brodwick, 1981).

Snail Neurone

Thomas (1974) finds that H^+ ions are not passively distributed across the snail neurone membrane. The pH_i regulatory mechanism in snail neurones works much faster in CO_2-containing Ringer than in CO_2- free Ringer (Thomas, 1976) and shows a requirement for Cl^- (Thomas, 1977). Using Cl^- sensitive microelectrodes Thomas (1977)

also noted that the pH_i recovery from an acid load was accompanied by a decrease in a^i_{Cl}. pH_i recovery was also sensitive to the stilbene derivative DIDS - a similar compound to SITS, see Cabantchik et al, 1978 - (Thomas, 1976) and these results suggested that in this tissue pH_i recovery was mediated by a Cl/HCO_3 exchange. However, the difference between this mechanism and that functioning in squid and barnacle lies in the Na-dependency of the system.

Thomas (1977) also found that the pH_i recovery was sensitive to the presence of Na_o. When Na was removed and replaced by BDA the pH_i recovery was inhibited. He found rises in a^i_{Na} during pH_i recovery from an acid load. After the presence of SITS, which blocks the recovery irreversibly in this tissue, Na-free solution showed no additive effect on inhibiting the pH_i recovery suggesting that both treatments affect the same process (Thomas, 1977).

Thomas therefore proposed that the pH_i regulatory mechanism in snail neurones involves a membrane carrier which exchanges external Na^+ and HCO_3^- ions for internal H^+ and Cl^- ions. The Na electrochemical gradient provides the energy to drive the other ion fluxes. Thomas has recently found (1981) that injection of vanadate into snail neurones does not inhibit pH_i regulation which suggests that the regulation in this tissue does not depend on ATP.

Cells possessing two separate pH_i regulatory mechanisms

Crayfish neurone

The average pH_i in this preparation measured by Moody (1981) was 7.12 (pH_o = 7.4 and a E_m of -76mV).

The intracellular pH recovered from various acid loading methods and the rate of this recovery was sensitive to $[HCO_3^-]_o$. Using CI^--sensitive microelectrodes Moody found that a^i_{Cl} decreased as pH_i recovered from an acid load. SITS partially inhibited the pH_i recovery process. These observations pointed to the existence of a Cl^-/HCO_3^- exchange mechanism which provided the influx of HCO_3^- to buffer the rise in H^+ ions brought about by the acid load. However,

in contrast to the results of Thomas (1977) SITS did not fully inhibit the pH_i recoveries, but slowed the rate to that normally seen in HCO_3^--free Ringer. Removal of Na almost completely blocked the pH_i recovery and using Na-sensitive microelectrodes Moody found that a rise in intracellular Na accompanied recovery of pH_i from an acid load. These results pointed to two separate mechanisms for pH_i regulation in crayfish neurone. Firstly, a Na/H exchange, whereby intracellular H^+ ions are exchanged for extracellular Na, the energy for the "uphill" H^+ ion extrusion provided by the large electrochemical gradient for Na entry. Secondly, a Cl^-/HCO_3^- exchange working in the direction already described which may be Na-dependent i.e. it also relies on the Na electrochemical gradient to drive the fluxes of ions against their respective electrochemical gradients.

Mouse soleus muscle

The distribution of H^+ ions is not in equilibrium across the membrane of mouse soleus muscle and the pH_i recovers from an acid load induced by CO_2 exposure or by NH_4Cl (Aickin & Thomas, 1977a)

The pH_i recovery system was further investigated by Aickin & Thomas (1977b) and they found that decreasing the $[Na]_o$ inhibited the pH_i recovery from NH_4Cl exposure. In addition amiloride also inhibited the rate of pH_i recovery to a similar extent as removal of Na_o. These results, together with the observation(carried out with Na-sensitive microelectrodes) that Na_i levels increase when the cell recovers from an acid load, suggest that pH_i recovery is mediated by a Na/H exchange working in a similar manner to that of crayfish.

However, Aickin & Thomas found that by application of amiloride or SITS a pH_i recovery could be split into two components. Amiloride applied on its own greatly inhibited the recovery from an NH_4Cl induced acid load, but did not completely block recovery. However, when both drugs were present pH_i recovery was completely inhibited. This additive effect suggests that there are two independent mechanisms controlling pH_i in mouse soleus. These are probably an Na/H exchange and a Cl/HCO_3 exchange. The Cl/HCO_3 exchange does not appear to be Na dependent, but Aickin & Thomas used Li as their

substitute for Na and as shown in this study Li can at least partially substitute for Na in pH_i recovery.

Cells with a single Na/H exchange system for pH_i regulation

Renal tubule cells

Boron & Boulpaep (1983a) have studied the pH_i regulation of the renal proximal tubule cell of the salamander. This is a very specialised cell which takes part in the renal secretion of acid. Although containing a HCO_3^- transport mechanism (Boron & Boulpaep, 1983b) pH_i recovery can be attributed wholly to the existence of a Na/H exchange mechanism. The pH_i recovery is insensitive to the stilbene derivatives and Cl^- removal and there is no change in a^i_{Cl} during pH_i recovery. Amiloride or removal of Na_o inhibits the pH_i recovery.

Cells with, as yet, unknown mechanisms for pH_i regulation

Smooth muscle cells

In smooth muscle cells (guinea-pig vas deferens) H^+ions are also not in equilibrium. Using double barrelled pH-sensitive microelectrodes containing the H^+- selective ligand of Ammann et al (1981), Aickin (1981) has found pH_i to be about 7.1 with pH_o 7.4 at 35°C and a membrane potential of about -65mV.

These results are in agreement with Stephens et al (1977) who used DMO to measure pH_i (7.2) in tracheal smooth muscle cells. Yamaguchi & Stephens (1977) also find pH_i to be 7.0 using a pH-sensitive glass microelectrode.

Intracellular chloride activity a^i_{Cl} in smooth muscle, like that in heart muscle is higher than that dictated by equilibrium conditions (Aickin & Brading, 1982b). Aickin & Brading (1982a) have also shown that Cl^- reaccumulation in Cl^- depleted tissues is inhibited by the nominal absence of CO_2 and HCO_3^- or by DIDS.

These results may indicate that, like heart muscle (Vaughan-Jones 1979a, b), there exists a Cl/HCO_3 membrane exchange system which keeps a^i_{Cl} high. The energy source for this Cl/HCO_3 exchange is at present unknown. Further experimentation is required to investigate whether the Cl/HCO_3 system plays any part in maintaining the high pH_i.

Crab Muscle

Aickin & Thomas (1975) find that the mean pH_i of crab muscle fibres is 7.2 at a pH_o of 7.5. The mean membrane potential was -65mV.

Exposure of the fibre to CO_2 containing Ringer produced an intracellular acidification. The pH_i slowly recovered from this CO_2-induced acidification and from an acid load applied by decreasing pH_o to 6.5

However, there is no information on how the recovery might be mediated.

Frog skeletal muscle

pH_i in frog skeletal muscle is about 6.9 in CO_2/HCO_3 buffered solution and about 7.1 in HEPES/O_2 buffered solution at pH_o of 7.2 at 25°C (Bolton & Vaughan-Jones, 1977). With an average E_m of about -88mV H^+ ions are again not in equilibrium. However, Bolton & Vaughan-Jones did not observe a pH_i recovery from a CO_2-induced acid load (i.e. pH_o constant but the bathing solution changed from O_2/HEPES buffered to one which is CO_2/HCO_3 buffered). This is in contrast to the other cell types previously mentioned.

Abercrombie & Roos (1981) have found that this muscle cell can recover from a CO_2-induced acid load when depolarized to between -20 and -30mV. The recovery under these conditions seems to be mediated by a Na/H exchange as it appears to be completely inhibited by Na^+-free medium or 0.5mM amiloride. More investigation is required to understand why this pH_i regulatory mechanism does not function in normal conditions.

Oocytes

In sea urchin eggs the measured pH_i was found to be about 6.9 with a E_m of -11mV (Shen & Steinhardt, 1978). At this E_m and with a pH_o of 8.0 passive equilibrium of H^+ ion would produce a pH_i of about 7.8. This unusual result, in comparison with other tissues, indicates that there may be a net transport of protons into sea urchin eggs. Unfertilized eggs do have a high input resistance (300kΩ) (Shen & Steinhardt, 1980) and the impalement of the egg by two microelectrodes may produce a large leak resistance so an apparently low E_m. If E_m is more negative the protons would be nearer a passive distribution. Other measurements of pH_i on eggs show that the pH_i values of Shen & Steinhardt are not wildly inaccurate. Johnson & Epel (1981) used the DMO technique and measured a pH_i of about 6.9. At fertilization there is a substantial increase in pH_i (Shen & Steinhardt, 1979) which may be mediated by the activation of a Na/H exchange because it requires small amounts of Na_o and is accompanied by a Na influx although strangely it does not appear to be markedly inhibited by amiloride.

Webb & Nuccitelli (1981) find that pH_i of frog oocytes also increases on fertilization but they find no evidence for an active pH_i regulatory system despite pH_i not being in equilibrium.

METHODS

General

Tension (force) transducer

Microelectrodes

- pH microelectrodes
- Na microelectrodes
- Ca microelectrodes

Signal Recording

The subtraction prodecure and calibration of electrodes

- Testing the subtraction

Solutions

Data handling

METHODS

General

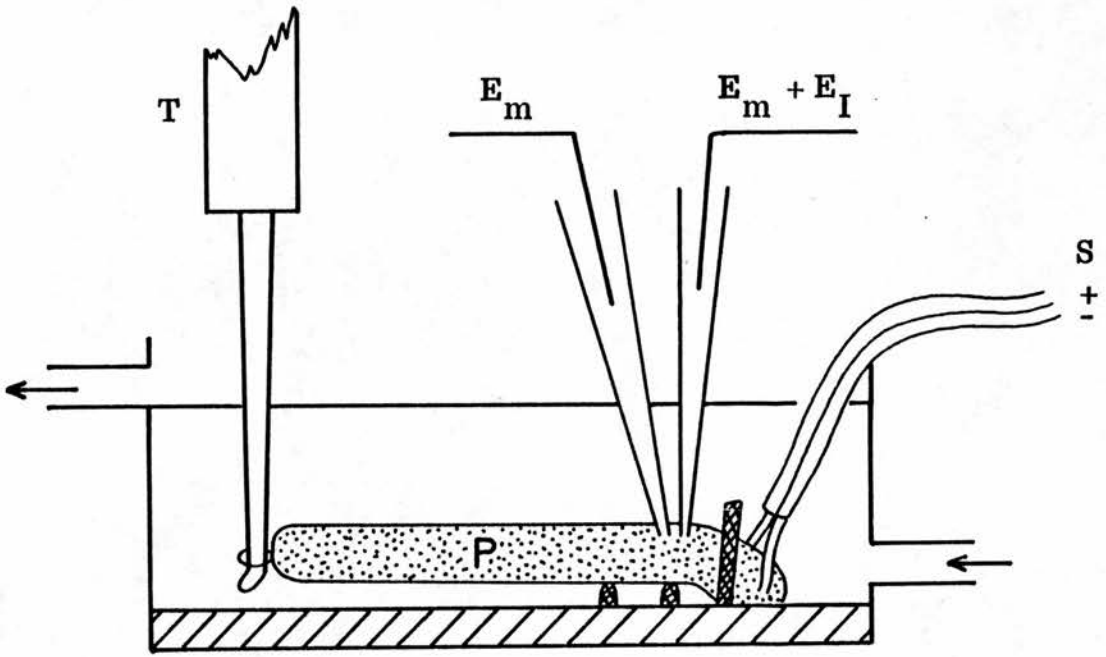
Fresh sheep hearts were obtained from the local slaughterhouse, the ventricles cut open, and transported to the laboratory immersed in a bicarbonate buffered Tyrode solution at ambient temperature and bubbled with a 95% O_2, 5% CO_2 gas mixture. Free running Purkinje fibre bundles (Jewell, 1980, for comment) were removed, usually from the left ventricle.

Ferrets and guinea-pigs were anaesthetised with an intraperitoneal injection of Pentobarbital Sodium (Sagatal, May & Baker, U.K.) and the hearts rapidly removed. Thin trabeculae or papillary muscles were isolated from either ventricle.

The experimental chamber (approximate volume = 0.2ml) was machined from a block of perspex and had a glass front giving good optical conditions for viewing the preparation. A 2mm diameter hole was drilled at the left end of the chamber to enable the bath electrode to be fitted. The bottom of the chamber was covered with a layer of silicone rubber (Sylgard, Dow Corning, Belgium). Two stainless steel wires (500μm thick) ran across the chamber and were slightly raised above the Sylgard base (see Fig. 2). Slightly stretching the preparation over the wires facilitated penetration with the microelectrodes (c.f. Ellis, 1977). One end of the preparation was pinned to the floor of the chamber with an entymological pin. A loop of fine nylon thread was tied on to the other end of the preparation allowing it to be connected to the arm of a force transducer constructed from piezo-resistive elements (see next section). Tyrode solution was run through the bath continuously. The temperature of the bath was maintained at 35± 1.0°C by means of a thermocouple, placed immediately at the entrance to the perfusion chamber. This was connected via a feedback circuit to a heating element surrounding glass tubing carrying solutions into the chamber. Solutions were changed by means of an eight way tap (similar to design of Partridge & Thomas (1975) except that the tap incorporated a drain). Oxygenated solutions were preheated to 35°C in a water bath and fed by gravity to the eightway tap by polythene tubing or stainless steel tubing when HCO_3/CO_2 buffered solutions were used. The

FIGURE 2

A schematic diagram of the experimental bath with the preparation (P) connected to the tension transducer (T). Superfusing Tyrode solutions flowed continuously over the preparation entering the chamber from the right and leaving from the left. The preparation could be stimulated by a pair of electrodes (S) and was impaled by two microelectrodes. One, (E_m) was a conventional 3M KCl filled microelectrode which measured membrane potential. The other, ($E_m + E_I$) was an ion-sensitive microelectrode which measured both the membrane potential and the potential due to the intracellular ion activity.


T
E_m
$E_m + E_I$
S
+
-
P

stainless steel tubing minimised any leak of CO_2 from the solutions. It was important to bubble the solutions with CO_2 at the experimental temperature because the solubility of the gas is dependent on temperature. The experimental chamber's exchange rate of solution was >10 volumes/min and the solution exchange time (as measured by the response of a liquid ion exchange Na sensitive microelectrode) was 90% complete in <10 sec.

All microelectrodes are prone to interference from the electrical supply, badly suppressed motors or switches and static electricity. In general, the higher the resistance of the microelectrodes the worse the interference. Thus a Faraday cage was used to shield the microelectrodes from interference. It was earthed to the metal conduit carrying the electricity supply. The preparation was lit from above and behind. The light from the source (Fort EF 150S light unit, France) being conveyed into the Faraday cage by fibre-optic light guides to minimise electrical noise.

A horizontally mounted microscope (Carl Zeiss, Germany) was used to view the preparation through a hole cut in the Faraday cage. It was earthed to the cage itself.

Stimulating electrodes were placed on either side of the preparation and used primarily to check the viability of the preparation and to elicit twitch contractions. The electrodes were connected to a Harvard Advanced Stimulator (SRI Ltd., Kent, England).

Tension (Force) Transducer

The tension transducer was constructed from piezo-resistive elements (Akers AE801, Aksjeselskapet Mickroelectronic, Horten, Norway). These elements are silicon beams (~5mm long, 2mm wide and 0.1mm thick) with planar piezo-resistive surfaces on either side of the beams. The beams are attached to a supportive 'head' to which electrical connections are made. A 21 gauge stainless steel tube ~22mm long was glued to the beam with rapid setting epoxy adhesive. A fine glass capillary with an end tapered to form a small hook was glued to the other end. This was hooked through the fine nylon loop tied to the preparation. This assembly had resonant frequency of ~75Hz. On deflecting the beam,

the value of the diffused resistances will change, one will increase, the other will decrease. This assembly was combined with another beam element providing two passive resistors to form a complete Wheatstone Bridge circuit. The whole Wheatstone Bridge assembly was inserted into a brass tube for protection. From the bottom end of the brass tube was mounted a miniature drill chuck. The drill chuck housed the active beam and its supportive head which was held in place by silicone rubber. The steel tube protruded through the chuck which was loosened by hand before an experiment and provided an overload protection for the fragile element when the transducer was not in use. The beams were energised by a stabilised 6V DC supply from a Bryans DC bridge amplifier (Model 40550). This bridge amplifier also provided the bridge balance and preamplification systems. The voltage output of the tension transducer was linear over and above the entire range of forces measured. The drift was <0.05μN/hr.

The preparation was stretched so that recorded twitch tensions were maximal.

Microelectrodes

Membrane potentials were measured with conventional 3M KCl filled glass microelectrodes[1]. These microelectrodes were pulled from lengths of 1.2mm i. d. 2.0mm o. d. borosilicate capillary tubing containing a glass fibre filament. (Clark Electromedical Instruments, Reading, England). They were filled with boiled 3M KCl and had resistances between 6 and 20 MΩ

Ion sensitive microelectrodes were of the recessed-tip design (Thomas, 1970, 1974, 1978). These consist of an ion-sensitive glass micropipette sealed at its tip and placed inside a larger diameter insulating glass micropipette. These micropipettes are then sealed together forming a recess inside the tip (see Figs. 1, 3).

1 Following the terminology of Thomas (1978), the fine-tipped tapering glass capillary tube will be called a micropipette. When filled with electrolyte solution and used for measuring potentials it becomes a microelectrode.

pH microelectrodes

The method for pH electrode assembly used was as described by Thomas (1978) and was as follows. pH sensitive glass tubing, 1.0mm outer diameter (Corning 0150 glass, CEM, Reading, England), was pulled to form long, sharp micropipettes. A batch of some 15-20 micropipettes were pulled and their outer diameters measured 62.5μm, 125μm and 500μm from the tips. The tips of these pH micropipettes were sealed under high power microscopic observation using heat from a microforge. The resulting heat-sealed micropipettes were used as the inner pH sensitive element. The outer micropipette was made from aluminosilicate glass tubing (Corning code 1720) o. d. 2.0mm i. d. $\geqslant$1.0mm. Aluminosilicate glass was found to be more durable (i.e. stayed sharper for longer) than outer pipettes made from borosilicate glass. The aluminosilicate glass tubing was cut into 3 inch lengths and the ends lightly fire polished (this made it easier to slide chlorided Ag wires into the back of the electrodes). Again a batch of some 15-20 insulating outer micropipettes were pulled and their respective inner diameters were measured 62.5, 125 and 500μm from their tips. Heat and pull settings on the microelectrode puller were adjusted so that the internal diameters of the insulating aluminosilicate pipettes were just larger than the corresponding outer diameters of the ion-sensitive pipettes. Such micropipettes had tips $<$1μm in diameter (see Fig. 4) and if filled with 3M KCl had resistances of 6-10 MΩ. The outer aluminosilicate micropipette was then set up in the microforge and the inner pH glass micropipette was fixed onto the end of fine steel tubing with sealing wax. The pH inner was carefully lowered into the insulating outer and under microscopic observation pushed down until the pH tip was about 10-20μm from the tip of the outer (see Fig. 3). Once in an appropriate position 200-500 lb/in^2 (~15-35 bar) of gas pressure was applied via the steel tubing to the inside of the pH micropipette. The pressure is necessary to prevent the pH-sensitive inner glass collapsing when heat is applied and to force it out to make contact with the outer glass so forming the glass/glass seal. The softening point of the pH glass is ~655°C while aluminosilicate glass softens at ~900°C. Initially a variety of gas pressures were used during the the sealing. Very high pressures (>800lb/in^2) give the advantage that the recess can be made smaller, so the electrode response to a change of ion concentration is faster (Moody, 1982) but these pressures, in

FIGURE 3

Procedures in constructing recessed tip pH-sensitive micro-electrodes. Firstly, the pH-sensitive glass micropipette is sealed at its tip with the heat from a microforge. The pH-glass micro-pipette is then attached to a steel tube with wax and lowered into the outer insulating glass micropipette. Gas pressure is applied to the inner pH glass via the steel tube and heat from the microforge softens the pH glass. The pressure "blows out" the pH glass and aids the formation of a glass-glass seal with the outer insulating glass.

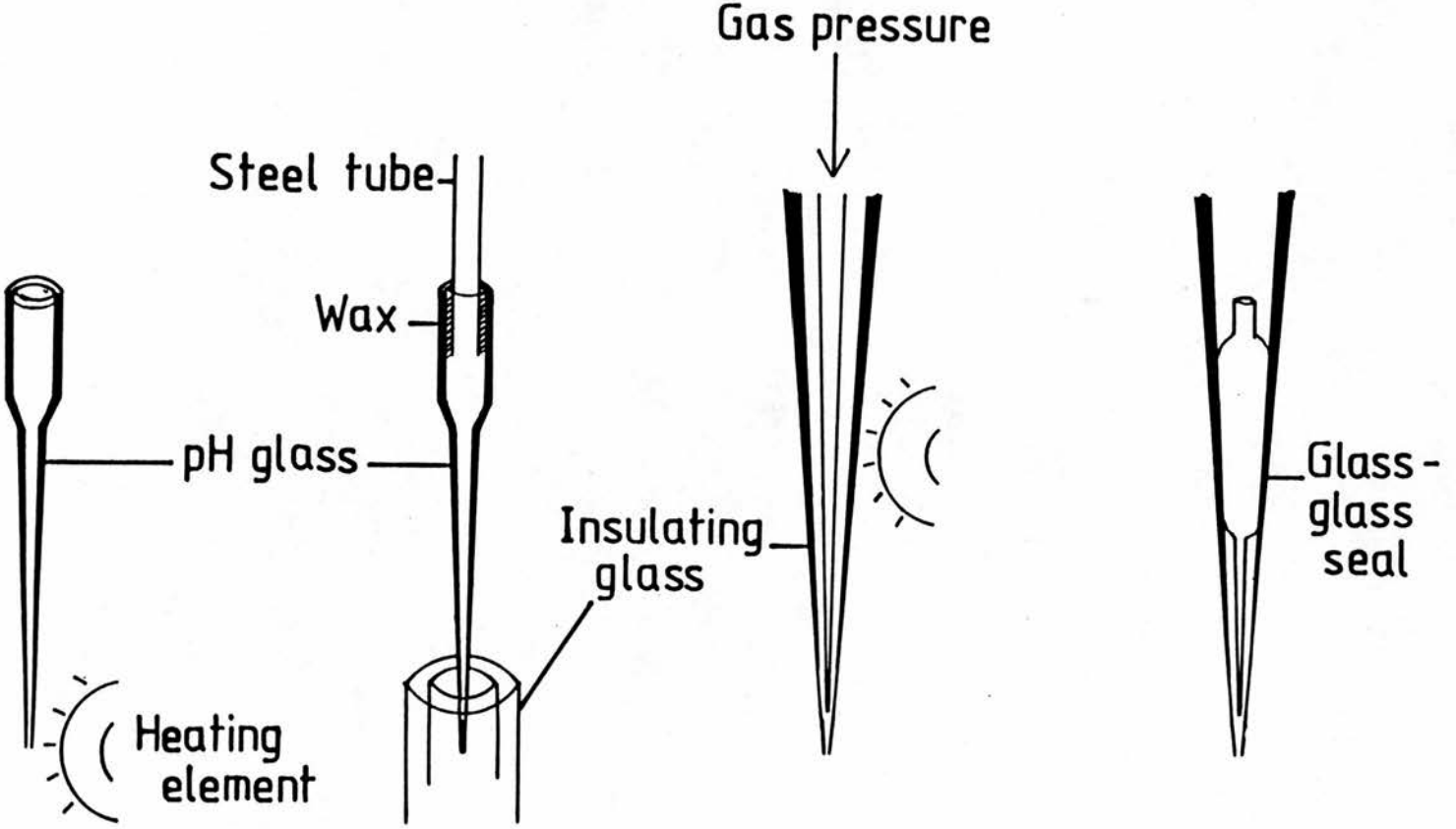
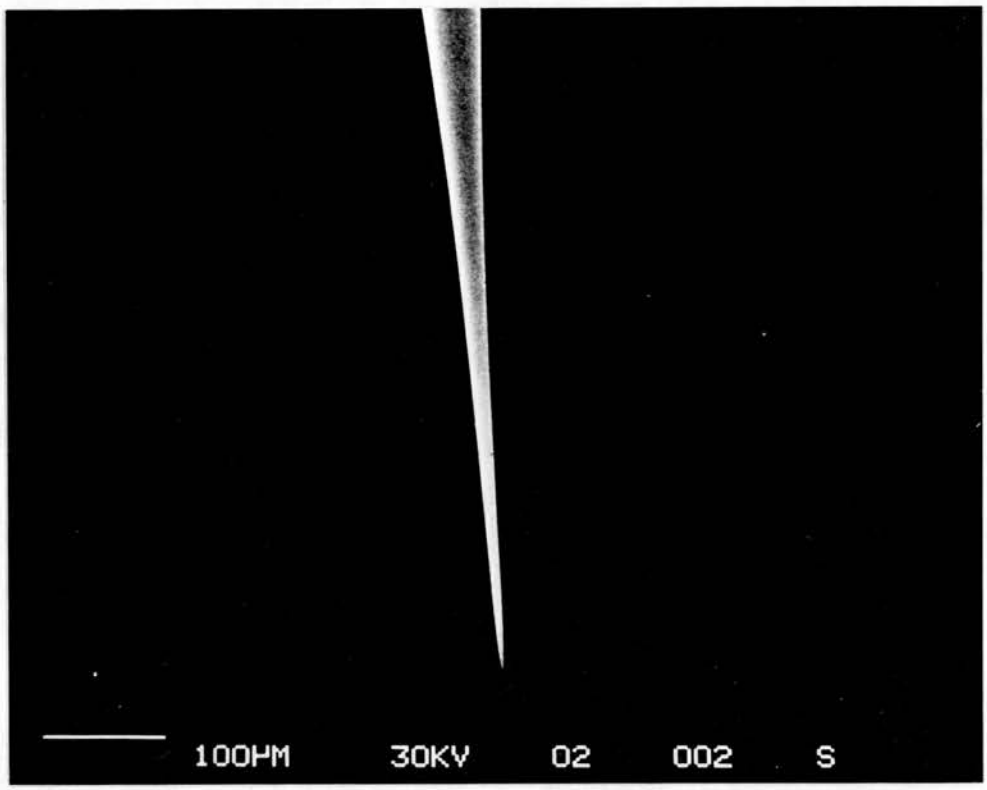
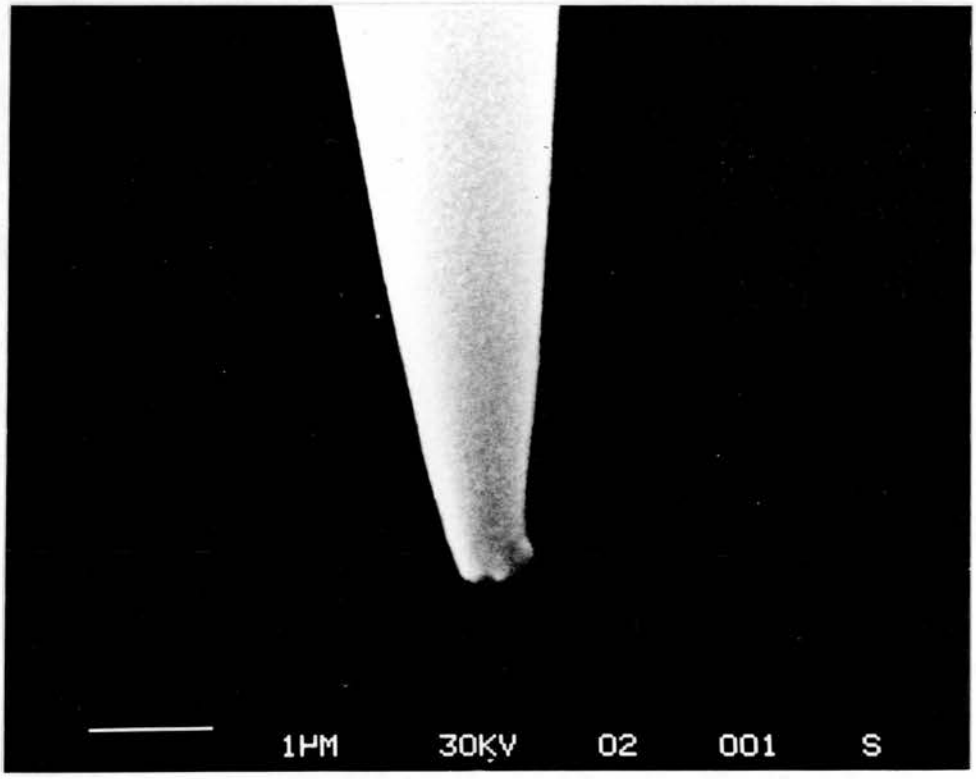

Gas pressure
Steel tube
Wax
pH glass
Insulating glass
Heating element
Glass - glass seal

FIGURE 4

Scanning electronmicrographs (taken by Mr. J. N. Brown, Dept. of Physiology, University of Edinburgh) of the tips of typical outer insulating micropipettes. These were taken to assess tip diameters which are beyond accurate resolution by a light microscope.

The bars indicate the scale and are 100μm in the top photograph and 1μm in the bottom photograph.


100UM 30KV 02 002 S


1UM 30KV 02 001 S

my experience, lead to cracking of the inner glass which results in a useless electrode. Low pressures (<150lb.in^2) are better but with too low a pressure it becomes difficult to keep the recess around the exposed length small with a consequent decrease in response time.

Finished pH sensitive microelectrodes were stored in dry air. Prior to use the pH sensitive microelectrodes require thorough hydration. This was carried out by various methods the most successful of which was mounting them on a holder, inserting the holder in a beaker of distilled water to which had been added a few drops of detergent, covering the beaker and cooking gently in an oven at 75-85°C overnight. After the water had cooled it was sucked out from each electrode and replaced with 0.1M NaCl buffered to pH 6.0 with 0.1M Na citrate. The filled electrodes were then stored with their tips in chromic acid or distilled water. However, keeping their tips immersed in distilled water for more than a few days leads to a deterioration of the tip (either a blunting or withering away of the tip). Chromic acid, provided it is made using saturated K_2CrO_4 solution, is best for keeping pH electrodes. The electrodes were usually kept for a few days in chromic acid before being used. Before this they were usually too noisy and had slow and small responses. Occasionally, the response of an electrode was helped by etching the tip in a solution of 4M NaOH and 0.1M EDTA (ethylenediaminetetraacetic acid tetrasodium salt) for a few hours (Thomas, 1978) but this treatment usually also resulted in a blunting of the tip.

All pH electrodes used for experimental purposes had response times which were 90% complete in <2 min. Their response to a unit change in pH was 52-58 mV. pH electrode responses, however, vary in speed according to the solution buffer concentration (Thomas, 1974, 1978). That used in the present experiments was normally 5mM HEPES. Since the cell buffer concentration is greater than this then the response of the electrode when inserted into the cell may be faster than when outside the cell.

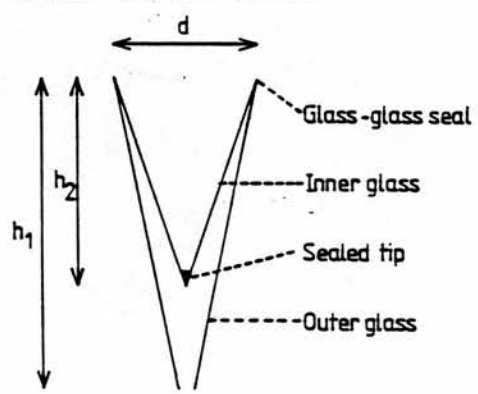
Na microelectrodes

The technique for recessed-tip Na microelectrode manufacture was first described by Thomas in 1970 and more thoroughly in 1978.

Briefly, Na sensitive glass (NAS 11-18, Microelectrodes Inc., U.S.A.) inners and aluminosilicate outers were pulled and measured as described for pH electrode construction. The Na glass inners had their tips sealed, were broken at the shank, separated from the rest of the micropipette and the small shank-to-tip piece carefully dropped down inside the outer insulating micropipette. If the measurements were made correctly the Na sensitive glass lodged near the tip of the outer. Since very high temperatures (~970°C) are required to soften the Na glass the aluminosilicate glass softens before the Na glass. Thus the glass-glass seal is formed by softening the outer which collapses around the inner Na glass. The internal geometry of the finished microelectrode is not as critical as for the pH electrodes. Adequately fast electrodes can be made by having recessed lengths of as much as 150μm. The completed microelectrode was removed from the microforge and tapped gently to break the excess NAS 11-18 above the seal. This surplus was removed and the electrode stored in dry air. Immediately before use the electrode was filled with 0.1M NaCl buffered to pH 6.0 with Na citrate. Na electrodes were used only once for even after 24 hours they became extremely noisy. In contrast to pH microelectrodes, Na electrode resistance increases with age. (Lee, 1979).

All Na electrodes used had response times which were 90% complete within 90 sec. and they all produced responses of 54-60 mV for a ten-fold change in Na concentration.

Assuming the recess inside the electrode approximates to the difference between the volume of two cones:-



then typical dimensions for h_1 = 50μm

h_2 = 35μm

d = <5μm

For the volume of a cone = $\frac{1}{3}\pi r^2 h$

then the volume of the larger cone = 327μm³

and the volume of the smaller cone = 229μm³

thus the recessed volume ≃100μm³

Eisenberg & Cohen (1983) find for dog Purkinje myocytes an average length of 80μm and an average diameter of 38μm. These values are similar to those measured by Muir (1957) for sheep Purkinje cells which he found had an average length of about 60μm and average diameter of about 40μm. Assuming the cell volume approximates that of a cylinder then a Purkinje cell volume approximately equals 100000μm³. In other words the recess volume of a pH microelectrode is about 0.001% of the total cell volume.

Ca microelectrodes

Ca-sensitive microelectrodes were manufactured as described by Tsien & Rink (1980). Thin walled borosilicate capillary tubing (o. d. 1.5mm) was pulled into micropipettes. These pipettes were preheated in an oven at 200°C for 15-20 min. in a glass container. Then a drop of tri-n-butylchlorosilane was added and the container covered. After 20 min. the cover was removed and after a further 15 min. the pipettes were removed from the oven and stored in dry air until used. The silanized micropipettes were filled on the day they were used. The micropipettes were first back-filled with a solution of 10μm $CaCl_2$ and 5mM HEPES. Then a column (~200μm) of a Ca selective resin (Oehme et al, 1976) was drawn by suction into the tip of the microelectrode.

Signal Recording

Chlorided silver wires were inserted into the backs of the conventional 3M KCl filled microelectrodes and the ion-sensitive microelectrodes. The silver wires were chlorided either by dipping them into molten AgCl or by electrolytic plating. Dipping produces a sturdy coating but electrolytic plating is an easier method and may produce more stable half cells. The purpose in chloriding silver wire is to produce an electrical contact between the microelectrode filling solution and the recording apparatus that is stable and will not drift during an experiment. This is accomplished by the Ag/AgCl half cell which is reversible:-

$$Ag^+ + Cl^- \rightleftharpoons AgCl + e^-$$

The "indifferent", bath or reference electrode was made by gently heating and stirring some Tyrode solution with 3-4% Agar (w:v) until the agar dissolved. This mixture was then injected into a glass tube (o. d. ~2.0mm) about 5cms in length, which contained a chlorided Ag wire.

With the bath grounded (i.e. at zero volts) by a chlorided silver wire running to earth, the output of the KCl microelectrode with respect to the Agar-Ringer reference electrode was displayed on one channel of a potentiometric pen recorder (Bryans BS 314) and on one channel of an oscilloscope (Tektronix 502A or 913). The signals from the microelectrode and the reference electrode were pre-amplified using RCA CA 3140 operational amplifiers wired for unity gain. A signal from the bath electrode was passed through a CA 3140 although the bath electrode resistance is low enough not to require an amplifier. In this way power supply and temperature changes and any other interference would affect each amplifier in a similar way and thus would be cancelled out at the differential input to the oscilloscope or pen recorder.

The conventional microelectrodes' resistances were measured continually by using a triangle generator (designed by Strickholm & Winston, see Thomas (1978) monograph). Triangle signals from a generator were fed to a capacitor (an air gap between the two wires) at the preamplifier input. This capacitor differentiated the triangles into square waves at the amplifier input. The microelectrode's resistance was proportional to the height of the square wave potential. The measurements were calibrated using known resistors. Ion sensitive microelectrode potentials were measured using a varactor bridge operational amplifier (Analog Devices 311J) wired for unity gain and filtered with a low pass filter (time constant 0.65 sec.). This amplifier has a very large input impedance (~10^{14} Ω) suitable for use with ion-sensitive microelectrodes. By recording the difference in signal between the KCl and the ion-sensitive microelectrode the intracellular activity of the relevant ion was obtained. This subtraction of voltages between one electrode and the other was accomplished at the pen recorder input.

FIGURE 5

A schematic diagram of the electrical connections from the microelectrodes to the recording apparatus. The "high" side of the conventional microelectrode which measures membrane potential also serves as the "low" side of the ion-sensitive microelectrode input.

The Subtraction Procedure and Calibration of Electrodes

A schematic diagram of the wiring is shown in Figure 5.

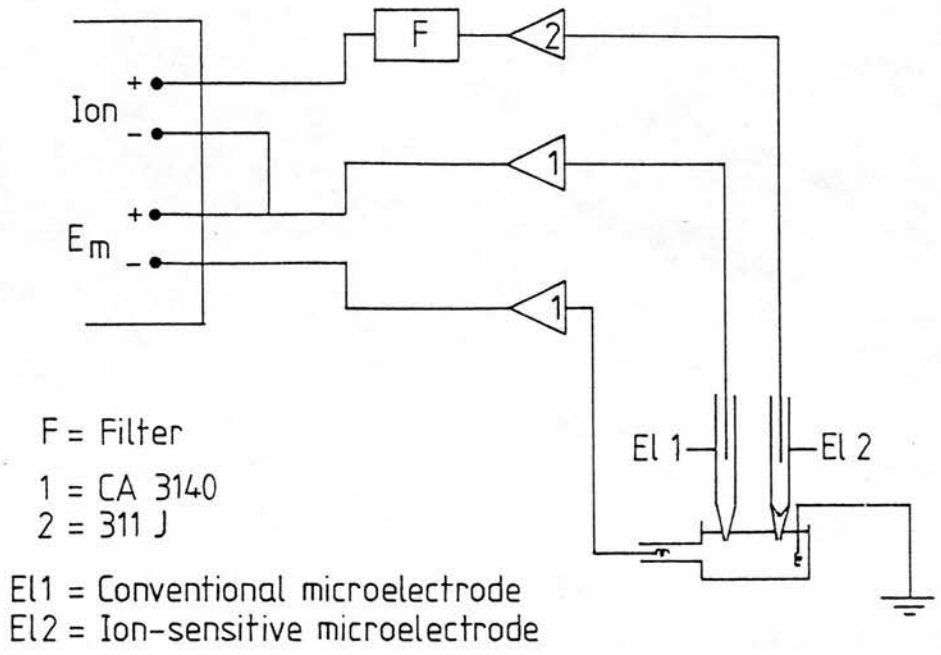


Figure 5

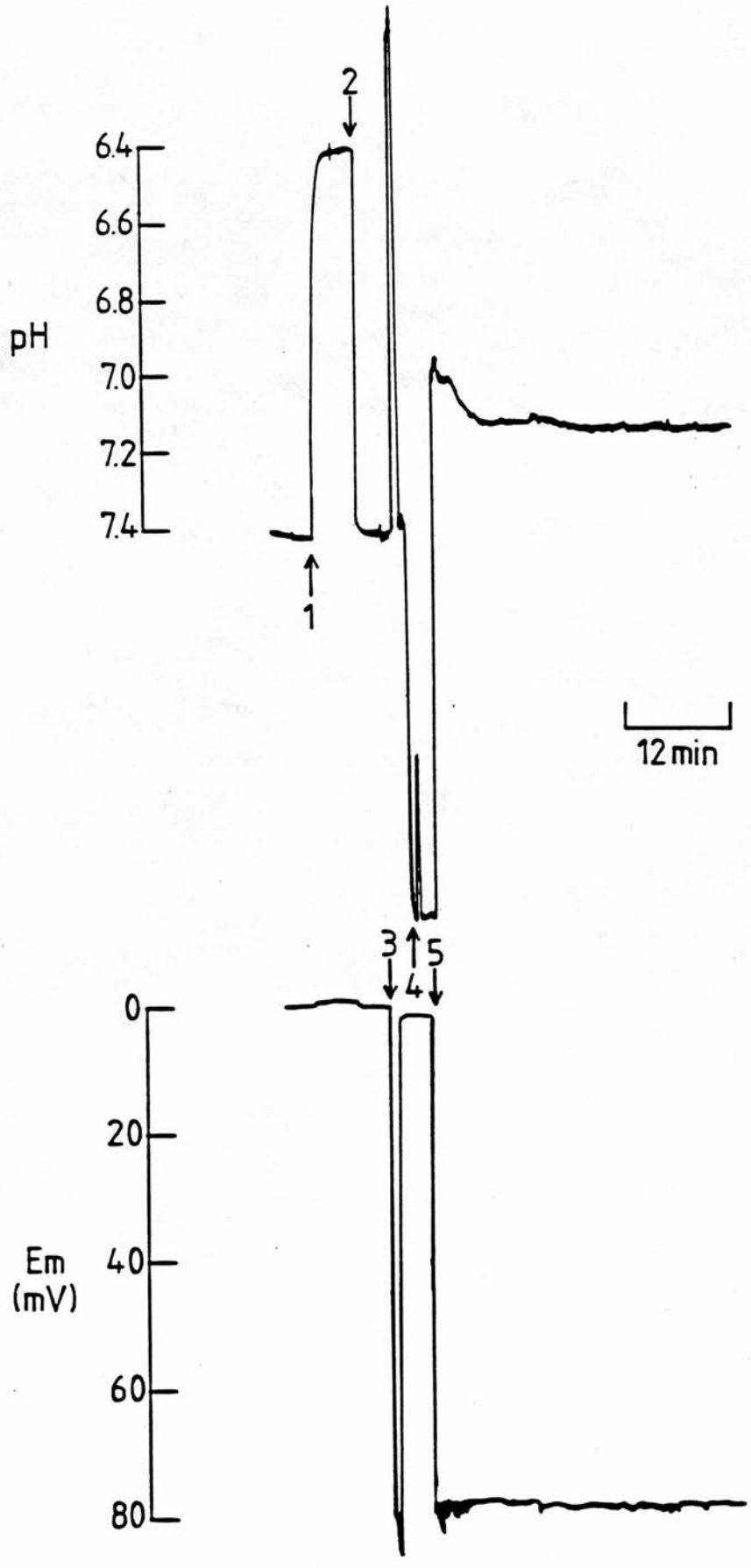
It is important to notice that the "positive" side of the conventional microelectrode input also serves as the "negative" to the differential input of the ion-sensitive microelectrode channel. With this in mind and with the use of Figure 6 and 30 the subtraction procedure and calibration of electrodes can be illustrated. The calibration procedure was carried out either at the beginning, or more usually, at the end of a Na experiment (Figure 30).

The preparation and microelectrodes were set up in the chamber and left to stabilise for at least 30 min. Figure 6 and 30 start after this stabilisation period. In Figure 6 at arrow 1, the superfusing Tyrode was changed from the normal pH of 7.4 to pH 6.4. This change in hydrogen ion activity was measured by the electrode and there was a Nernstian response of 58mV for a tenfold change in the hydrogen ion activity, 90% of the response complete in <2 min . At arrow 2 the solution pH was changed back to pH of 7.4 and the pH electrode potential was restored to the initial value. Because Purkinje

FIGURE 6

The calibration procedure and the insertion of a pH-sensitive microelectrode into a cell. The top trace is pH and the bottom trace is membrane potential (E_m).

At arrow 1 both electrodes are extracellular and the pH of the superfusing Tyrode is changed from 7.4 to 6.4. The pH electrode responds with a change of about 58mV. At arrow 2 the pH of the solution is changed back to 7.4. At arrow 3 the conventional microelectrode is inserted into a cell. The E_m trace moves downward (negative) and measures a E_m of about -80mV. The pH trace moves a similar amount in the opposite direction. A few minutes later the conventional microelectrode was withdrawn from the cell. At arrow 4 the pH-sensitive microelectrode is inserted into a cell. The electrode starts to come out the cell a few minutes later but is re-inserted and after several minutes it records a steady potential. At arrow 5 the conventional microelectrode is inserted into another cell and the E_m was about -80mV. The pH trace now becomes the pH_i trace as both electrodes are now intracellular. The pH_i trace moves in an equal but opposite direction from the E_m trace and records an initial pH_i of about 7.0 which, after 5 - 10 min. stabilises at about 7.2.

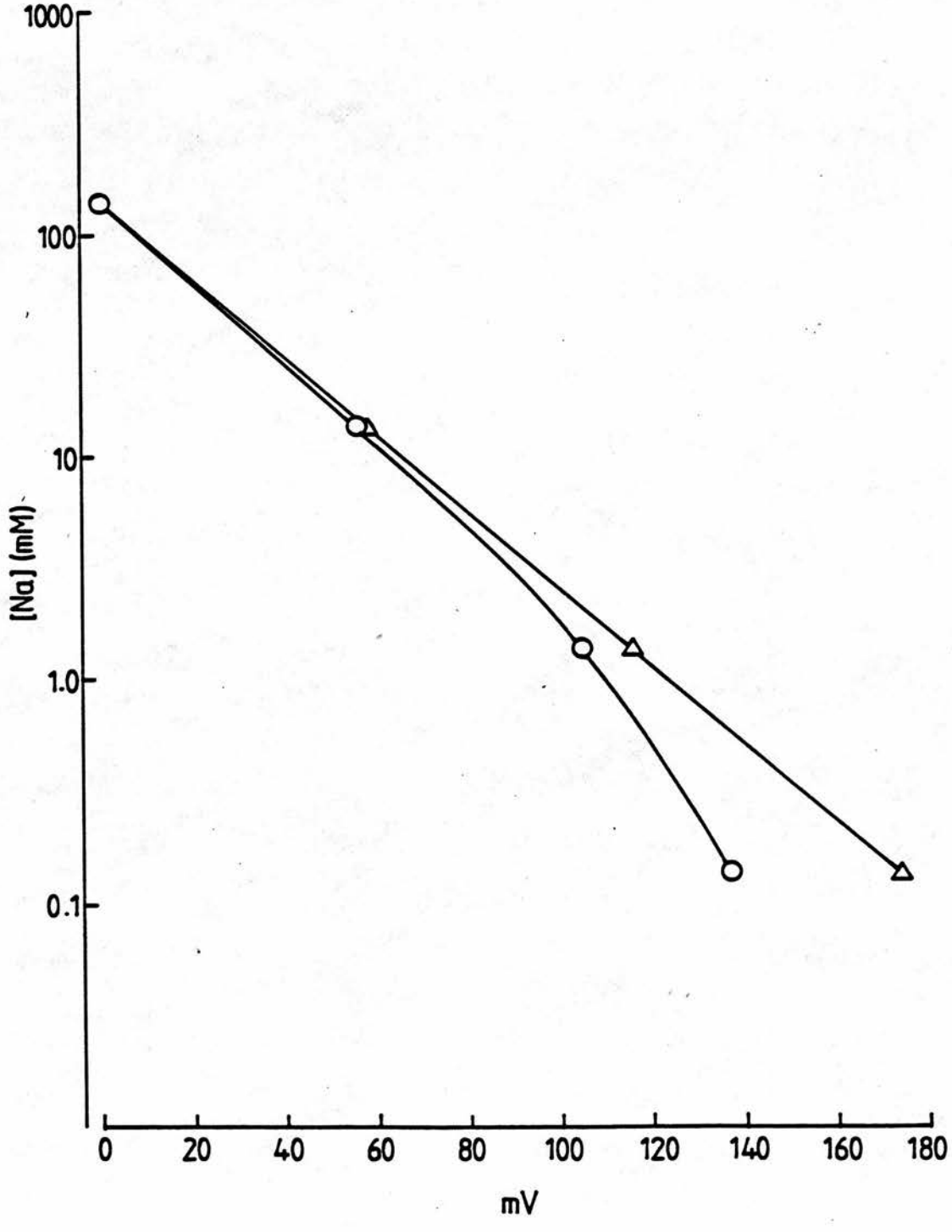


fibres are very tough to penetrate, an initial impalement was made using the conventional microelectrode to find an area which could be penetrated easily. At arrow 3 such a protocol was carried out, the conventional microelectrode recorded a resting membrane potential (E_m) of almost -80mV. Notice that at this time the pH trace moves in an equal and opposite direction because the ion-sensitive electrode's negative input is also the membrane potential electrode's positive input. Several minutes later the conventional microelectrode was removed and again both microelectrodes were now extracellular. At arrow 4 the pH microelectrode was inserted into the cell. The trace deflection was also negative (downward) because on entering a cell the pH electrode also measures the membrane potential in addition to the change in potential due to the difference in hydrogen ion activity. In order to subtract the membrane potential from the combined E_m and E_h measured by the pH sensitive microelectrode the conventional microelectrode was inserted at arrow 5. The E_m electrode was almost definitely not in the same cell but owing to the syncytial nature of heart cells this does not pose substantial problems (page 17). The downward negative going trace of E_m is accompanied by the same amount of upward deflection on the pH trace. Both electrodes were now intracellular and the pH trace becomes the pH_i trace which, after about 5 minutes, stabilised at a pH_i of 7.2. The E_m electrode thus also forms the reference electrode for the pH measuring circuit. In Figure 30 initially both electrodes are extracellular. After about 5 minutes the Na electrode was inserted into the cell and there was a large downward deflection due to the summing of the E_m and the dramatic change in Na activity seen by the electrode as it entered the cell. The upward deflection on the Na trace about 12 min. later was the E_m being subtracted from the total potential measured by the Na electrode as the conventional microelectrode entered the cell.

At the end of the experiment the electrode was calibrated in solutions of known Na concentration. 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 with Tyrode solutions where Na was replaced by K. At very low Na concentrations the electrode response departs from a theoretical Nernstian slope. Figure 7 shows the observed mV output for

FIGURE 7

The response (measured in mV) of a Na-sensitive glass microelectrode to changes in Tyrode [Na]. The theoretical Nernstian slope is plotted by the line joining the triangles (Δ). The actual response of the elctrode is plotted by the circles (O). When Na was removed it was replaced by K.


1000
100
10
1.0
0.1
[Na] (mM)
0
20
40
60
80
100
120
140
160
180
mV

a change in [Na] produced by the same electrode as that used in the experiment illustrated in Figure 30, and the deviation from the Nernstian slope. The Na^- sensitive glass is very selective and does not seem to be influenced significantly by other ions except by a small effect of K (the glass is 200 times more selective for Na than K solutions) but as discussed already this problem is alleviated if the calibration is carried out using high K solutions. The intracellular Na electrode potentials were then converted into ion activities using the calibration curve. It seems appropriate to briefly discuss the differences between the use of the terms 'activity' and 'concentration'.

Activity (a) of an ion (I) is related to its concentration (c) by the equation

$$a_i = f \times c_i$$

where f is the activity coefficient of I. The activity of a substance may be thought of as the 'effective concentration'. For electrolytes in very dilute aqueous solutions, activity is very nearly numerically equal to the molal or molar concentration, that is -

$$a_i \simeq c_i$$

For solutions of 0.1M concentration however, activity and concentration can differ by 10-50% depending on the electrolytic concentration and the charge of ions. The reason for this is as follows. NaCl is an example of a strong electrolyte, i.e. it is fully ionised in solution, forming freely mobile Na^+ ions and Cl^- ions. However, mutual electrostatic attraction between the cations and anions tends to restrict their freedom or mobility. The magnitude of the interaction is determined by the ionic charge, and the concentration and charges of the other ionic species present in the solution. It is intuitively obvious that the more concentrated the solution then the more ions are inactive, e.g. a 0.1 molal solution of NaCl has an f of 0.778 and a 1.0 molal solution of NaCl has an f of 0.657.

In the case of Na measurements the [Na] for the appropriate measured electrode potential was read off the calibration graph and multiplied by the Na activity coefficient of 0.75. It is assumed that the f for Na is the same inside and outside the cell. Arguments in favour of expressing results in ion activity rather than concentration are presented in Thomas' monograph (1978). The use of activities

seems reasonable for two reasons. Firstly, because the potential of an ion-sensitive microelectrode varies with the logarithm of the ion activity not concentration. Secondly, the ion activity is physiologically and biochemically the more important parameter. For pH electrodes the calibration procedure is undoubtedly much more straightforward than that for Na electrodes. Since pH is the negative logarithm of the H ion activity then there is a linear relationship between potential changes by the electrode and pH. The pH glass is very selective and there are no known significantly interfering ions throughout the pH range measured in this study. The electrodes were calibrated in Tyrode solutions of known pH. The pH values of these solutions were accurately measured with a pH meter which was calibrated every day before use by electrometrically standardised buffers of pH 6.50 and pH 8.00 (B.D.H. Chemicals, Poole, England).

At the end of the experiment the microelectrodes were removed from the cells and the E_m and pH_i or a^i_{Na} traces returned to their original "baseline" (now extracellular) levels. This was not always the case, however, and usually this was caused by the conventional microelectrode developing, after several hours of cell impalement, a significant (>5mV) tip potential. Rarely was the problem due to drift in the ion-sensitive microelectrode. If the baseline changes were greater than 5mV then the experiment was rejected for quantitative measurements. Qualitative assessment was made and the experiment repeated.

Testing the subtraction

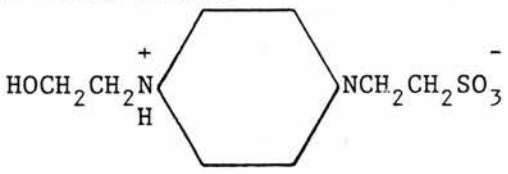
Since the E_m is subtracted from the ion-sensitive microelectrode's total recorded potential then in order that the correct intracellular ion activity be measured it is essential the two microelectrodes measure the same E_m. Most KCl filled microelectrodes with small tip potentials will measure the same $E_m \pm 2$mV but it is difficult to be certain that the ion-sensitive microelectrode will also measure the E_m with a similar accuracy. What is perhaps more important, however, is that both microelectrodes measure the same change in E_m. A good test is to impose a change of E_m upon the preparation. The E_m electrode should register the change and if subtraction is accurate then the ion activity trace should not respond to the change (except

if the ion levels themselves are changed by the change of E_m (see Results)). This was done on many occasions but see for example Figure 27. Although E_m was depolarized by ~30mV little change in pH_i occurs. It is important also to emphasise that both electrodes were almost definitely not in the same cell for most of the experiments shown in this work. Ideally, both electrodes should be in the same cell and this is theoretically possible for Purkinje cells. However, it is, in practice, difficult to obtain stable recordings when the interelectrode distance is <100μm. This form of recording is dependent on good electrical coupling between cells. Although not directly tested, some of the experimental protocols in this work would presumably uncouple cells (see Introduction section 3). However, this would not change the validity of the results as long as the two microelectrodes subsequently measured the same change in E_m. If the two microelectrodes measured different E_m's then the absolute value of the ion levels would be inaccurate but as long as they measured the same changes in E_m then the rates of change of the ion levels would be accurate.

Solutions

The normal Tyrode solutions were composed of Analar grade chemicals. The normal Tyrode contained (mM) Na, 140; K, 6; Ca, 2; Mg, 1; Cl, 152; Glucose, 10; HEPES (N-2-hydroxyethylpiperazine-N-2-ethane-sulphonic acid)[1], 5; and was titrated with NaOH to give a pH of 7.40 ± 0.05 at 35°C. The solution was equilibrated with 100% O_2. When measurements were carried out at room temperature the

1 HEPES belongs to one of a series of buffers originally synthesised by Good et al (1966) who, at the time, were seeking more acceptable buffers for H^+ ion between pH 6 and 8. In designing new buffers the authors laid down a considerable list of criteria which should ideally be met by the substances. They recognised that not all of their criteria were likely to be met in all instances but HEPES fulfils most requirements like others in the Good series. HEPES is a zwitterion with a pK_a at 20°Cof 7.55. Its $\Delta pK_a/°C$ = -0.014 and its structure is shown below:



It binds metal cations to a negligible degree and according to Good et al does not readily pass through biological membranes.

normal Tyrode was titrated to give a pH of 7.40 ± 0.05 at 22°C. The pH of the Tyrode solutions were generally measured by a pH electrode which had a separate reference electrode with a free flowing liquid junction. The use of the combination type electrode with a reference half cell connected to the external solution via a ceramic porous plug was avoided. The combination type of assembly can give rise to incorrect pH measurements due to errors arising from substantial liquid junction potentials associated with the porous ceramic plug, which vary with the ionic composition of the solution under test (Illingworth, 1981). 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[1]; BDA (Bis (2-hydroxy-ethyl) dimethyl ammonium), 140; TMA (tetramethyl ammonium), 140 or K, 140. Bicarbonate buffered Tyrode, equilibrated with nominally 95% O_2, 5% CO_2, lacked HEPES but contained (mM) Na, 140; K, 6; Ca, 2; Mg, 1; Cl, 128; HCO_3, 24; Glucose, 15; and its pH was 7.40 ± 0.1 at 35°C. Na free bicarbonate buffered solutions were produced by replacing Na with 116 mM of one of the above substitutes and 24mM KCO_3 to buffer the CO_2. When solutions of intermediate Na concentration were required they were made by mixing Na-free Tyrode with Na containing Tyrode in appropriate amounts.

When NH_4Cl was added an equivalent amount of NaCl was removed.

Amiloride was added as solid directly to the Tyrode solution and the mixture well stirred before use. SITS (4-acetamido-4-isothiocyanatostilbene-2, 2'-disulphonic acid disodium salt) was added as solid to the Tyrode just prior to use giving a final concentration of 100μM.

Strophanthidin (Boehringer Mannheim) was dissolved in 50% ethanol (v:v) 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%.

Solutions of very low Ca concentration were produced by

1 The osmotic coefficient of Tris is 1.1286

adding 1mM EGTA (Ethyleneglycol-bis-(β-aminoethylether N, N'-tetra-acetic acid)) to nominally Ca free Tyrode. An extra 1 or 2 mM Mg were added to replace the Ca. Such solutions had a Ca concentration of $<10^{-8}$M. The Ca concentration in these solutions was estimated by the use of Ca-selective microelectrodes. These electrodes had tip diameters circa 10μm, were manufactured as detailed in the micro-electrodes section and calibrated by using standards as detailed in Tsien & Rink (1980).

Data Handling

All illustrations of actual recordings are direct Xerographic copies which have been subsequently photographed.

All statistical calculations were performed on a Hewlett-Packard 9815 A desk top calculator.

All experimental numbers (n) refer to separate preparations. i.e. the experimental numbers do not include repeat protocols on the same preparation.

RESULTS

Section 1 - Initial observations

Section 2 - Removal of Na_o on pH_i recovery

Section 3 - Temperature effects

Section 4 - Na dependence of pH_i recovery

Na measurements

Section 5 - Ca dependency

Section 6 - Further interactions of Ca and H

Section 7 - Alkali recoveries

RESULTS

The results have been divided into seven sections.

Section 1 - INITIAL OBSERVATIONS

In general, from these experiments, it has been found that in normal Tyrode buffered with 5mM HEPES to give a pH_o of 7.40, pH_i lies between 7.00 and 7.40. The mean ± S.D. of 83 experiments is 7.16 ± 0.12. The mean E_m from these experiments is -73mV ± 4. These results are in good agreement with those of Ellis & Thomas (1976 a, b). Deitmer & Ellis (1980) and de Hemptinne (1980) who have previously used glass ion-sensitive microelectrodes to record pH_i in heart muscle which was also superfused with Tyrode buffered with HEPES and equilibrated with 100% O_2 i.e. similar buffer conditions to those used in this study. In Tyrode buffered with HCO_3^- and bubbled with 5% CO_2/ 95% O_2 the pH_i measured is slightly less than that measured in HEPES buffered solution (see Figure 8 & 27). This is perhaps due to the negative membrane potential driving the efflux of HCO_3^- (de Hemptinne, 1981) which subsequently leaves H^+ in the cell. In either of the two buffer conditions, however, pH_i is far from equilibrium.

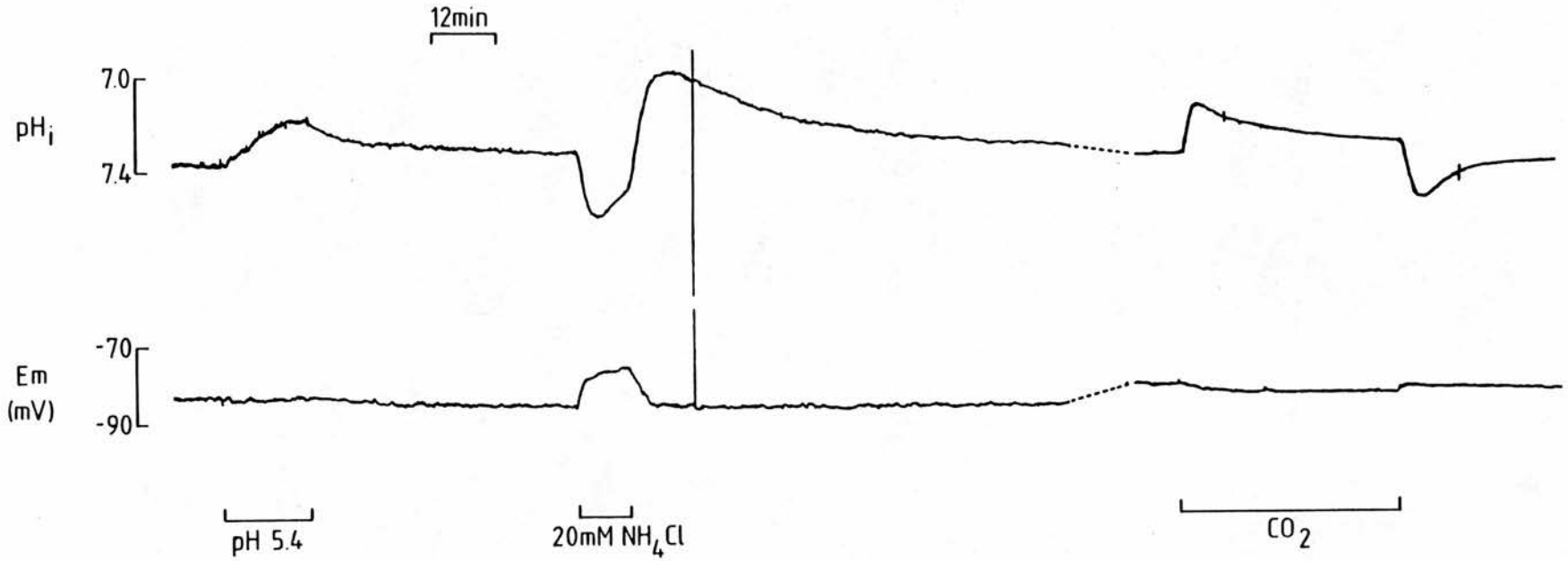
$$E_H \text{ (equilibrium potential for } H^+ \text{ ions)} = -61 \log \frac{[H]_i}{[H]_o} \text{ mV}$$

$$= -12\text{mV}$$

With an E_m of -70mV and an extracellular pH of 7.40 the Nernst equation would predict pH_i to be about 6.2. pH_i is thus far more alkaline (by an order of magnitude) than is dictated by a system in equilibrium. A cardiac cell must therefore possess a mechanism(s) which accomplishes what is in effect a net efflux of H^+ from the cell. The method adopted to investigate the problem of pH_i regulation in heart muscle was firstly to acidify (acid load) the cell and then study the subsequent pH_i recovery as this should be equivalent to an "uphill" movement of H^+ ions against their electrochemical gradient out of the cell. The cells were acid loaded by (1) the addition and removal of NH_4Cl, (2)exposure to CO_2 containing solutions or (3) changing to a more acid Tyrode solution. These three types of acid loading are

FIGURE 8

The effects on pH_i (top) and membrane potential (bottom) 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.


12min
7.0
pH$_i$
7.4
-70
Em
(mV)
-90
pH 5.4
20mM NH$_4$Cl
CO$_2$

shown in Figure 8. Application of Tyrode at a pH of 5.4 caused an intracellular acid load. The relationship between the steady-state level of pH_i and pH_o is linear over the range of 5.4 - 8.4 and shows a change of about 0.23 pH units for a unit change in pH_o (Deitmer & Ellis, 1980).

Exposure of the fibre to NH_4Cl initially produces an alkalinization. Cell membranes appear to be highly permeable to NH_3 which rapidly crosses the cell membrane and its subsequent hydration forms NH_4^+ and OH^-. This is followed by a slow acidification presumably due to a slower entry of NH_4^+ ions which provide a source of protons (Boron & de Weer, 1976). The slow acidification in NH_4Cl may also be due to an active recovery of the cell from the alkalinization (Vaughan-Jones, 1981, 1982a, b). Removal of NH_4Cl causes a large and rapid intracellular acid load as NH_3 rapidly leaves the cell. The depolarization during the NH_4Cl exposure is a consistent phenomenon and is presumably brought about by the entry of NH_4^+ perhaps through K channels (Boron & de Weer, 1976).

Exposure to CO_2-containing solutions rapidly acidifies the cell. CO_2 will rapidly cross the membrane producing H^+ and HCO_3^- after hydration and subsequent dissociation. The intracellular acidification is transient, i.e. there is a substantial amount of pH_i recovery. On returning from a CO_2/HCO_3^- buffered to a HEPES, CO_2-free Tyrode the pH_i rapidly becomes alkaline and overshoots its initial value. This overshoot is, in itself, indicative of a net active acid extrusion mechanism during the period of exposure to CO_2. The amplitude of the overshoot is a measure of the net loss of protons which occurs during the CO_2 exposure (Boron & de Weer, 1976).

Recovery of pH_i to a more alkaline value after the initial acidification takes place from all three types of acid loading. It is the ability of the cell to recover from acid loading which is of interest in this section.

The pH_i recoveries from these types of acid loading all follow a time course which is rarely a pure exponential but approximates one. The non-exponential recovery of pH_i is mentioned by Boron (1983).

Translating the pH_i values into H^+ ion activity (a_H^i) and plotting these recoveries yields recoveries which also approximate to an exponential. Theoretically, only a linear pH_i recovery should produce a purely exponential a_H^i recovery. However, in the example shown in Table 1 below both ways of measuring a pH_i recovery (i.e. pH units or a_H^i yield acceptable correlation coefficient values (r) of 0.988 and 0.993 respectively. The data in Table 1 are taken from an experiment (shown in Figure 9a) which measures the pH_i recovery from a CO_2-induced acid load.

These results are plotted semi-logarithmically in Figure 9b. Recovery from an acid load seems to lie somewhere between an exponential pH_i recovery and an exponential a_H^i recovery. All the recoveries from acid loads were therefore measured in terms of pH_i and in terms of the equivalent a_H^i measurements. The time constants for recovery were worked out in both units. Although the absolute values of the results were different (in general the time constants for recovery calculated using hydrogen ion activities were faster) a similar pattern of results was obtained and the statistical relationship between different groups of results remained the same.

The time constants are different according to which type of acid loading procedure is used. Table 2 shows the differences in time constants between each method of acid loading calculated (a) by assuming pH_i follows an exponential recovery and (b) by assuming a_H^i follows an exponential recovery. The results from (b) are expressed graphically in Figure 10.

One of the reasons why pH could be a more suitable way of expressing the results in some cases, is that the amount of hydrogen ion available to cell processes is dependent upon pH_i and the cell buffering power. The cell buffering power is also measured in pH units (i.e. how much acid (or alkali) one has to add in order that the solutions pH be changed by one pH unit). However, the experimental results of this study can be better fitted by assuming a_H^i recovers in an exponential manner.

In Figure 10 the unshaded columns indicate the mean ± S.E. of

FIGURE 9

(a) A pH_i recovery from a CO_2-induced acid load. pH_i was measured every 1.2 min. and the corresponding a_H^i calculated. These are shown in Table 1.

(b) The pH_i differences and a_H^i differences from the baseline or final value were calculated as shown in Table 1 and plotted, assuming both measurements follow an exponential. The better fit to the line is achieved by assuming the a_H^i recovers exponentially.

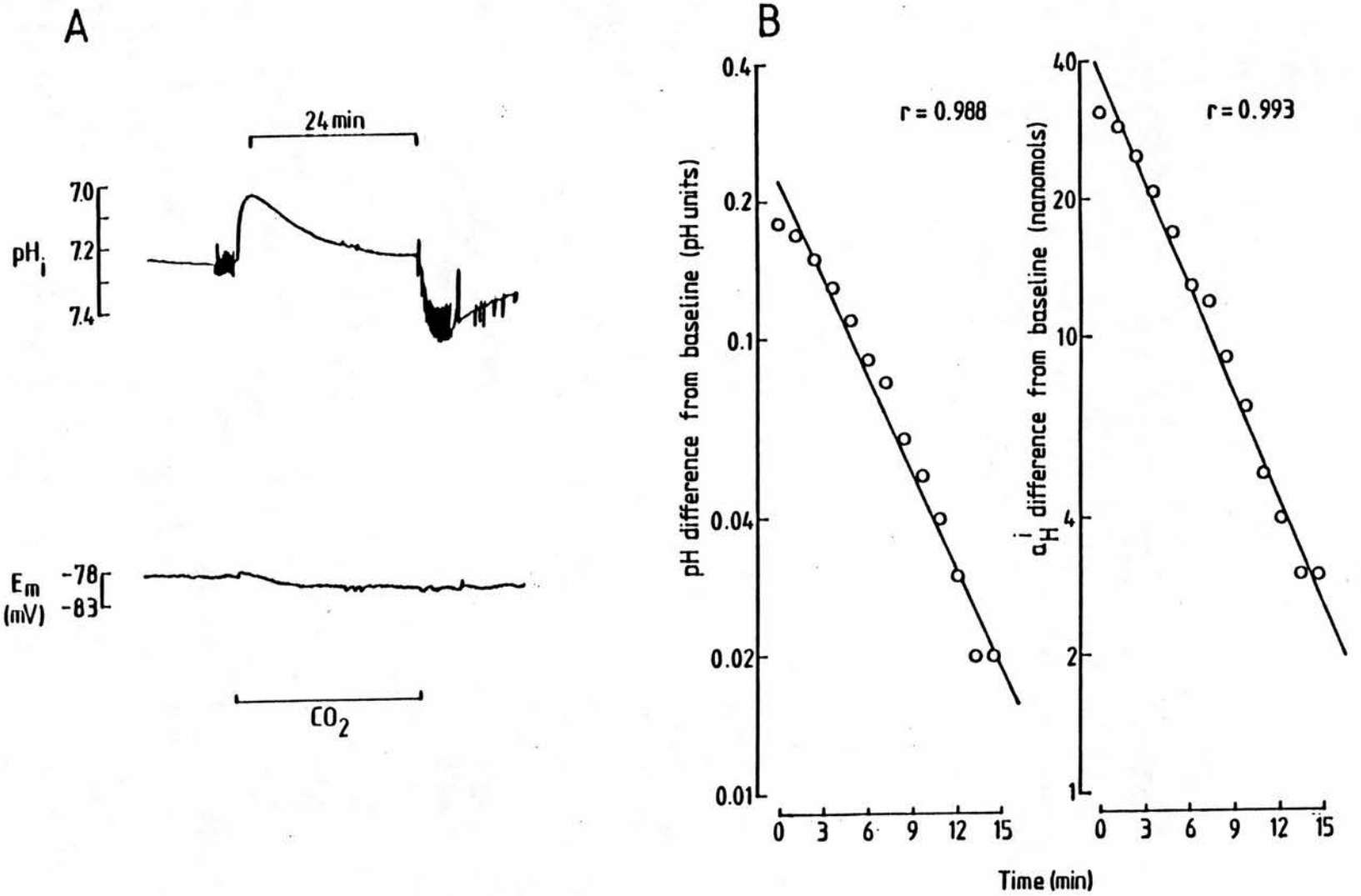

A
24 min
7.0
7.2
7.4
pH$_i$
-78
-83
E_m
(mV)
CO_2
B
r = 0.988
r = 0.993
pH difference from baseline (pH units)
a_H^i difference from baseline (nanomols)
0.4
0.2
0.1
0.04
0.02
0.01
40
20
10
4
2
1
0 3 6 9 12 15
Time (min)

TABLE 1

pH_i VALUES AND a_H^i TAKEN FROM THE EXPERIMENT SHOWN IN FIGURE 9A

	pH_i (units)	a_H^i (nmol)	pH_i (difference from baseline) (units)	a_H^i (difference from baseline) (nmol)
START	7.03	93	0.18	31
	7.04	91	0.17	29
	7.06	87	0.15	25
	7.08	83	0.13	21
	7.10	79	0.11	17
	7.12	75	0.09	13
	7.13	74	0.08	12
	7.15	71	0.06	9
	7.16	69	0.05	7
	7.17	67	0.04	5
	7.18	66	0.03	4
	7.19	65	0.02	3
	7.19	65	0.02	3
	7.20	63	0.01	1
	7.20	63	0.01	1
BASELINE	7.21	62	-	-

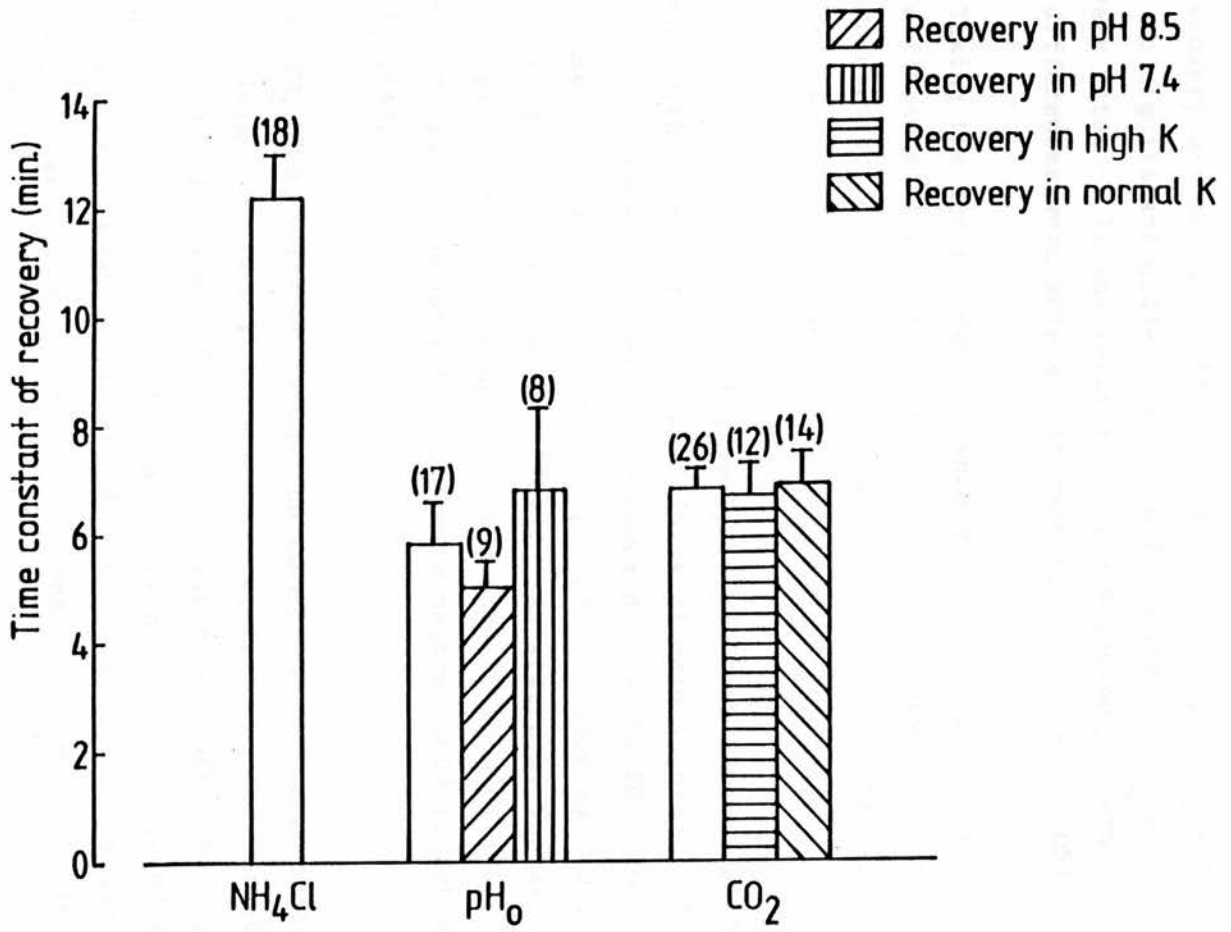
TABLE 2

TIME CONSTANTS OF RECOVERY

TYPE OF ACID LOAD	SUPERFUSING SOLUTION DURING RECOVERY PERIOD (NT = NORMAL TYRODE)	NUMBER OF EXPERIMENTS	(a) ASSUMING pH_i RECOVERY EXPONENTIAL MEAN ± S.E. (min.)	(b) ASSUMING a_H^i RECOVERY EXPONENTIAL MEAN ± S.E. (min.)
NH_4Cl	NT	18	14.3 ± 1.0	12.2 ± 0.8
ACID pH_o	Grouped Results	17	6.9 ± 0.9	5.8 ± 0.8
	pH 8.5	9	5.5 ± 0.5	5.0 ± 0.5
	NT	8	8.6 ± 1.6	6.8 ± 1.5
CO_2	Grouped Results	26	7.9 ± 0.5	6.8 ± 0.4
	High $[K]_o$	12	8.5 ± 0.8	6.7 ± 0.6
	NT	14	7.3 ± 0.6	6.9 ± 0.6

FIGURE 10

The time constants of the rates of recovery of a_H^i following three methods of acid loading. The unshaded columns indicate the means + S. E. of the time constants of recovery from NH_4Cl, acid Tyrode (pH_o 5.4) and CO_2 (5%) induced acidifications. The number of separate experiments for each type of acid load is given above the columns. The shaded columns indicate the different recovery conditions used in some experiments. The number of these experiments is also shown above the columns. The normal $[K]_o$ was 6mM, the high $[K]_o$ 20-26mM.


Recovery in pH 8.5
Recovery in pH 7.4
Recovery in high K
Recovery in normal K
Time constant of recovery (min.)
0
2
4
6
8
10
12
14
(18)
(17)
(9)
(8)
(26)
(12)
(14)
NH4Cl
pHo
CO2

the time constants of recovery from experiments where the three different types of acid loading procedures were used. The number of separate experiments for each type of acid load is given above the columns. The shaded columns illustrate the different recovery conditions used in some experiments. Following acid loading with acid pH_o the recovery was measured either in an external pH of 7.4 or 8.5. There was no significant difference ($p > 0.1$, Student's T-test) between the recovery rates. If the recoveries were measured in terms of pH then the differences were more significant ($p < 0.1$; $p > 0.05$).

Taking the grouped data (unshaded columns) there was a significant difference between the rate constant of recovery from acid loading produced by acid pH_o compared with that produced by NH_4Cl exposure from both types of measurement ($p < 0.01$, Student's T-test).

It is not easy to quantitatively compare the recoveries from CO_2 exposure with those from the other forms of acid loading. This is because the intracellular buffering power during the CO_2 exposure will be greater than during the other types of acid loading (Woodbury, 1965; Thomas, 1976a; Roos & Boron, 1981). As stated by Thomas (1977), "The higher the buffering power the more H^+ ions will have to be transported out of the cell (or otherwise neutralized) to achieve a given pH_i change".

In CO_2/HCO_3 buffered Tyrode the total intracellular buffering power is larger because it consists of two terms. The 'non-CO_2' or intrinsic buffering power (β_I) and the 'bicarbonate/CO_2' buffering power (β_{CO_2}). The intrinsic buffering is probably accomplished by (1) the intracellular weak acids and bases which minimise the direction of a pH_i change by combining with H^+ ions in accordance with the equilibrium(s)

$$H^+ + A^- \rightleftharpoons HA$$
$$H^+ + B^- \rightleftharpoons HB$$

where A is the weak acid and B is the weak base and (2) the possible cell compartmentalization of acid. The values of β_I for Purkinje fibres and ferret ventricular muscle have been estimated by Ellis &

Thomas (1976b) and are, respectively, 35 and 69mM per pH unit per litre (or slykes).

When CO_2/bicarbonate buffer is used then the cell's buffering capacity increases because the equilibrium

$$H^+ + HCO_3^- \rightleftharpoons H_2CO_2 \rightleftharpoons H_2O + CO_2$$

is set up inside the cell. β_{CO_2} thus depends on the $[HCO_3^-]_i$ and so can vary with pH_i.

Thus the buffering power during a CO_2 exposure is not constant as pH_i is changing.

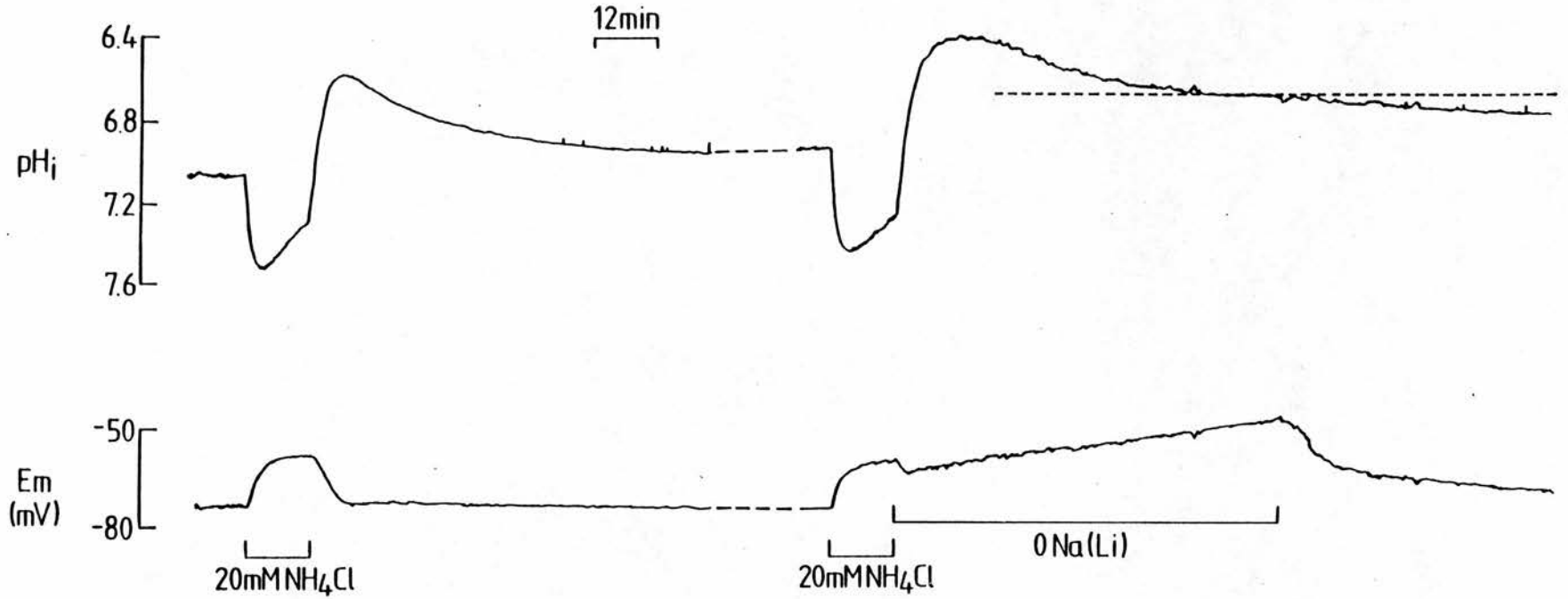
It is possible to compare changes in recovery time constant in normal and high $[K]_o$ because these experiments were all carried out in CO_2/HCO_3 buffered Tyrode and over a similar pH_{ii} range. Following acid loading with CO_2 the recovery was measured in either normal $[K]_o$ (i.e. 6mM) or in high $[K]_o$ (i.e. 20 - 26mM) (with $[Na]_o$ reduced by an equivalent amount). The high $[K]_o$ used in these experiments produced a fall in membrane potential from about -70mV to about -40mV. There was no significant difference between recovery rates in normal compared with depolarized cells measured by the two different methods ($p > 0.1$; Student's T-test)

Section 2 - REMOVAL OF Na_o 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% of normal (i.e. 14mM) slowed the rate of recovery from an NH_4Cl induced acid load. In order to investigate this further I have tried to completely inhibit the recovery from an acid load by removing all Na_o. Figure 11 shows the effect on pH_i recovery from acid loading when all the Na_o is substituted by lithium (Li). The rate of recovery in normal Tyrode has a time constant of 23 min. (measured in terms of pH_i recovery). The acidification in Na-free (Li substituted) solution is larger and the recovery delayed but the rate of recovery is only slightly slower than in normal

FIGURE 11

The effect of removal of all Na_o (Li substitution) on pH_i recovery from an NH_4Cl induced acid load. The dotted line has been drawn to facilitate comparison of the steady-state levels of pH_i attained in Na-free Tyrode with that in normal Tyrode. The break in the recording was for a period of about ½ hour.


12min
6.4
6.8
7.2
7.6
pHi
-50
-80
Em
(mV)
20mM NH4Cl
20mM NH4Cl
0 Na (Li)

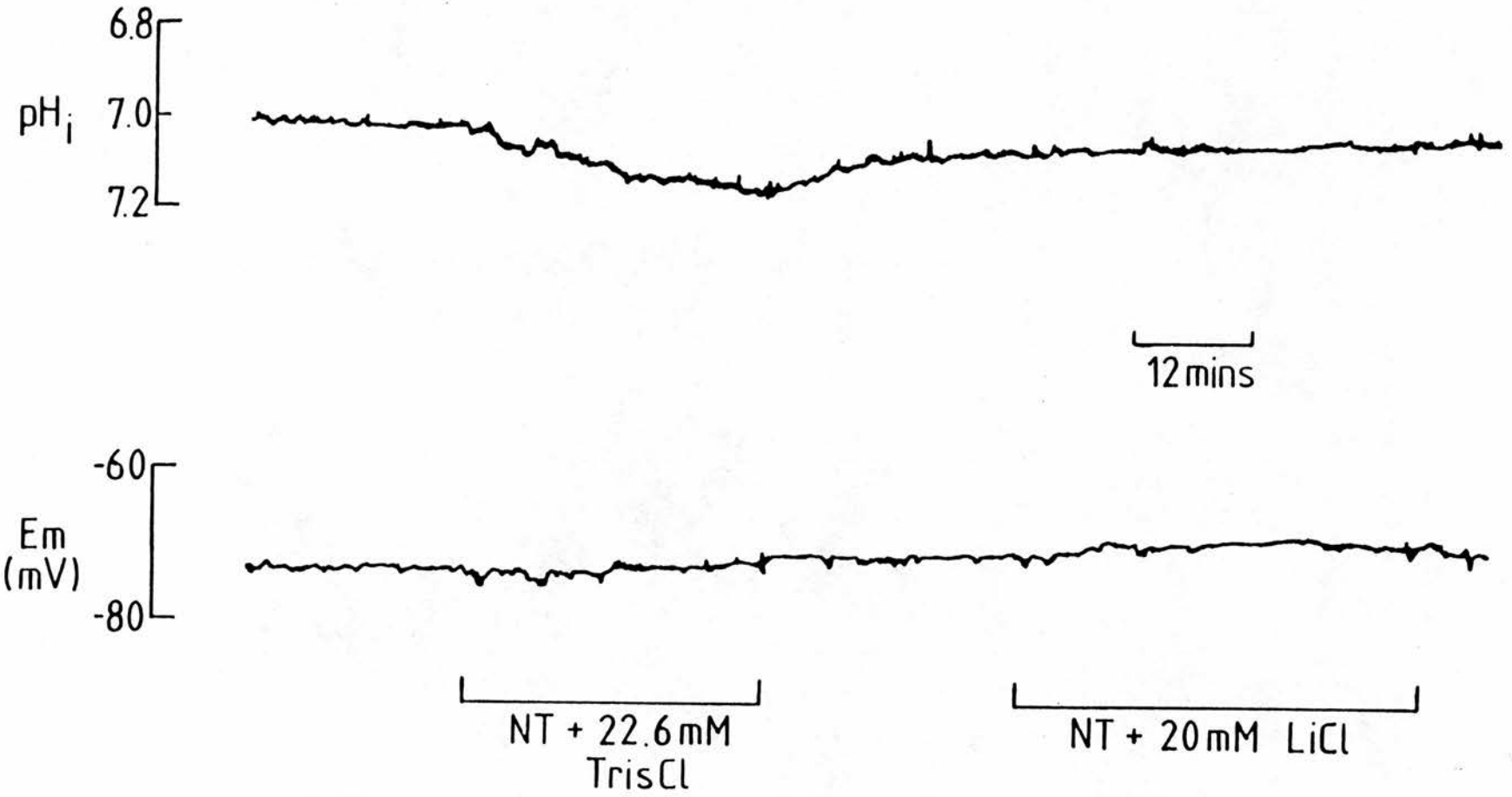
Tyrode (NT) (time constant = 24 min.). Intracellular pH returns to a new steady-state level. The new steady state level of pH_i (pH_i^{∞}) in Na-free solution (dotted line) is approximately 0.1 units more acid than the steady-state level of pH_i in normal Tyrode.

The delayed recovery and slightly larger acidification could be due to (1) an inhibition of the Na^+/H^+ exchanger brought about by Na removal and/or (2) an extra displacement, or reduced uptake of protons at intracellular buffering sites that are shared with Ca^{2+}. In a Tyrode of low $[Na]_o$ the $[Ca]_i$ will be higher than normal due to an altered Na gradient (Reuter & Seitz, 1968; Baker et al, 1981; Glitsch et al, 1970) and this raised $[Ca]_i$ can directly change pH perhaps because of competition at buffering sites shared between H^+ and Ca^{2+} (Meech & Thomas, 1977; Ellis, Deitmer & Bers, 1981; Bers & Ellis, 1982; Vaughan-Jones et al, 1983). This interplay between H^+ and Ca^{2+} will be discussed at greater length in Sections 5 and 6 of the Results section.

If low $[Na]_o$ solutions inhibit a sarcolemmal Na/H exchange, why did Na-free Tyrode not inhibit the rate of recovery from an acid load? One possibility is that Li can substitute for Na on the putative Na^+/H^+ exchanger. This has been recently suggested by Kinsella & Aronson (1981) working with rabbit renal microvillus membrane vesicles and Weinman & Reuss (1982) using *Necturus* gall-bladder. In order to test this possibility, other Na substitutes were used. Tris is commonly used as a Na substitute. However, I have found that this can produce alkaline pH_i changes and such an effect is shown in Figure 12. Tris Cl (22.6mM) was added to the normal Tyrode (pH readjusted to 7.4) during the period shown by the first bar. To ensure that this was not an effect of the hypertonic solution an osmotically equivalent amount of LiCl was added to the normal Tyrode. This produced no change in pH_i. It has been documented (see Nahas, 1962) that as much as 30% of Tris can remain unionised at pH 7.4 and can penetrate cells rendering the cytoplasm more alkaline. I have, therefore, preferred to use either quaternary ammonium compounds (which appear to be fully dissociated in solution) or K as Na substitutes.

FIGURE 12

The effect of Tris on pH_i. At the first bar the normal Tyrode (NT) was changed to one which had 22.6mM Tris added and the pH readjusted to 7.4. At the second bar the Tyrode had an osmotically equivalent amount of LiCl added (note that the osmotic coefficient for Tris = 1.129).


6.8
pH$_i$ 7.0
7.2
12 mins
-60
Em
(mV)
-80
NT + 22.6 mM
TrisCl
NT + 20 mM LiCl

In Figure 13 acid loading is again brought about by exposure to, then removal of 20mM NH_4Cl. After the first NH_4Cl exposure the superfusing Tyrode is changed to one where all the Na has been replaced by bis(2-hydroxyethyl) dimethylammonium (BDA). The Na-free solution completely inhibits the pH_i recovery from the acid load. Approximately 20 min. later the Tyrode is changed to one which also lacks Na but has Li as the Na substitute. Recovery of pH_i now proceeds (time constant = 19 min.) but at a slower rate than that in NT (time constant = 14.4 min.) This demonstrates two important points concerning pH_i regulation in cardiac Purkinje fibres. Firstly, removal of all Na_o and the use of an appropriate Na substitute can completely inhibit pH_i recovery and secondly, Li can support pH_i recovery. It was for this reason that Li was not regularly used as a Na substitute. The passive (tonic) tension increases as the cell goes alkaline and then gradually decreases again as the cell recovers from the alkalinization. Increases in twitch tension during the NH_4Cl alkalinization have been observed by Eisner et al (1983a).

Using K as the Na substitute gives similar results to those with BDA. Figure 14 shows a similar experiment to that in Figure 13. Here K is used as the Na substitute and the recovery from NH_4Cl induced acid load is completely inhibited. When the Tyrode is changed to one where Li substitutes for Na once again pH_i recovers.

Similar inhibitory effects of Na-free solutions on pH_i recovery can be demonstrated when the cell is acid loaded by exposure to CO_2-containing solutions (see e.g. Figure 24). In this figure the cells have been exposed to CO_2 at the point denoted by the bar. The first exposure to CO_2 and subsequent pH_i recovery takes place in 140mM Na. When the second exposure to CO_2 is made the solution is changed to one which lacks Na the Na being replaced by K. This accounts for the large depolarization accompanying the change of solution. (In Section 4 it will be shown that although depolarization can change steady-state pH_i in the acid direction this cannot account for the inhibition of pH_i recovery). The overshoot on CO_2 removal does not occur after Na-free Tyrode, indicating that no acid extrusion took place (Boron & de Weer, 1976).

FIGURE 13

The effect of removal of all Na_o (BDA substitution) on pH recovery from an NH_4Cl induced acid load. Immediately after NH_4Cl exposure the Tyrode 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.

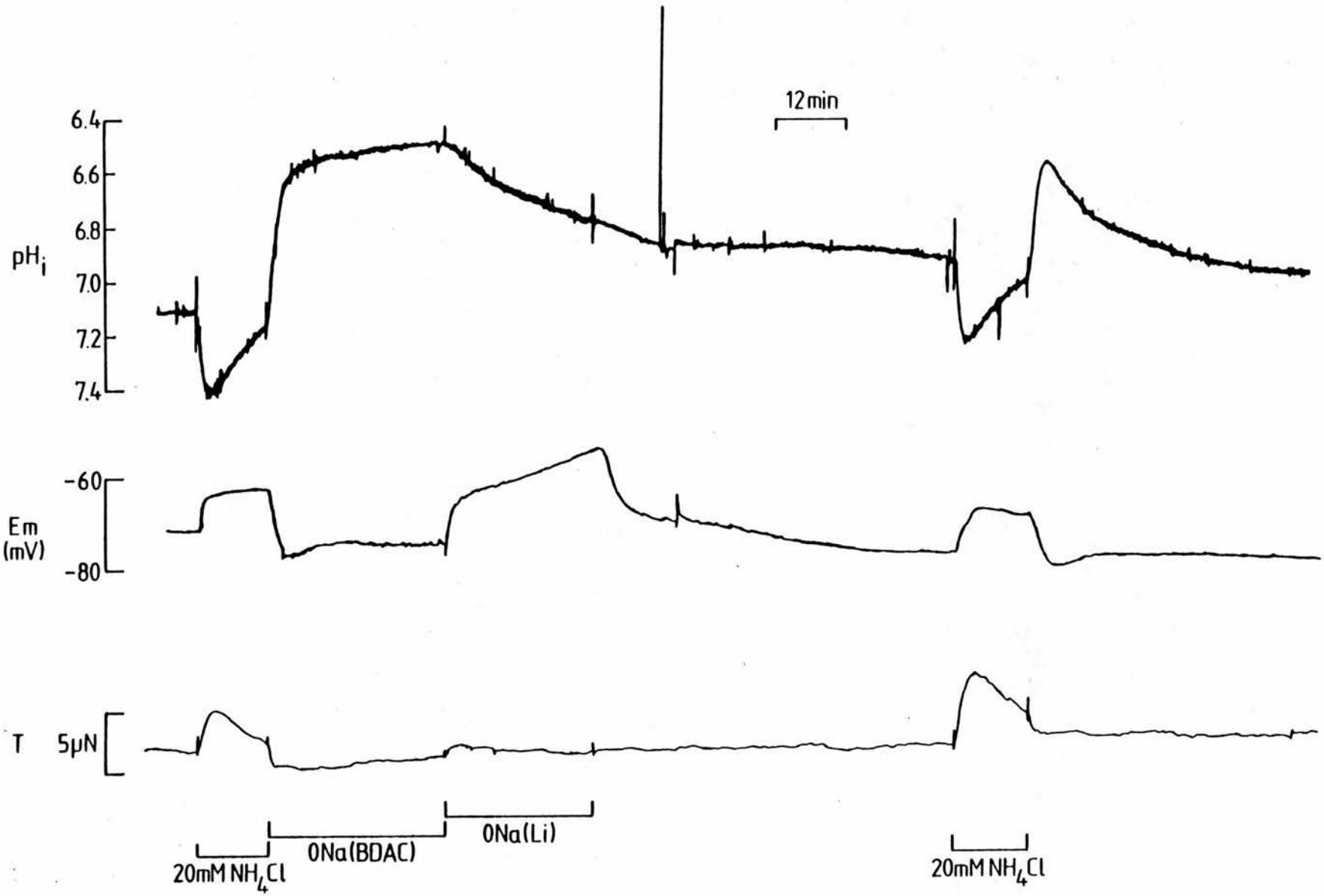
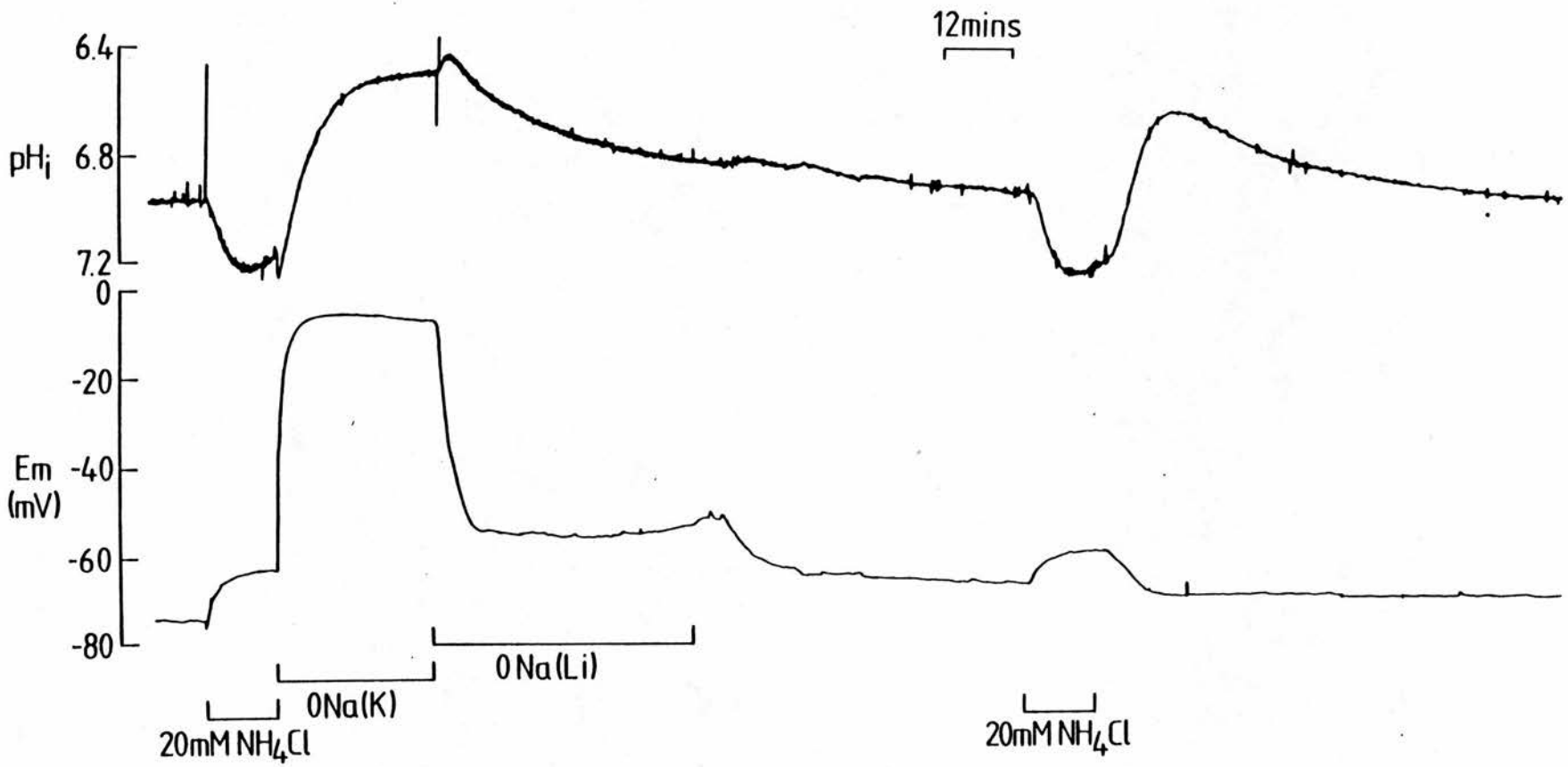

12 min
6.4
6.6
6.8
7.0
7.2
7.4
pH$_i$
-60
-80
Em
(mV)
T
5μN
20mM NH$_4$Cl
0Na(BDAC)
0Na(Li)
20mM NH$_4$Cl

FIGURE 14

The effect of removing all extracellular Na on the pH_i recovery from an acid load induced by exposure of the fibre to 20mM NH_4Cl. On removal on NH_4Cl the fibre was superfused with a Tyrode solution which contained no Na. The Na was substituted by K. After about 30 min. the Tyrode was then changed to one which still lacked Na, but the Na was substituted by Li. The small "bumps" on the pH_i trace on changing to or from the high K solution are probably due to imperfect signal subtraction. The recovery from the acid load in normal Tyrode is shown on the right.


12mins
6.4
pHi 6.8
7.2
0
-20
Em -40
(mV)
-60
-80
0 Na (Li)
0 Na (K)
20mM NH4Cl
20mM NH4Cl

The inhibition of pH_i recovery by removing all the $[Na]_o$ can also occur when the cell is acid loaded by changing the external pH to e.g. 5.4 as in Figure 15. In this example the acid load is carried out in a Na-free solution (TMA substituted). The degree of inhibition of Na-free solution on recovery after this type of acid loading is, however, quite variable between preparations. Only (60%) of preparations showed complete inhibition of recovery in Na-free solution. Figure 16a shows a partial pH_i recovery in Na-free (BDA substituted) Tyrode. It is interesting to note that small, usually transient alkalinizations (which looked like partial recoveries) are also sometimes observed from CO_2 and acid pH-induced acid loads in Na-free solutions (Figure 16a,b). These small transients were initially attributed to slow Na washout from the extracellular space. This may not, however, be the reason because exposure of the fibre to Na-free solution prior to, and during the acid load, still did not completely block the recovery. No small, partial recoveries are observed in Na-free solutions (BDA, TMA, or K substituted) following the NH_4Cl induced acid loading (e.g. see Figures 13,14).

Since recovery from an acid load can be almost completely blocked by removing Na_o, with the proviso that an appropriate Na substitute is used, then simple removal of the Na_o should promote an intracellular acidification because there should be a continuous 'leak' entry of protons into the cells in addition to metabolic production of protons by the cells. An intracellular acidification on Na removal is demonstrated in Figure 17, (and also in Figures 18, 20 and 33). When Na was replaced by BDA pH_i decreased by 0.08 pH unit in 12 minutes. Exposure to Na-free (K or TMA substituted) Tyrode for prolonged periods of time (i.e. > 1 hr.) also produced slow continuous decreases in pH (Figures 18, 20 respectively). Average rates of pH_i decreases for K, BDA and TMA substituted Na-free Tyrode are similar (0.0062 ± 0.0012, 0.0060 ± 0.0007, 0.0060 ± 0.0004 pH units/min. for $n = 8$, 4 and 15 respectively). These rates are also shown in a comparison with Li and the effect of amiloride (see next paragraph) in Figure 19. In all cases when Na was added back pH_i recovered. Even small amounts of Na_o stopped pH_i from going acid. For example, in the experiment shown in Figure 20 only 7mM of Na was required to halt the Na-free induced acidification.

FIGURE 15

The effect of reduction of $[Na]_o$ on pH_i recovery from acid loading (brought about by changing external pH). The top trace shows pH_i, middle trace membrane potential and bottom line pH_o. The bottom bars show when Na was completely removed from the Tyrode (ONa) or was reduced to 7mM (7Na). The Na substitute was TMA.

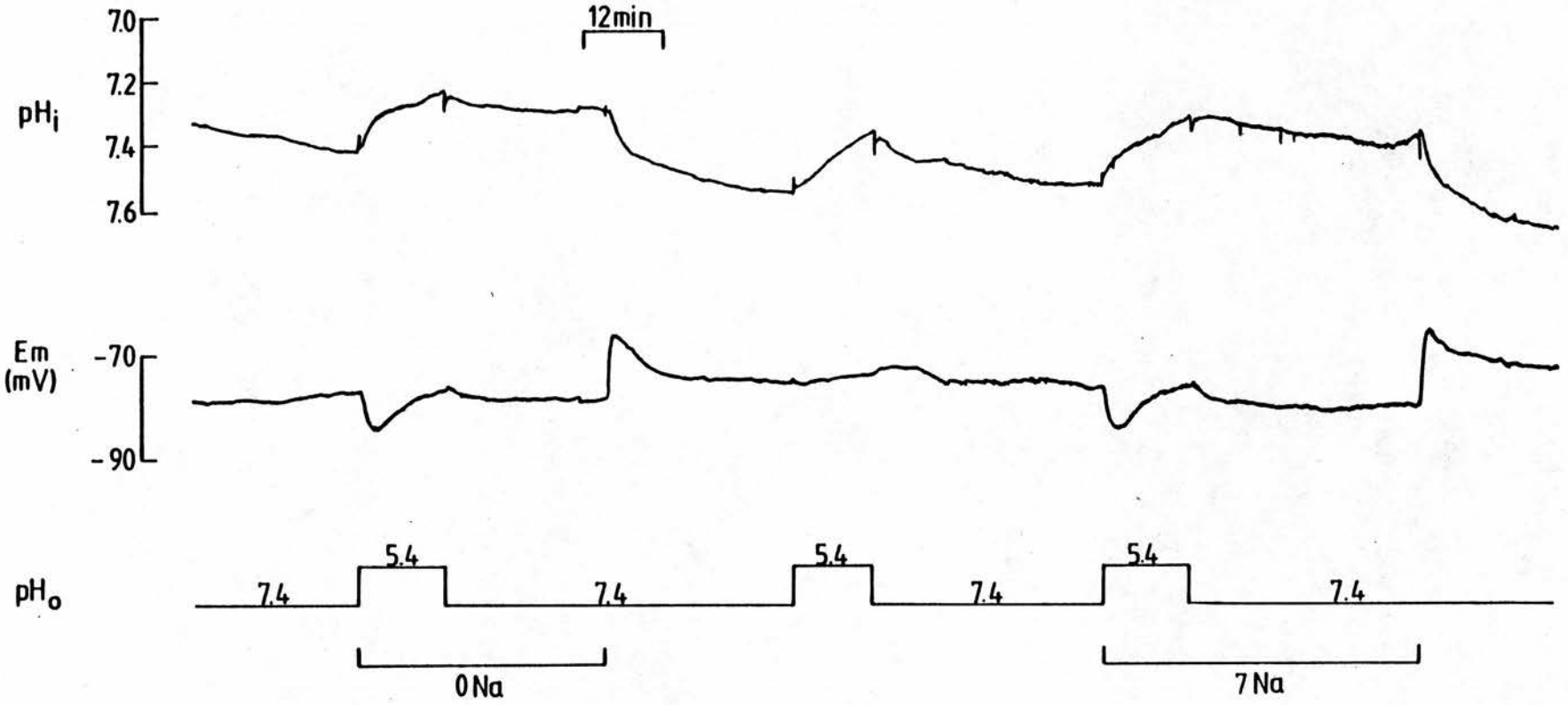

12min
7.0
7.2
7.4
7.6
pH$_i$
Em
(mV)
-70
-90
pH$_o$
7.4
5.4
7.4
5.4
7.4
5.4
7.4
0 Na
7 Na

FIGURE 16

Two examples of the partial recoveries occasionally observed in Na-free solution.

(a) The first intracellular acidification was brought about by changing the pH of the superfusing Tyrode from 8.5 to 5.5. After acid loading the pH of the Tyrode was then changed back to 8.5. Both Tyrode solutions contained 140mM Na. The second acid load was produced by a similar method, but when the Tyrode was changed back to 8.5, Na was also removed and replaced with BDA. The dotted line indicates a break in the recording of about 30 min. During this time a leak in the perfusing system beyond the preparation which gave rise to a "noisy" pH_i trace was corrected.

(b) Both acid loads were brought about by CO_2-containing solutions. The first CO_2 solution was buffered with 20mM Na HCO_3, and the second CO_2 solution by 20mM $KHCO_3$. The second CO_2 solution was also Na-free, the Na being replaced by TMA.

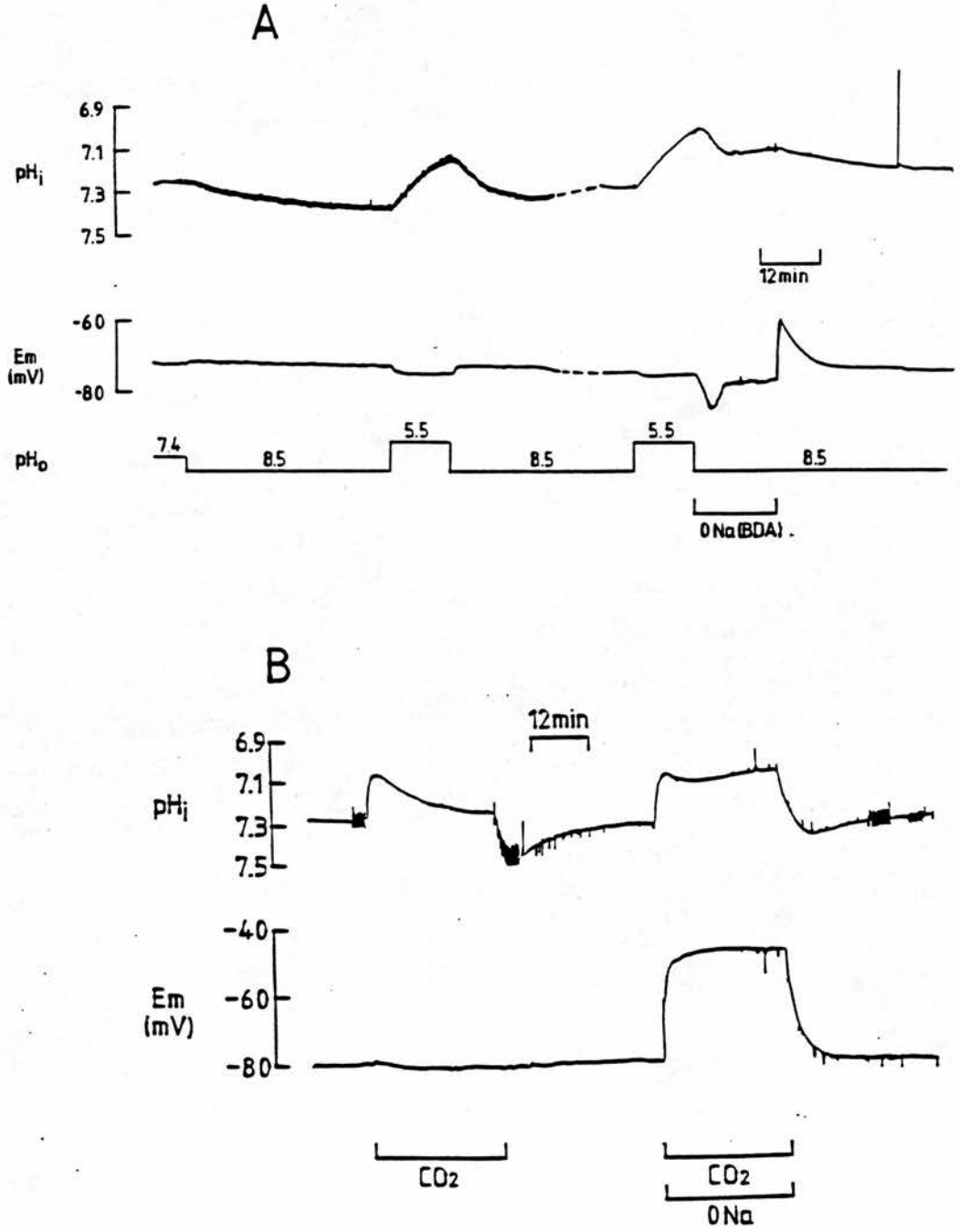

A
6.9
7.1
7.3
7.5
pHi
12min
-60
-80
Em
(mV)
pHo
7.4
8.5
5.5
8.5
5.5
8.5
0 Na (BDA)
B
12min
6.9
7.1
7.3
7.5
pHi
-40
-60
-80
Em
(mV)
CO2
CO2
0 Na

FIGURE 17

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

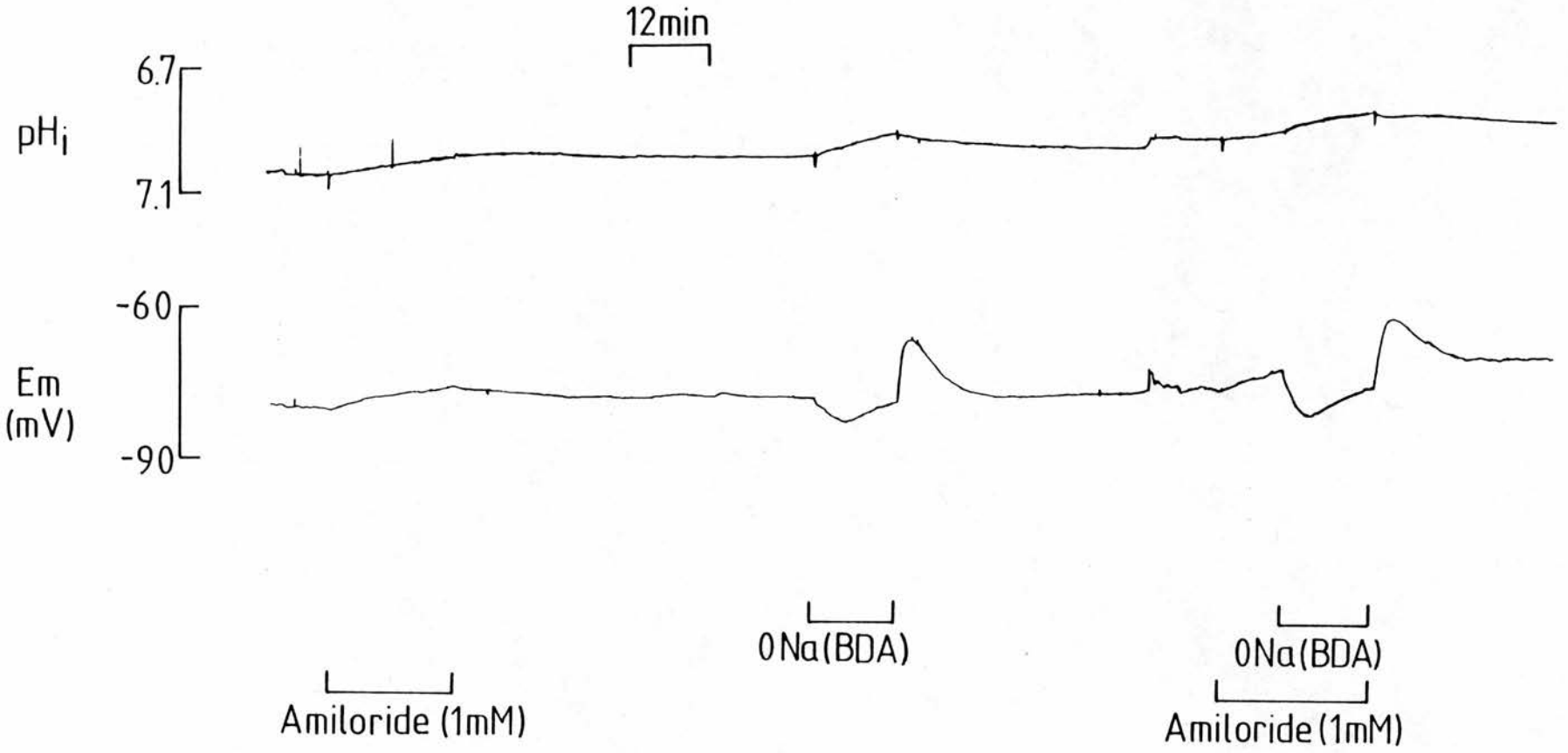

12min
6.7
pHi
7.1
-60
Em
(mV)
-90
0Na(BDA)
0Na(BDA)
Amiloride (1mM)
Amiloride (1mM)

FIGURE 18

The effect on pH_i of a prolonged exposure (ca. 1 hr.) to Na-free solution. The Na substitute was K.

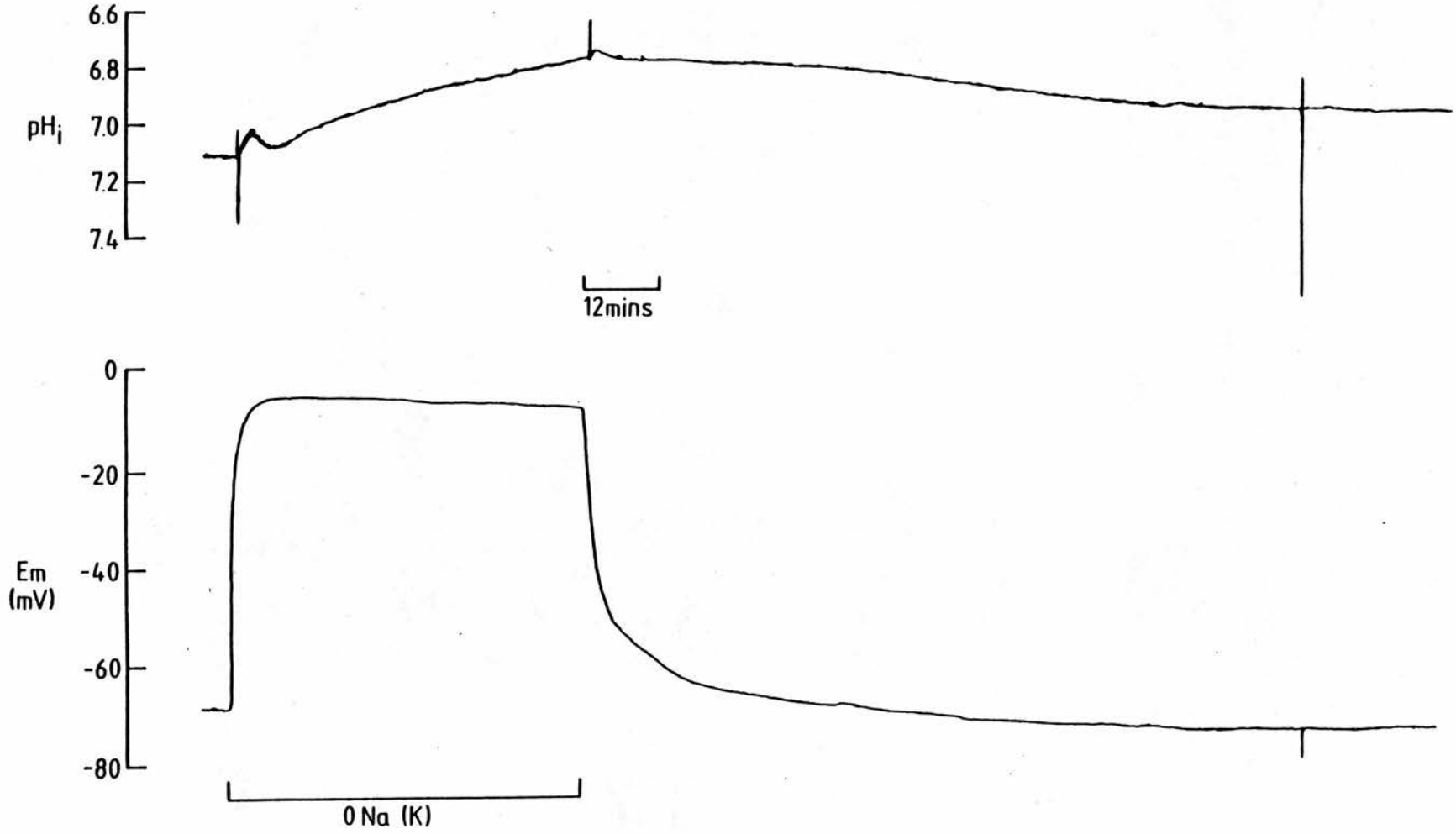

6.6
6.8
pH$_i$ 7.0
7.2
7.4
12mins
0
-20
Em
(mV)
-40
-60
-80
0 Na (K)

FIGURE 19

The relative rates of intracellular acidification induced by Na-free Tyrode solutions. The Na substitutes are respectively K, TMA, BDA and Li. Also shown for comparison is the rate of acidification observed in normal Tyrode with 1mM amiloride added. The respective number of experiments are shown above the columns. The error bars represent the S.E.M.

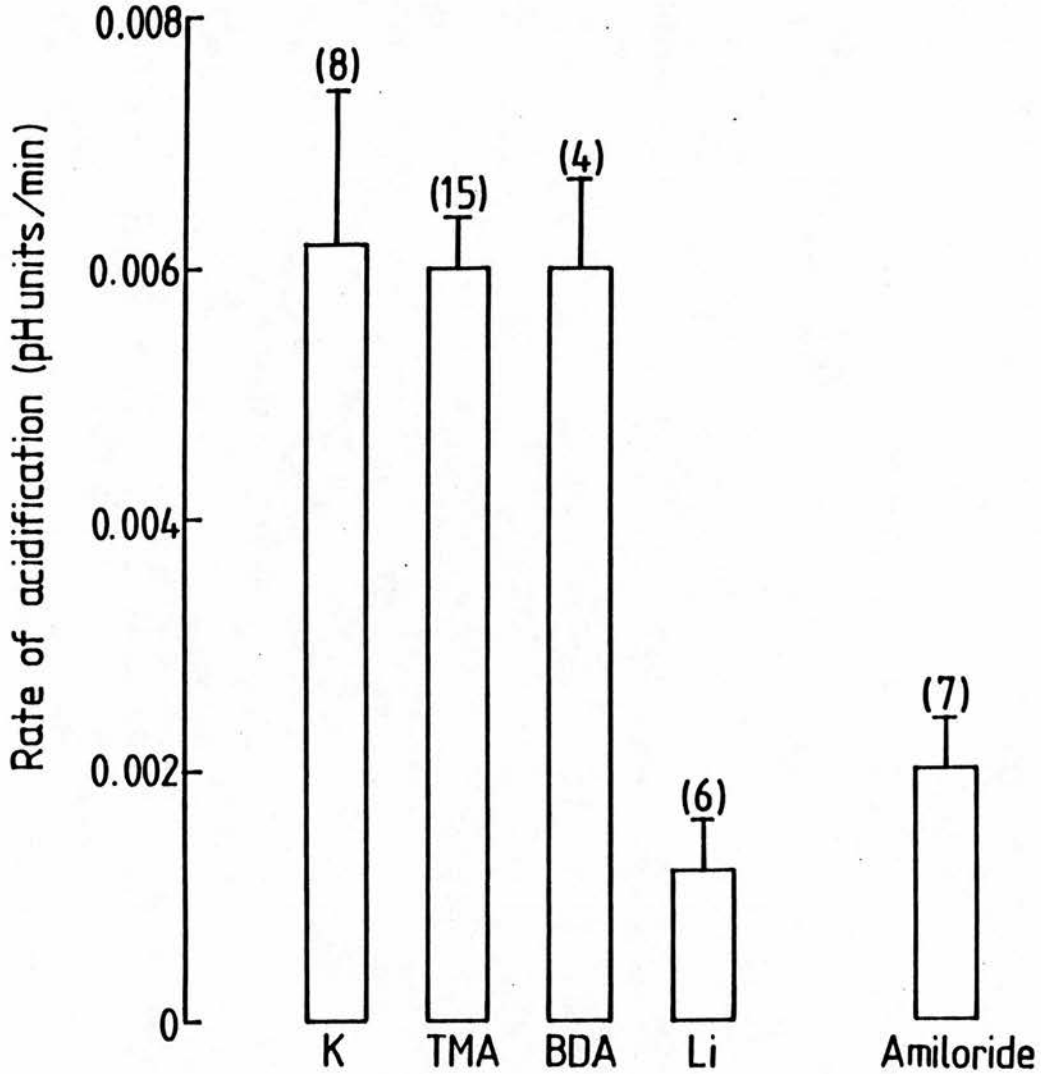
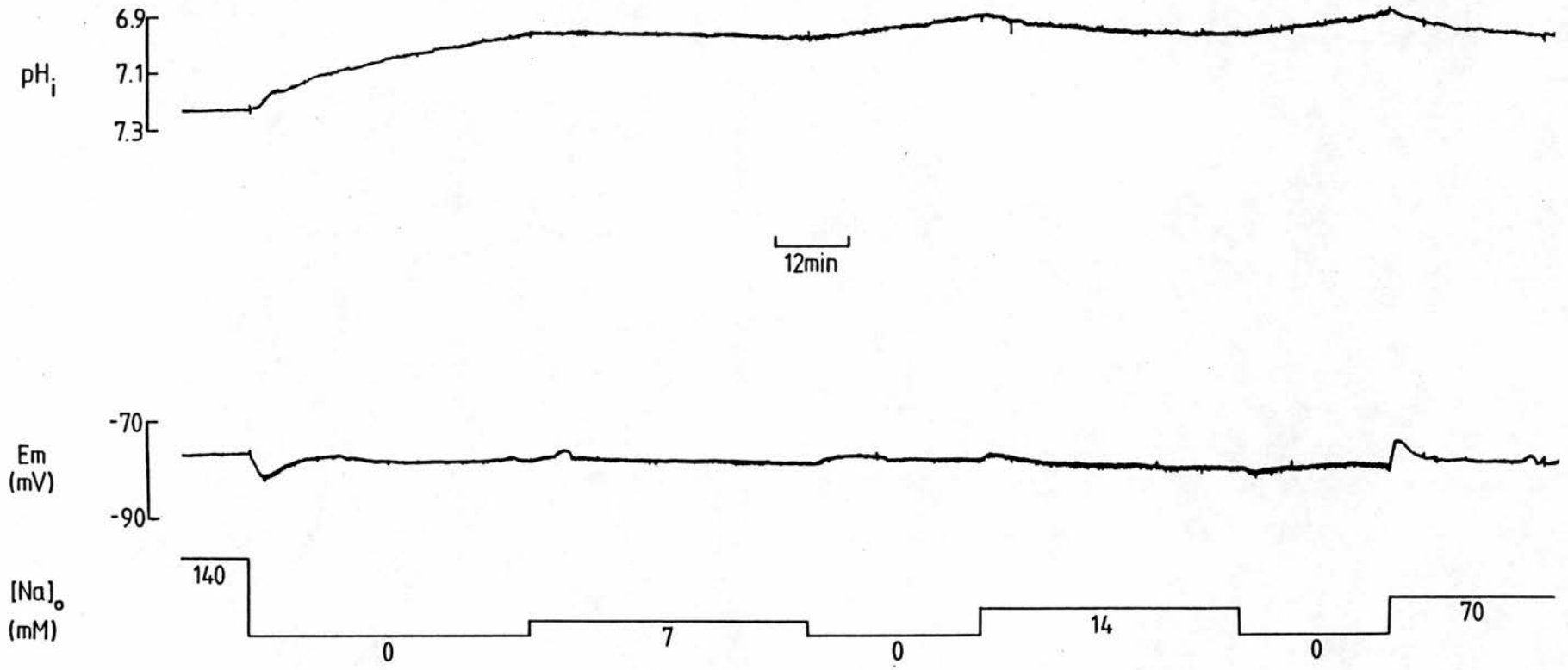

Rate of acidification (pH units/min)
0.008
0.006
0.004
0.002
0
(8)
(15)
(4)
(6)
(7)
K
TMA
BDA
Li
Amiloride

FIGURE 20

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


6.9
7.1
7.3
pH$_i$
12min
-70
-90
Em
(mV)
140
[Na]$_o$
(mM)
0
7
0
14
0
70

Removal of Na from the superfusing Tyrode also produces an intracellular acidification in ventricular muscle. Several experiments have been carried out using ferret and guinea pig papillary muscle. Figure 21 shows a comparison of the effects of substitution of Li, K and BDA for Na in ferret ventricle. In this tissue removal of Na from the bathing Tyrode also caused intracellular acidifications. Substitution of Na by Li causes less of an acidification than when Na is substituted by K or BDA. This is consistent with the results from Purkinje fibres if Li can substitute for Na on a Na/H exchange.

Exposure of the fibre to 1mM amiloride (a putative Na/H exchange inhibitor) also decreases pH_i but more slowly than removal of all Na_o. Amiloride has been used as a Na transport inhibitor in various tissues and clinically as a K sparing diuretic (see Bull & Laragh, 1968). Two general Na transport mechanisms appear to be inhibited by the drug. Amiloride can inhibit Na transport across epithelia (e.g. Bentley, 1968) and the Na/H transport pathway in *Necturus* bladder (Weinman & Reuss, 1982), sea urchin eggs (Johnson et al, 1976), soleus muscle (Aickin & Thomas, 1977b)and salamander proximal tubule (Boron & Boulpaep, 1983a). The observed rate of pH_i fall with 1mM amiloride in sheep heart Purkinje fibres was about 33% of that observed in Na-free solution. (Rate of acidification in normal Tyrode + 1mM amiloride = 0.0020 ± 0.0004 pH units/min., n = 7). No additive effect of 1mM amiloride and Na-free (BDA or TMA substituted Tyrode) could be detected (Figure 17). Amiloride could slow but not completely inhibit recovery from an acid load by CO_2-exposure (Figure 22) and has already been demonstrated to have similar effects on recovery from NH_4Cl induced acidifications. (Deitmer & Ellis, 1980; Vaughan-Jones, 1982a).

In the present experiments HEPES was normally used as the pH buffer. These Tyrode solutions were normally CO_2 and HCO_3^--free. It is possible that exposure to CO_2/HCO_3^- buffered Tyrode could allow a HCO_3^-/Cl^- transport mechanism to assist pH_i regulation. Such a Cl^-/HCO_3^- exchange mechanism appears to aid pH_i regulation in e.g. snail neurone (Thomas, 1977) and mouse soleus muscle (Aickin & Thomas, 1977b). No evidence of an effect of the stilbene derivative, SITS, an inhibitor of Cl^-/HCO_3^- exchange (e.g. Knauf & Rothstein, 1971) on

FIGURE 21

The effect on the pH_i of ferret ventricular muscle of removing extracellular Na. The bars indicate when Na was removed from the superfusing Tyrode solution and the Na substitutes used during these times are also shown. The first upward pointing arrow indicates when the pH-sensitive microelectrode started to come out of the cell, and the second upward arrow indicates when it was put back into another cell. The downward pointing arrow indicates when the conventional microelectrode started to come out of the cell. At this point the pH_i trace moves an equal but opposite amount because the full membrane potential signal is no longer being subtracted.

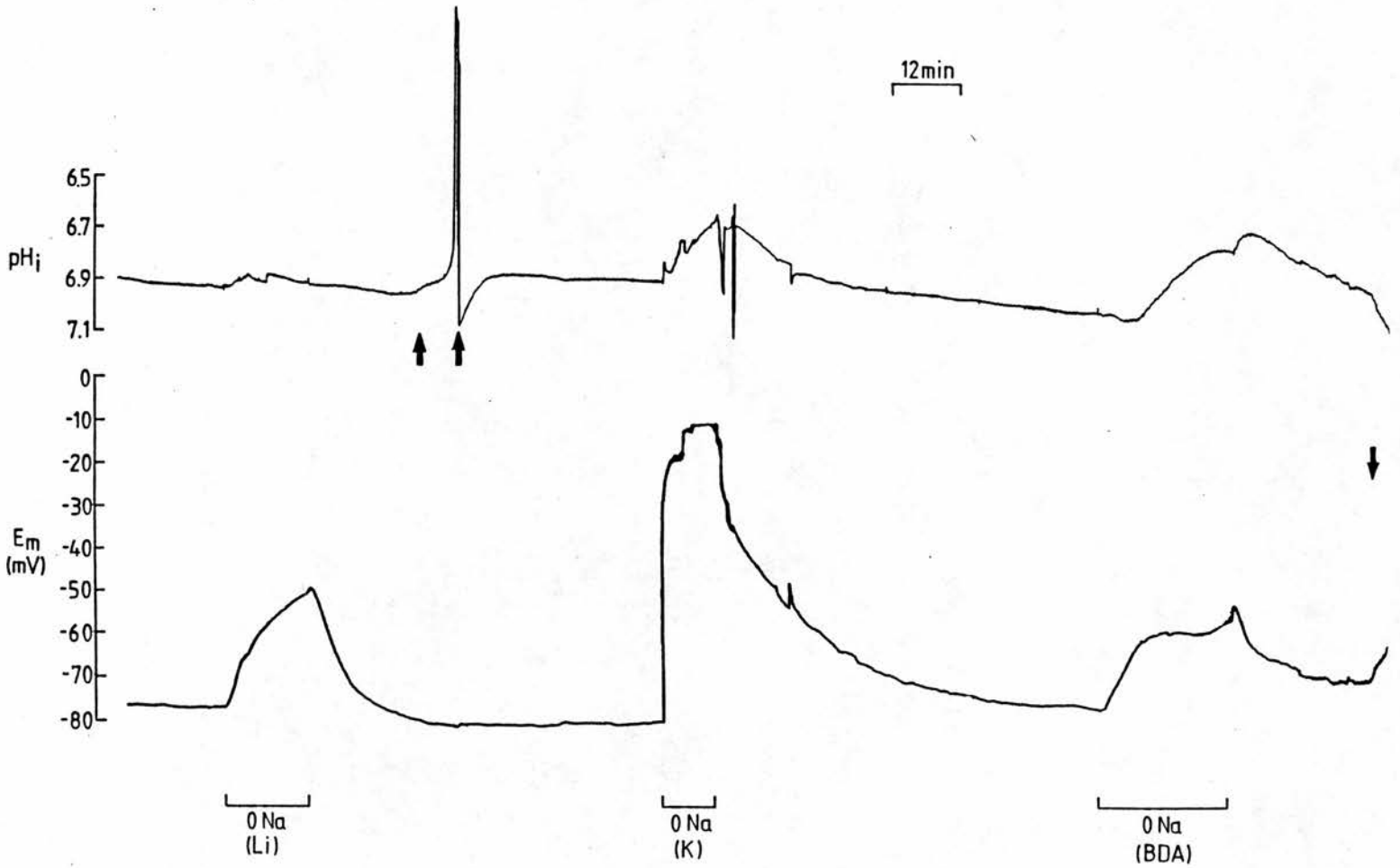
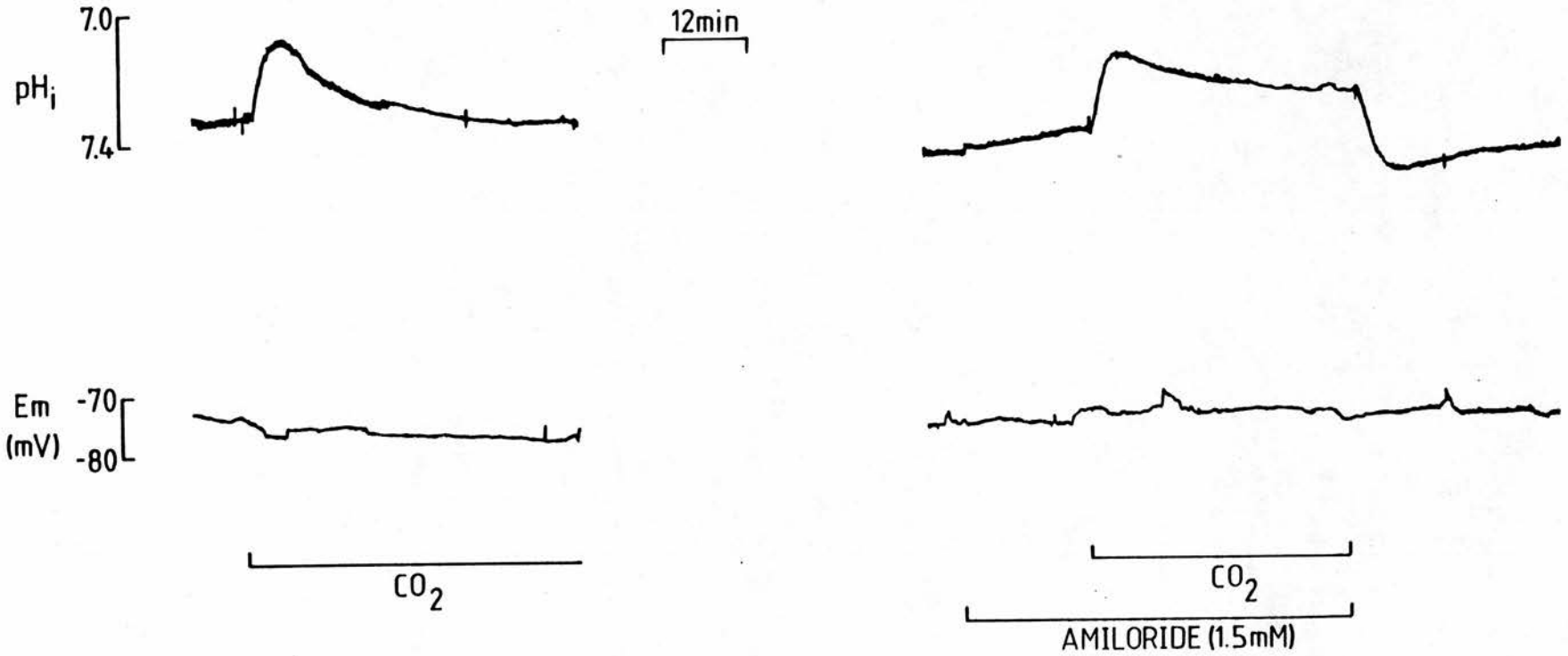

12min
pHi
6.5
6.7
6.9
7.1
Em
(mV)
0
-10
-20
-30
-40
-50
-60
-70
-80
0 Na
(Li)
0 Na
(K)
0 Na
(BDA)

FIGURE 22

The effect of amiloride (1.5mM) on pH_i recovery from a CO_2 (5%) induced acid load. The break in the recording was for a period of about 1 hour.


7.0
pH$_i$
7.4
12min
Em
(mV)
-70
-80
CO$_2$
CO$_2$
AMILORIDE (1.5mM)

the recovery from a CO_2 induced acid load could be found in these experiments.

The experiments described in this Section show that Na_o is required for pH_i recovery from an acid load and point to the existence of a Na/H exchange. The Na concentration dependence of pH_i recovery is investigated in Section 4. pH_i recovery from an acid load does not seem to depend on a Cl^-/HCO_3^- exchange mechanism as is the case for some other types of cell.

Section 3 - TEMPERATURE EFFECTS

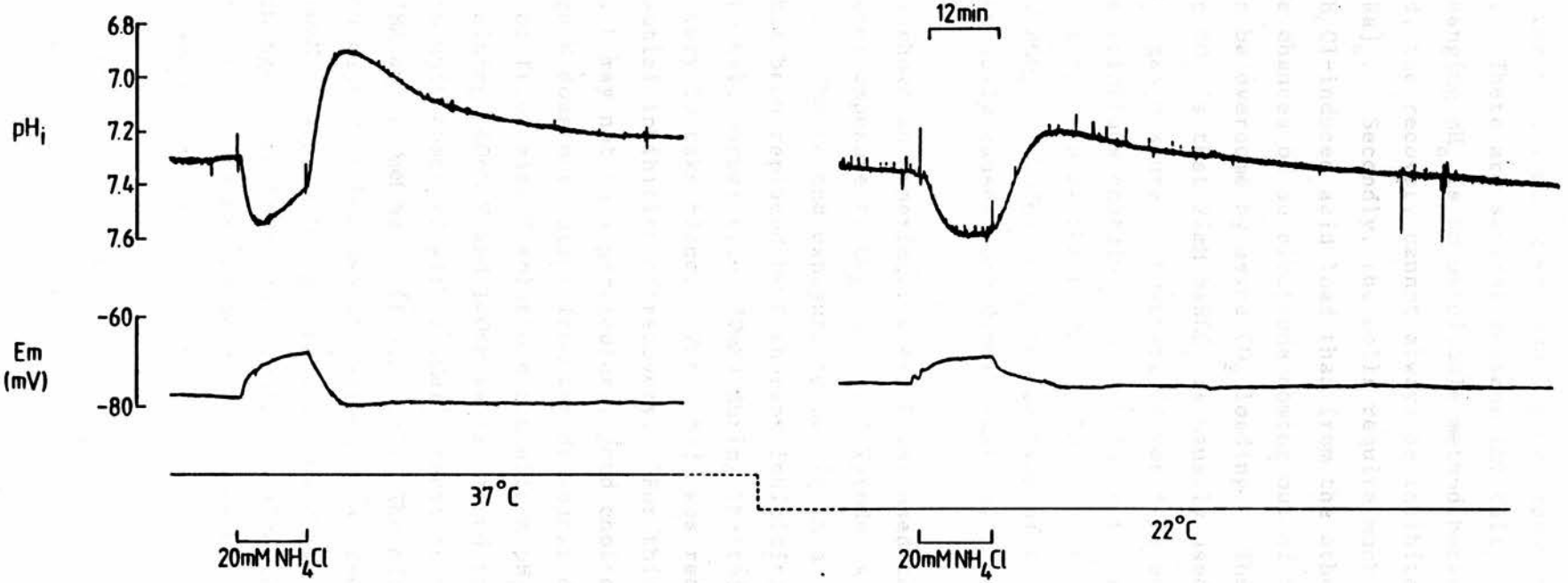
Figure 23 shows part of an experiment illustrating the effects of temperature on recovery from an acid loading which was induced by exposure to NH_4Cl. This form of acid loading was used in these experiments because it could be easily repeated at low temperature. CO_2-exposure would have required a different amount of HCO_3^- to be present at low temperature in order to keep the pH_o constant. Decreasing temperature from 37° to 22°C produces a decrease in the rate of recovery of pH_i by an average of 60% and an increase in steady-state pH_i by an average of 0.21 ± 0.06 units (mean ± S.E. of 4 experiments). These results are similar to those of Aickin & Thomas (1977b). From 4 experiments an average Q_{10} for pH_i recovery over a temperature range of 21–35°C was calculated to be 2.65. This is higher than the Q_{10} for the component of pH_i regulation due to Na/H exchange in mouse skeletal muscle (Aickin & Thomas, 1977b) which was calculated to be 1.4. At decreased (i.e. room) temperature acid loading is not as great. The recovery from alkalinization during the NH_4Cl exposure was also slowed and this is further discussed in Section 7. In this connection it is interesting to note that the depolarization presumably associated with NH_4^+ influx, is less.

Section 4 - Na DEPENDENCE OF pH_i RECOVERY

In Section 2 it was shown that removal of Na_o inhibited pH_i recovery from acid loading and this is consistent with a role of a sarcolemmal Na/H exchange in pH_i recovery. In this Section the $[Na]_o$ dependence of pH_i control is more closely examined.

FIGURE 23

The effect of temperature on pH_i recovery from an NH_4Cl induced acid load. After the recovery at 37°C the heating unit was switched off and the Tyrode was changed to one which had a pH of 7.4 at 22°C. The break in the trace was for approximately 2 hours.


12min
6.8
7.0
7.2
7.4
7.6
pH$_i$
-60
-80
Em
(mV)
37°C
20mM NH$_4$Cl
22°C
20mM NH$_4$Cl

In this series of experiments the cells were acid loaded and the subsequent pH_i recovery studied in solutions of varying [Na]. In these experiments the acid loading was usually by exposure to CO_2-containing solutions. There are several reasons for this. Firstly, acid loading by changing pH_o is an unreliable method because, as already demonstrated, the recovery cannot always be inhibited completely by removing all $[Na]_o$. Secondly, the cells require much longer to recover from an NH_4Cl-induced acid load than from the other two types. This increases the chances of an electrode coming out of the cell. These problems can be overcome by using CO_2 loading. The main disadvantage in using CO_2 is that 24mM $NaHCO_3$ is usually used to buffer the 5% CO_2 (95% O_2) gas mixture. However, it was frequently necessary to use Tyrode solutions containing < 24mM Na in these experiments so $NaHCO_3^-$ could not be used as the buffer. The most readily available other form of HCO_3^- is $KHCO_3$, but the addition of the extra K to these solutions obviously causes some depolarization.

Figure 24 shows an experiment where K was used to replace Na entirely. A control exposure to CO_2 in normal Tyrode is shown on the left of the figure. The second exposure to CO_2 is in a solution where all the Na has been replaced by K thereby inhibiting recovery. Decreasing $[Na]_o$ to half normal (i.e. 70mM) during the third CO_2 exposure allows recovery to take place. When $[Na]_o$ was reduced to 7mM there is a substantial inhibition of recovery. For this type of experiment, however, K may not be a particularly good choice of Na substitute. Although K does not suffer from the drawbacks described previously for Li or Tris, high K solutions can affect pH_i. Hypertonic Tyrode, containing 140mM K and 140mM Na is found to decrease pH_i by up to 0.1 pH unit compared with a small transient pH_i change induced by 140mM TMA and 140mM Na (Figure 25). The effect appears to be concentration dependent because it is found that reducing the $[K]_o$ from 140 to 70mM (again in the presence of 140mM Na) produces a smaller acid pH_i change. De Hemptinne (1981) has also found that depolarization with high K induces an acid pH_i change which he suggested was due to release of Ca_i (see Section 5).

It is interesting that in this type of experiment (Figure 24) pH_i tends to return to a more acid baseline as Na is progressively

FIGURE 24

The effect of lowering the $[Na]_o$ on pH_i recovery from acid loading induced by exposure to CO_2-containing solutions. Top trace, pH_i; lower trace, membrane potential (E_m). The variations of $[Na]_o$ throughout the experiment are shown on the lowest line. When Na was removed it was replaced by K giving rise to the large depolarizations. The CO_2 bars show when the superfusing Tyrode was changed from one which was HEPES buffered (equilibrated with 100% O_2) to one which was HCO_3 buffered (equilibrated with 95% O_2, 5% CO_2).

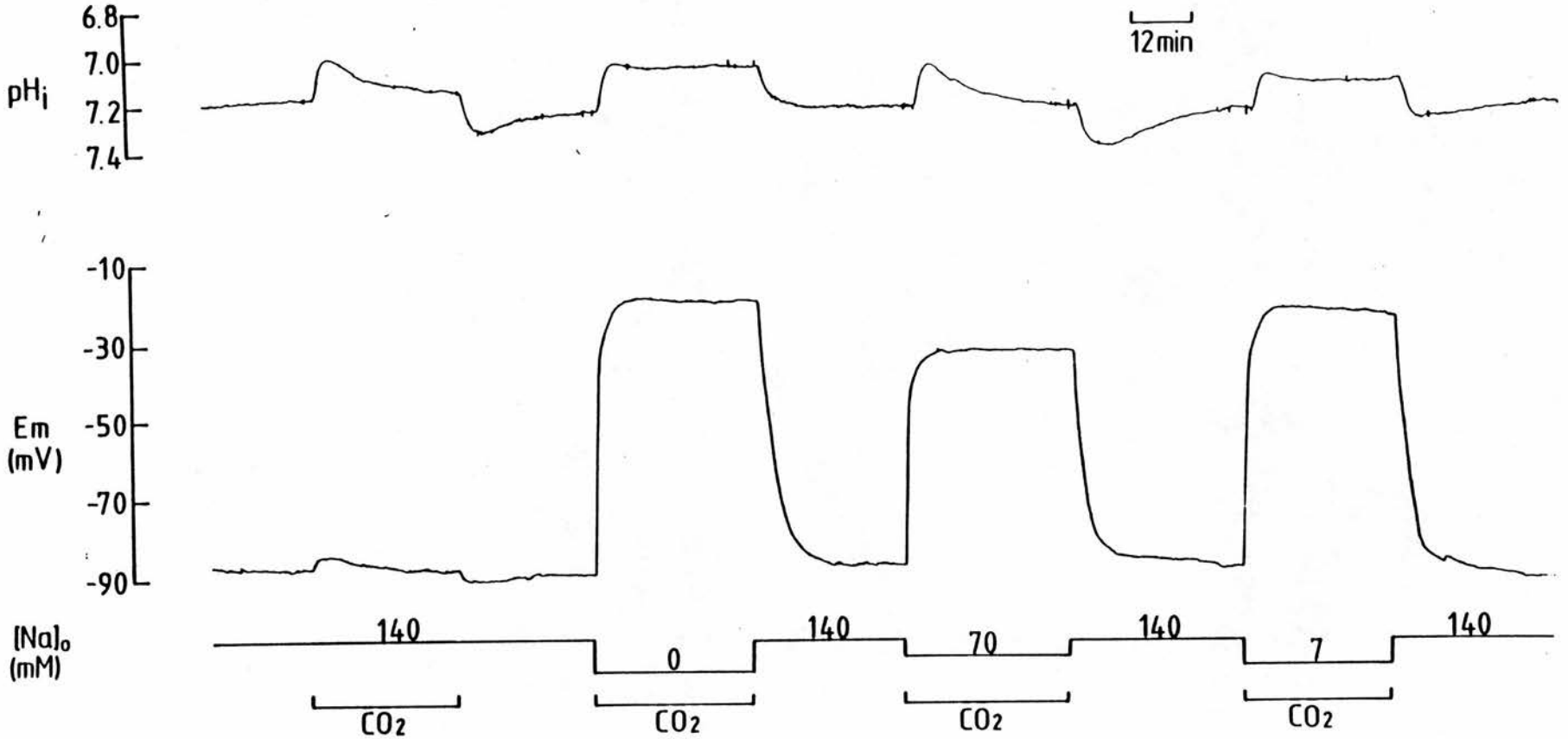
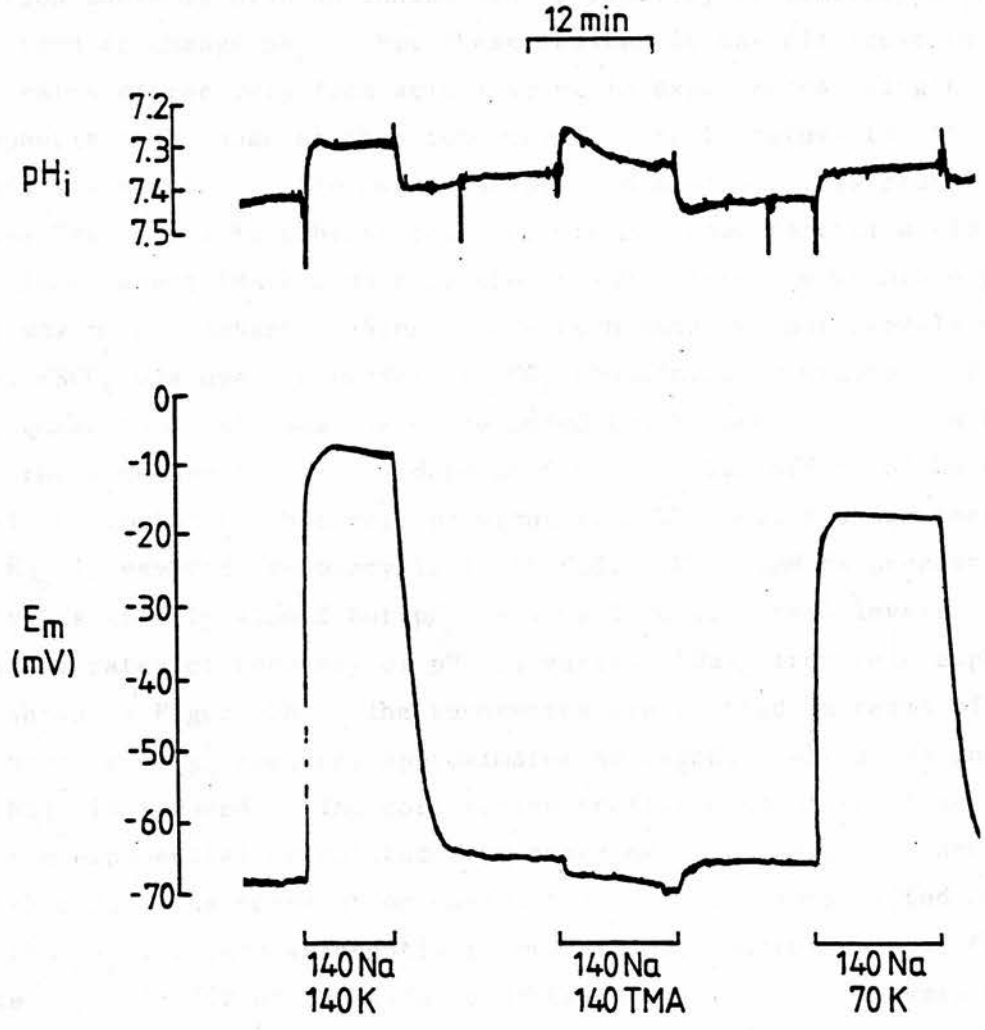

6.8
7.0
7.2
7.4
pHi
12min
-10
-30
-50
-70
-90
Em
(mV)
[Na]o
(mM)
140
0
140
70
140
7
140
CO2
CO2
CO2
CO2

FIGURE 25

The effect of depolarization on pH_i. At the first bar the normal Tyrode was changed to a hypertonic solution with 140mM K. (i.e. normal Tyrode + 140mM K). During the time period denoted by the second bar the Tyrode was again made hypertonic, but with 140mM TMA added. The third bar indicates the time during which the preparation was exposed to normal Tyrode + 70mM K.


12 min
pH$_i$
7.2
7.3
7.4
7.5
E$_m$
(mV)
0
-10
-20
-30
-40
-50
-60
-70
140 Na
140 K
140 Na
140 TMA
140 Na
70 K

reduced and replaced by K. This tendency has been further investigated and is illustrated in Figure 26. In this Figure the change in steady-state pH_i (ΔpH_i^{∞}) has been measured as illustrated in the inset and plotted against the $[Na]_o$. Note that the steady-state pH_i returns to a more acid level in CO_2 solutions even in normal $[Na]_o$ (see Section 1). When $[Na]_o$ is lowered to very low levels i.e. < 20mM ΔpH_i^{∞} starts to change appreciably. This appears to be related to the progressively larger accompanying depolarizations which, as already noted, are associated with an intracellular acidification. In addition there is also an inhibition of recovery by removing Na which will tend to change pH_i. For these reasons it was difficult to measure rates of recovery from acid loading in experiments using K as the Na substitute because of this tendency for pH_i to return to a new steady-state level. The method adopted to overcome this problem was to use TMA as the Na substitute. Figure 27 shows part of a similar experiment where TMA was used as the Na substitute and membrane potential was more constant. 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 Na removal is similar to that seen previously. The figure shows that when all Na_o is removed, recovery is inhibited. With 7mM Na present, recovery is greatly slowed but pH_i returns to near normal levels. The relative rates of recovery of pH_i in various $[Na]_o$ from this experiment are shown in Figure 28. The recoveries are plotted in terms of a_H^i. The process of a_H^i recovery approximates an exponential and is inhibited as $[Na]_o$ is reduced. The correlation coefficients of the lines fitting the exponential calculated from experiments of this kind are given in Table 3. The correlation coefficients of the lines fitted assuming the pH_i recovers exponentially are given in parenthesis. On the whole a better fit of the data is achieved assuming a_H^i recovers exponentially. In the experiment shown the rate of recovery in 7mM Na is 58% of that in 122mM Na. Figure 29 shows the combined results from a group of similar experiments. The rates of recovery (expressed as a percentage of that in normal $[Na]_o$, which in these experiments was 120 - 124mM Na) plotted against $[Na]_o$. In five experiments a 50% inhibition of the rate of recovery was produced by lowering the $[Na]_o$ to ~7mM. The curve drawn through the points is a rectangular

FIGURE 26

The effect of decreasing $[Na]_o$ on steady-state pH (pH_i^{∞}). These plotted points are means of at least 5 experiments where K was used as the Na substitute. The error bars indicate the S.E.M. Only 2 experiments were carried out with 70mM Na. These two points are thus plotted separately.

Steady-state pH_i was calculated as shown in the inset.

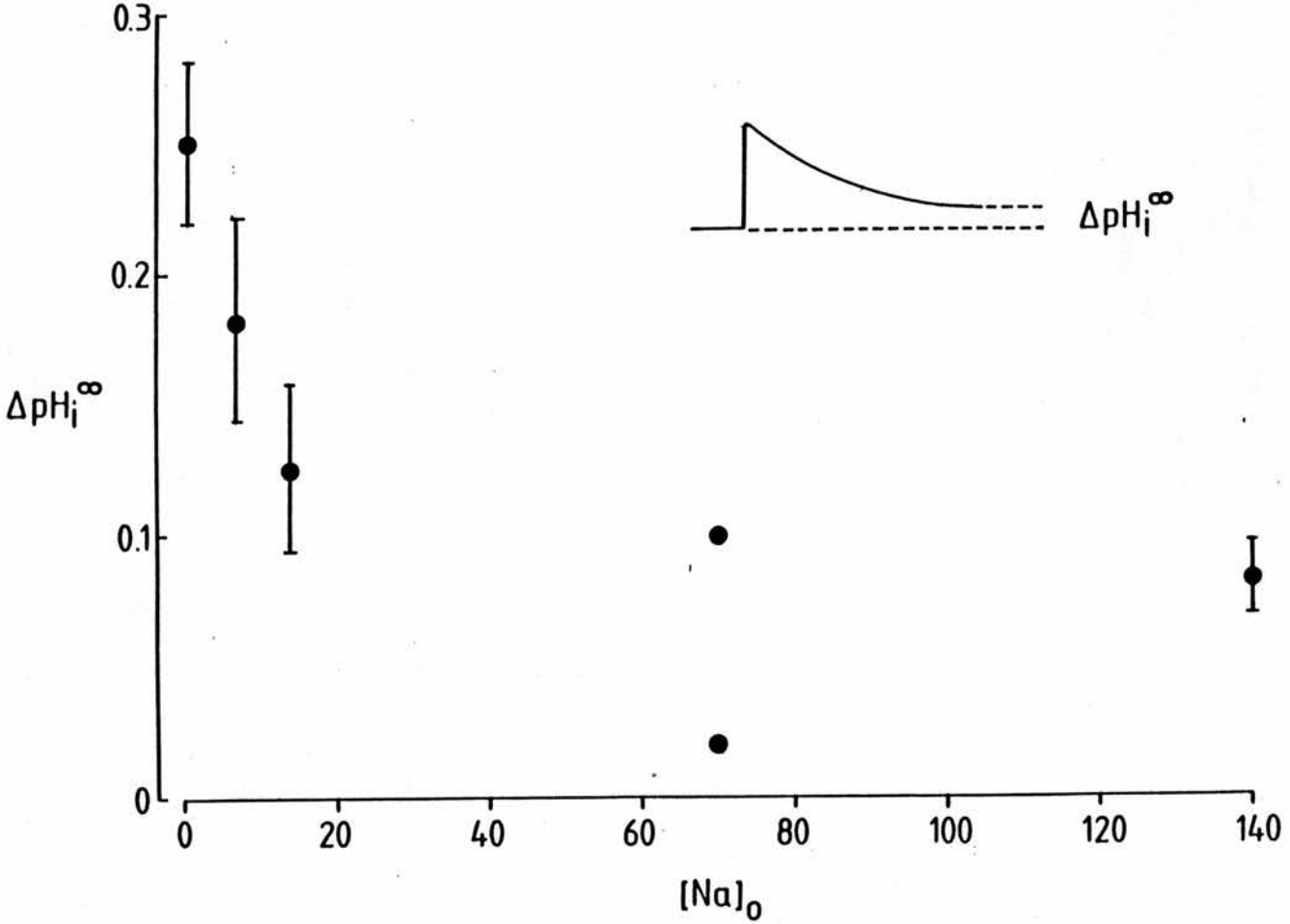

0.3
0.2
0.1
0
ΔpH_i^∞
0
20
40
60
80
100
120
140
$[Na]_o$
ΔpH_i^∞

FIGURE 27

The effect of lowering the $[Na]_o$ on pH_i recovery from acid loading induced by exposure to CO_2-containing solutions. When the $[Na]_o$ was reduced it was replaced by TMA. Approximately 24mM KCO_3 was used to buffer the CO_2-containing solutions. The same quantity of KCl 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 hrs. during which time a similar pH_i recovery was measured in 70mM Na.

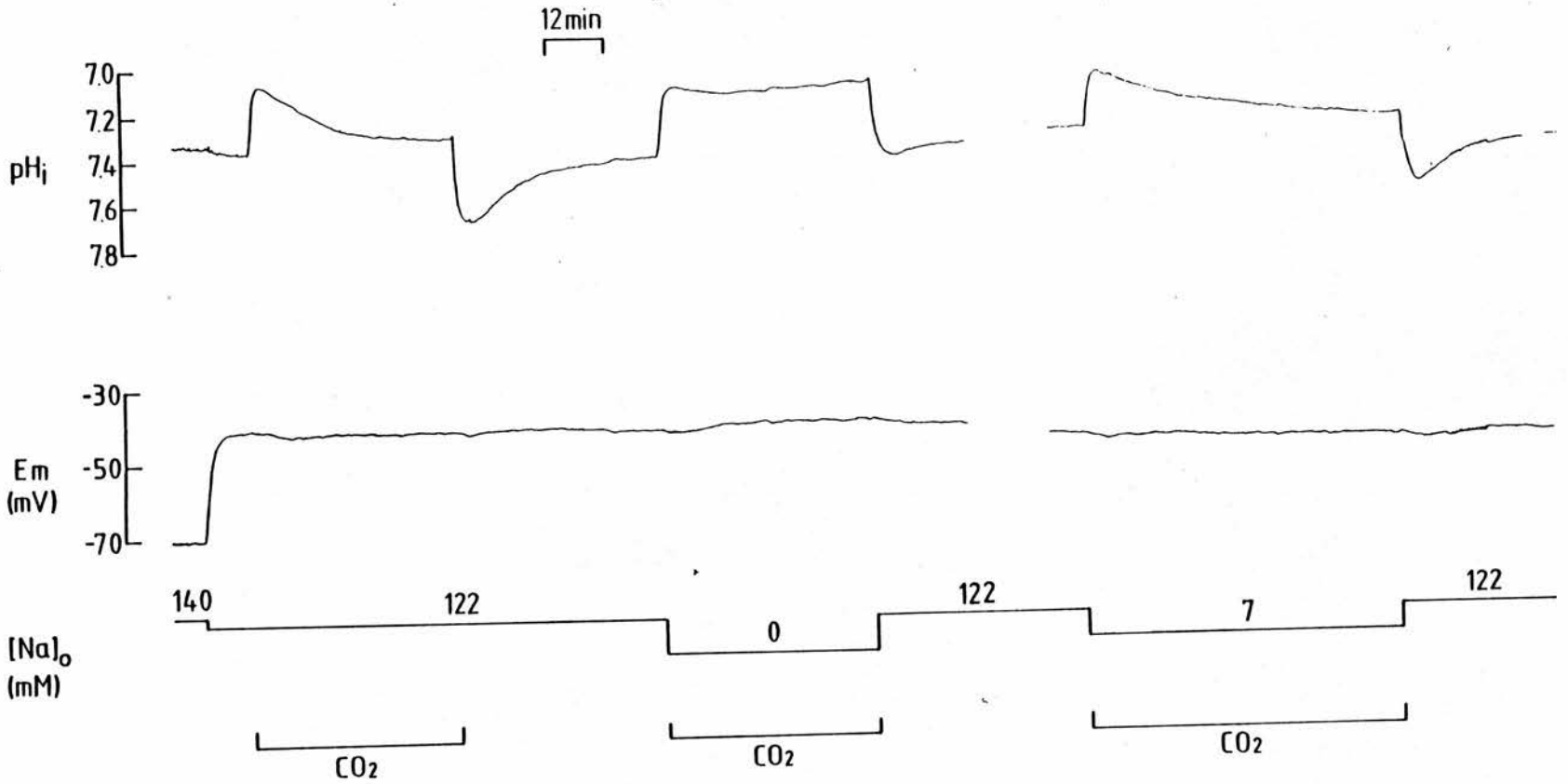

12 min
7.0
7.2
7.4
7.6
7.8
pHi
-30
-50
-70
Em
(mV)
140
122
0
122
7
122
[Na]o
(mM)
CO2
CO2
CO2

FIGURE 28

The rate of a_H^i recovery back to its baseline value in solutions of varying $[Na]_o$, following acid loading with CO_2-containing solutions. The data points were taken from the experiment in Figure 27 The lines drawn through the points were fitted by regression analysis. The initial points of each line should all lie at zero minutes but have been offset for clarity.

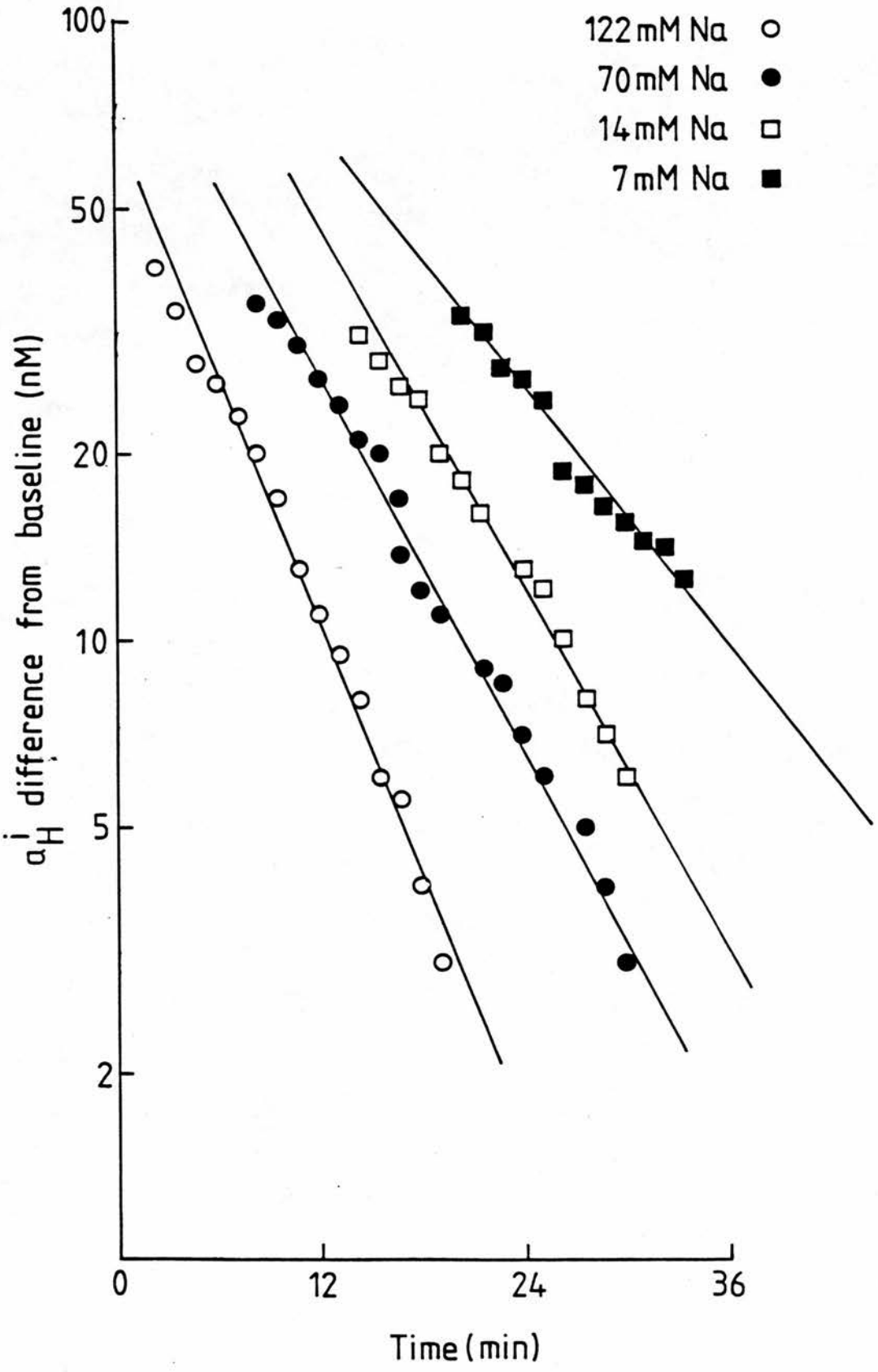

122 mM Na
70 mM Na
14 mM Na
7 mM Na
100
50
20
10
5
2
0
12
24
36
Time (min)
a_H^i difference from baseline (nM)

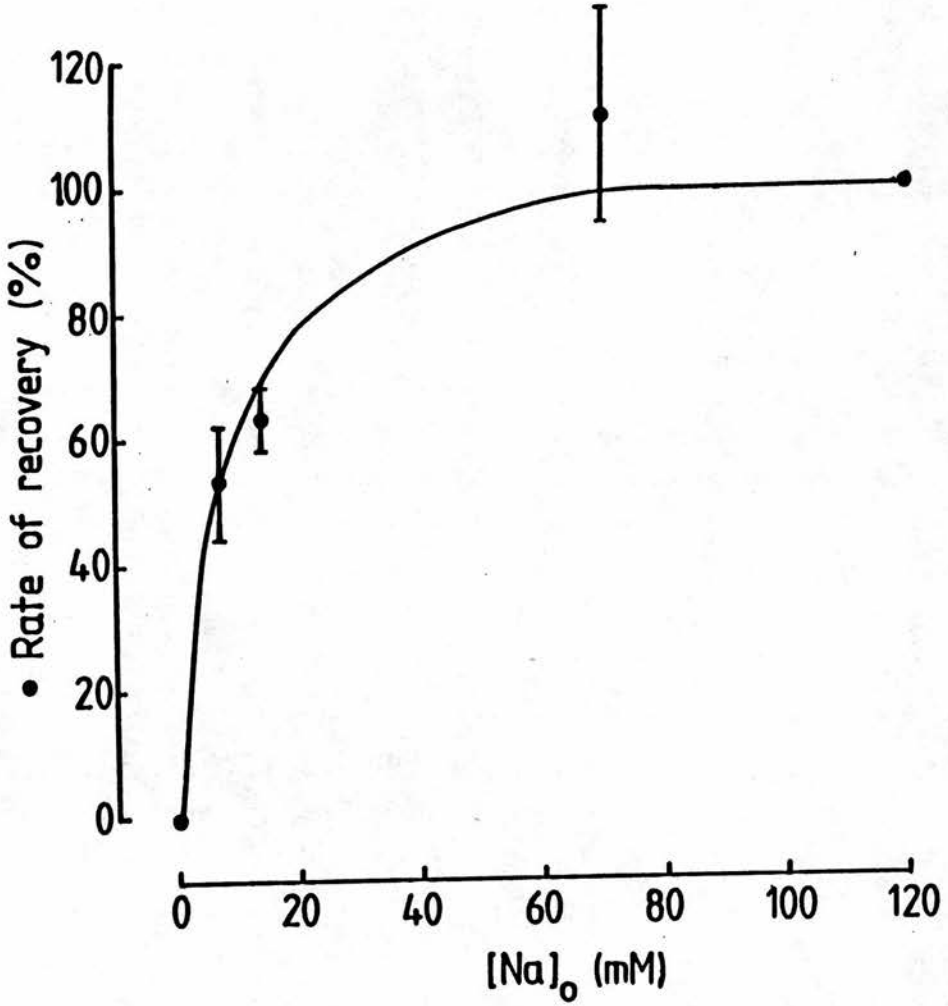
TABLE 3

CORRELATION COEFFICIENTS

EXPERIMENT DATE	$[Na]_o$ (mM) 120	70	14	7
26/10/82	0.991 (0.977)	0.995 (0.992)	0.992 (0.974)	0.990 (0.992)
10/ 1/83	0.983 (0.984)	0.977 (0.970)	0.988 (0.988)	-
27/ 2/83	0.996 (0.995)	-	0.991 (0.997)	0.997 (0.995)
7/ 3/83	0.994 (0.995)	0.992 (0.982)	0.988 (0.986)	0.992 (0.992)
28/ 3/83	0.990 (0.987)	0.989 (0.994)	0.993 (0.993)	0.980 (0.962)
5/ 4/83	0.995 (0.984)	0.988 (0.994)	-	0.981 (0.981)

FIGURE 29

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 120mM $[Na]_o$. The line through the points is a rectangular hyperbola drawn according to equation (see text).


120
100
80
60
40
20
0
• Rate of recovery (%)
0
20
40
60
80
100
120
[Na]o (mM)

hyperbola corresponding to the equation:-

$$\text{Rate of recovery (r) (as \% of normal)} = \frac{1}{\frac{a}{[Na]_o} + b} \qquad \text{——— 1}$$

where a and b are constants with values of 0.073mM, and 0.0091 respectively. The equation is derived from the Michaelis plot of $1/r$ against $1/[Na]_o$. The correlation coefficient (r) of the line drawn through the points of this graph was 0.974.

The other method of measuring the recoveries (i.e. assuming pH_i recovery to be exponential) gives a similar graph but yields values of 0.052mM and 0.0100 for constants a and b respectively and r = 0.893. It is interesting to note that in three out of five experiments the recovery in 70mM Na was faster than in 120mM Na. It is difficult to estimate from these experiments what $[Na]_o$ would still promote extrusion of H^+ ions. Extrusion of H^+ ions will continue as long as the energy gradient for Na entry is great enough.

For a Na/H exchange mechanism, the stoichiometry of which is 1:1 then
Assuming the Na energy gradient can be represented by

$$E_m - E_{Na}$$

then the energy required for transport of H^+ is similar

$$E_m - E_H$$

Equilibrium will be reached when $(E_m - E_{Na}) = (E_m - E_H)$ or simply when

$$E_H = E_{Na}$$

No thorough measurements of a^i_{Na} at < 14mM $[Na]_o$ have been made in Purkinje fibres and so no reliable estimates of E_{Na} can be made at low levels of extracellular Na. There should be H^+ ion extrusion as long as

$$E_{Na} > E_H$$

E_H in the steady-state is ~-12mV. Unless $pH_i \geqslant 7.4$ then E_H will be $\leqslant$ zero. In order to find reliable values of E_{Na} at low levels of extracellular Na a series of measurements of a^i_{Na} was undertaken using Na-sensitive glass microelectrodes.

Na Measurements

These experiments were designed to measure the a^i_{Na} at very low $[Na]_o$. Previous measurements of a^i_{Na} during changes in $[Na]_o$ were limited to $[Na]_o$ of between 14 and 140mM (Ellis, 1977; Ellis & Deitmer, 1978; Sheu & Fozzard, 1982). An example of such an experiment carried out in this study is shown in Figure 30. For the first 25 minutes the preparation was superfused with normal Tyrode (140mM Na, 6mM K). The Tyrode was then changed to one with a higher [K] (120 Na, 26 K) and the cells depolarized to ~-45mV i.e. conditions similar to those during the CO_2 recovery experiments. From this point, when Na was removed there was an isotonic amount of TMA substituted. Tonic tension rises progressively as $[Na]_o$ is reduced. On each decrease of $[Na]_o$ there was a transient tension rise sometimes followed by a relaxation to a new steady-state. The combined results of five such experiments are shown in Figure 31. The intracellular Na activity varies linearly with $[Na]_o$ down to extracellular Na values of ~14mM. This agrees well with the results of Ellis (1977), Ellis & Deitmer (1978) and, more recently, Sheu & Fozzard (1982). The straight line drawn through the data points between 14 and 120mM was fitted by regression analysis and has equation:-

$$a^i_{Na} = 0.030\,[Na]_o + 1.43 \qquad r = 0.813 \qquad \text{——— 2}$$

At lower $[Na]_o$ the graph no longer remains linear but falls away steeply to yield values of 0.34 ± 0.12 (mean ± S.E.) mM intracellular Na in Na-free Tyrode. (The Na-free Tyrode was analysed for Na and the concentration of contaminant Na was < 50μM). The values of a^i_{Na} from experiments of this type have been used to calculate E_{Na}. E_{Na} is plotted against $[Na]_o$ in Figure 32 . E_{Na} falls to zero when $[Na]_o$ is between 3.5 and 0.05 ("Na-free Tyrode") mM. From Figure 32 it can be estimated that the $[Na]_o$ will have to be decreased to $\lesssim$ <~1.0mM before ion extrusion is halted. As pH_i decreases from the

FIGURE 30

The effect of changes of $[Na]_o$ on a^i_{Na}. Top trace, intracellular Na activity (a^i_{Na}); middle trace, membrane potential (E_m); bottom trace, tension. 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 120mM). 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.

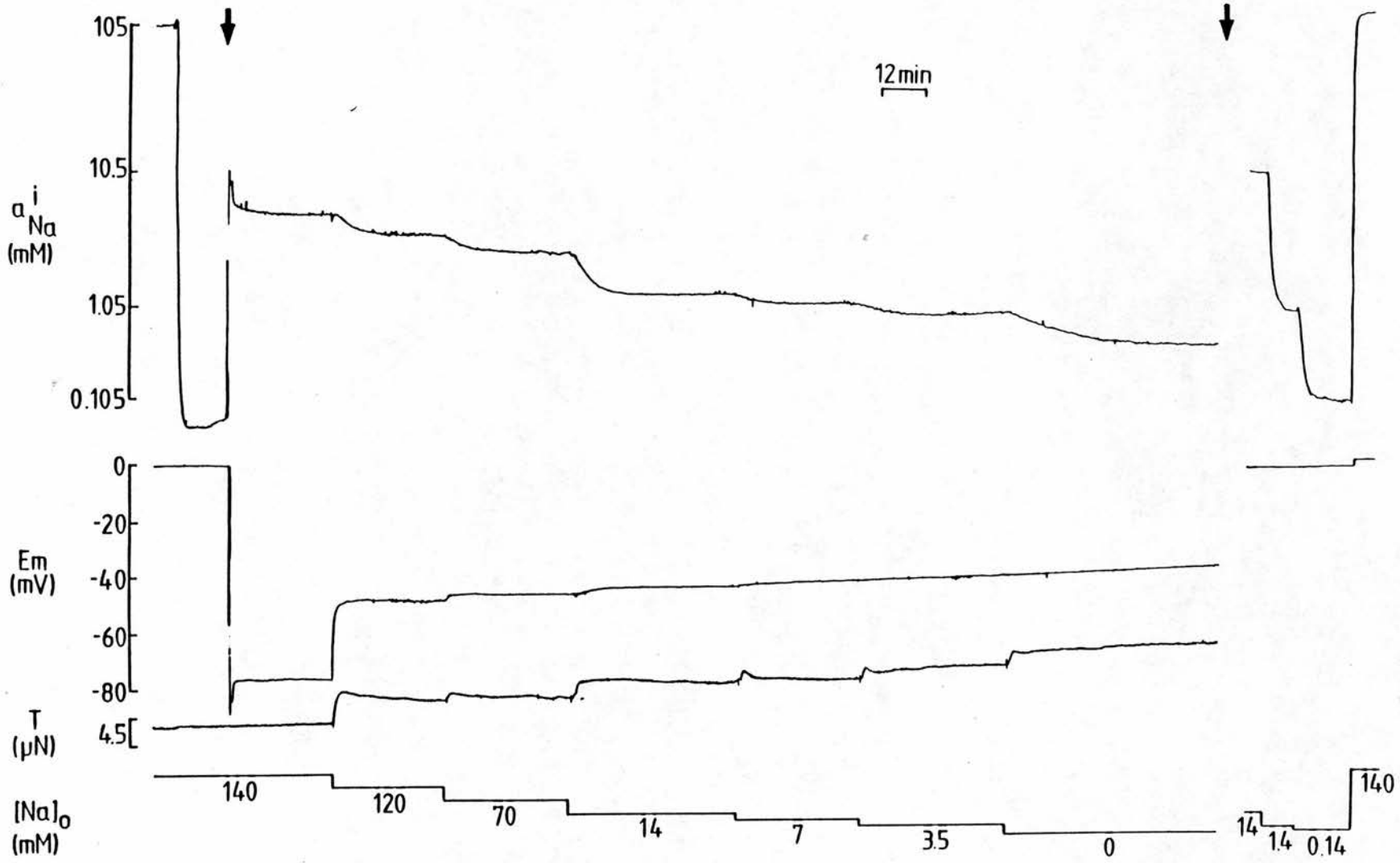

105
10.5
1.05
0.105
a^{i}_{Na} (mM)
12 min
0
-20
-40
-60
-80
Em (mV)
4.5
T (μN)
140
120
70
14
7
3.5
0
14
1.4
0.14
140
$[Na]_o$ (mM)

FIGURE 31

The relationship between the a^{i}_{Na} and the $[Na]_o$ obtained from experiments like that shown in Figure 30. The filled circles are measurements from experiments carried out in 26mM K while the open circle is from measurements made in 6mM K. The points are means ± SE of five preparations. The straight line drawn through the points between 14 and 120mM $[Na]_o$ was fitted by linear regression analysis.

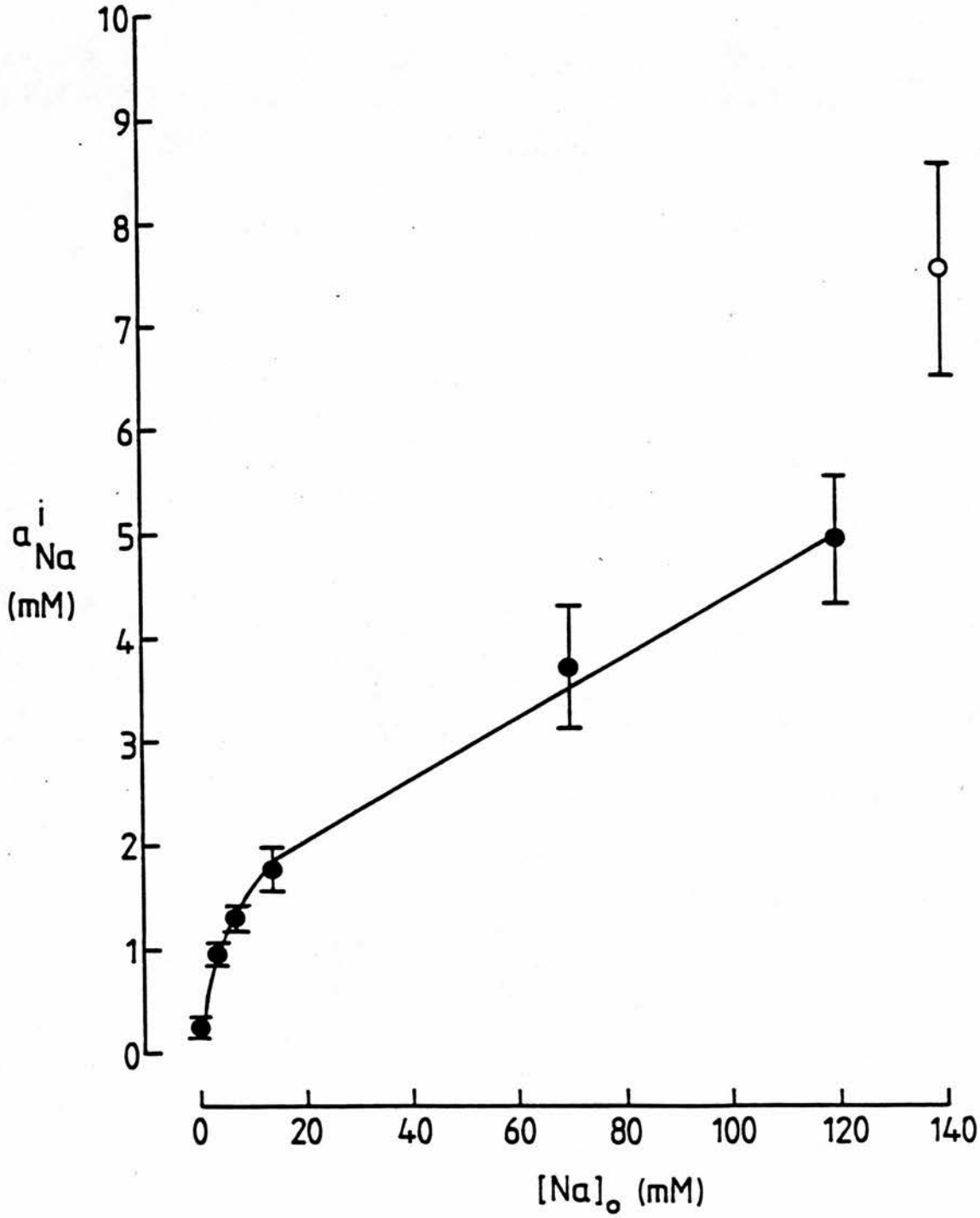
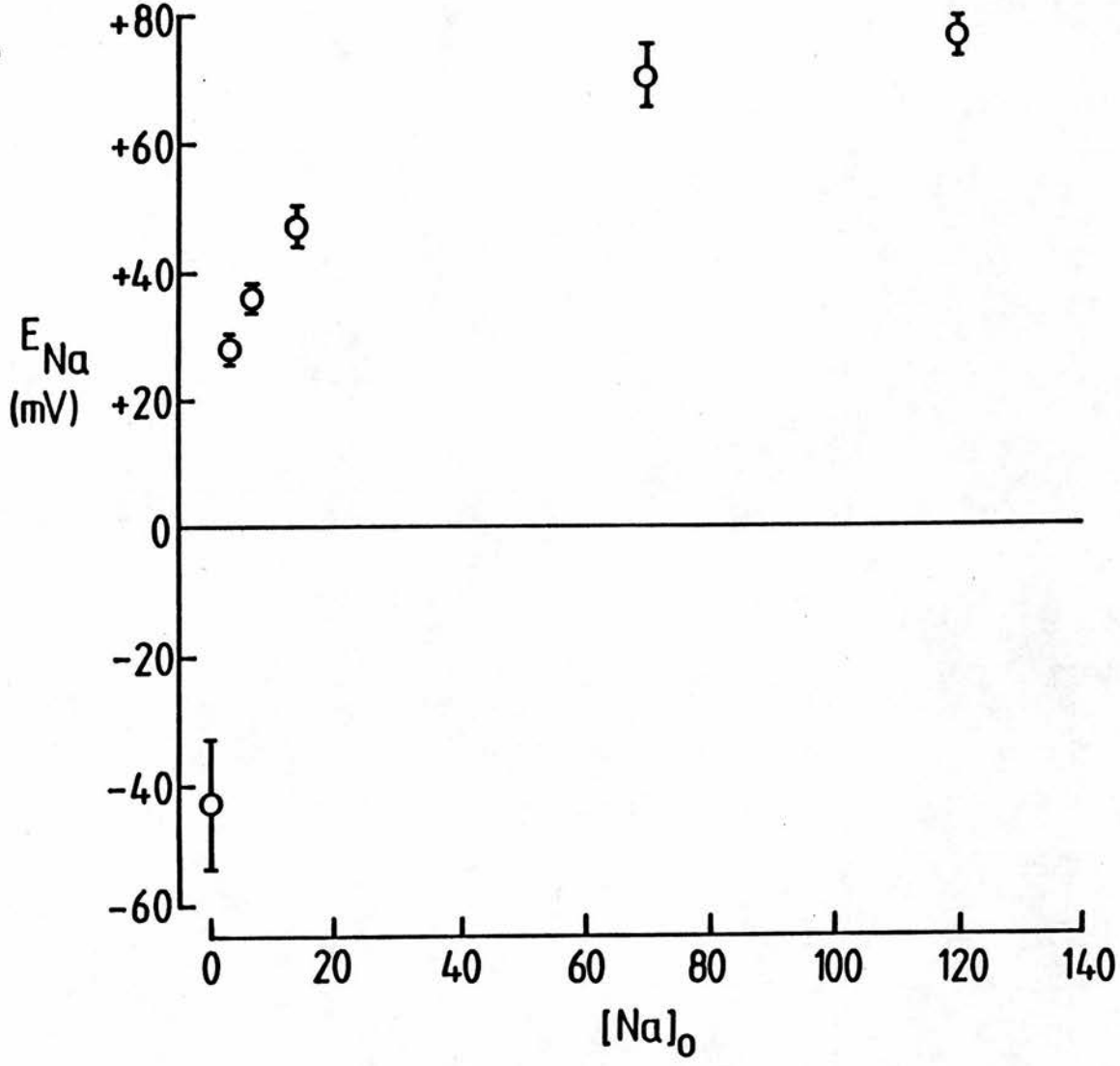

10
9
8
7
6
5
4
3
2
1
0
a^{i}_{Na}
(mM)
0
20
40
60
80
100
120
140
$[Na]_o$ (mM)

FIGURE 32

The relationship of E_{Na} to the $[Na]_o$. E_{Na} was calculated from the Nernst equation using the data from Figure 31.


+80
+60
+40
+20
0
-20
-40
-60
E_{Na}
(mV)
0
20
40
60
80
100
120
140
$[Na]_o$

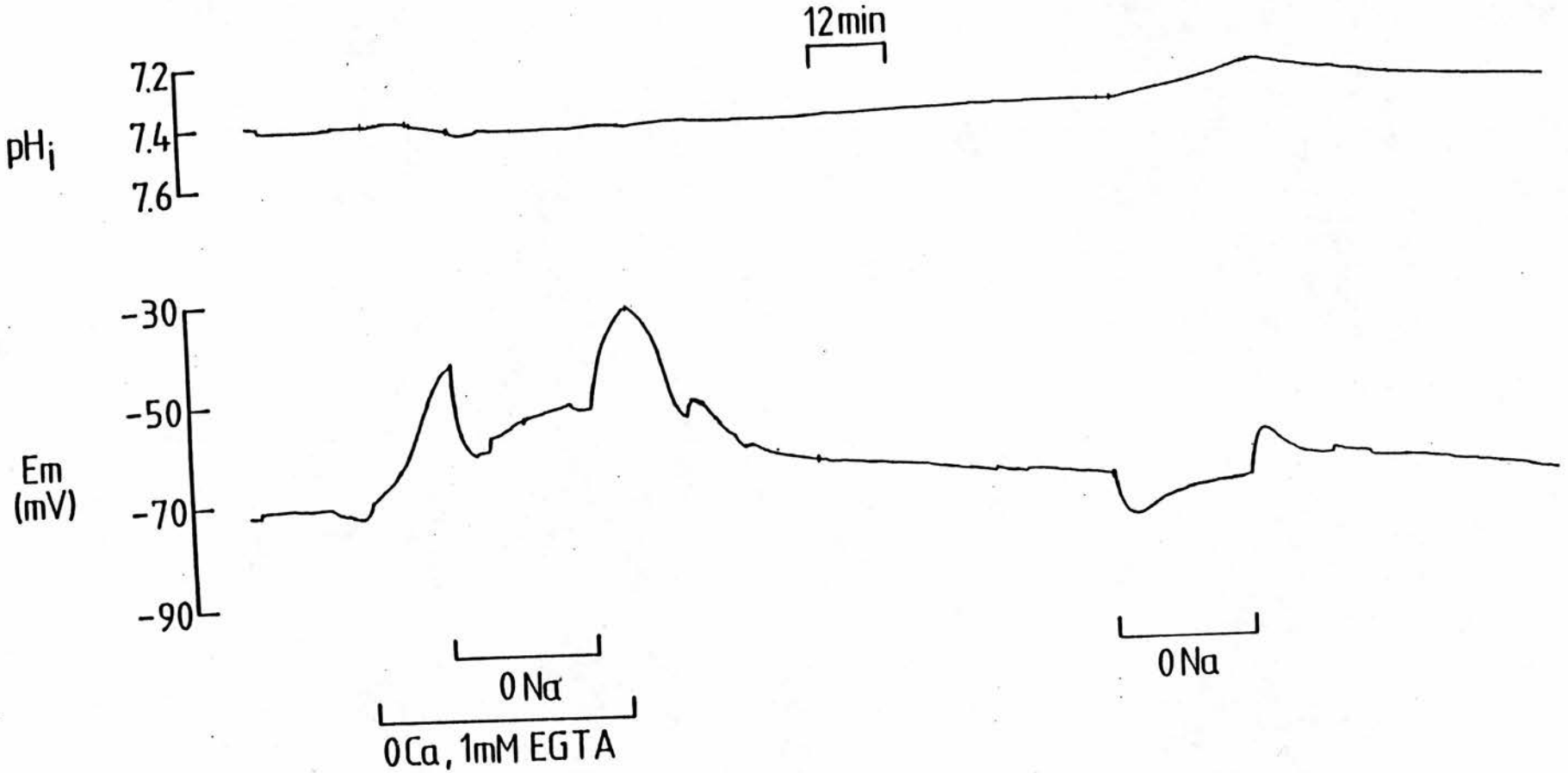
steady-state value of about 7.2, E_H becomes more negative and so the $[Na]_o$ which will be required to stop H^+ ion extrusion will be less.

Section 5 - Ca DEPENDENCY

The apparent inhibition of pH_i recovery in low Na_o could be mediated (as briefly mentioned in Section 2) not only via a direct effect on a Na/H exchange but also by an indirect process whereby a rise in $[Ca]_i$, brought about by reducing $[Na]_o$, affects pH_i. When Na_o is decreased a rise in $[Ca]_i$ is brought about probably via the sarcolemmal Na/Ca exchanger (Luttgau & Neidergerke, 1958; Neidergerke, 1963; Reuter & Seitz, 1968; Baker et al, 1969; Glitsch et al, 1970). If Ca_i were to rise under conditions of low Na_o the observations that (i) pH_i decreases in Na-free Tyrode and (ii) pH_i recovery from an acid load is inhibited in low Na_o, could be explained by the rise in Ca_i being buffered and thus giving rise to an increase in free protons within the cell. Meech & Thomas (1977) have found that an injection of Ca^{2+} into snail neurones was followed by an intracellular acidification. They suggested that this was due to mitochondria taking up Ca in exchange for protons. This result, together with several more recent studies on heart muscle (Ellis, Deitmer & Bers, 1981; Fry & Poole-Wilson, 1981; Fry et al, 1981; Bers & Ellis, 1982; Vaughan-Jones et al, 1983, Fry et al, 1983) points to the mitochondria as loci for significant interactive Ca/H buffering. The next series of experiments were therefore undertaken using very low $[Ca]_o$ solutions (buffered with EGTA) to prevent large rises of $[Ca]_i$ when $[Na]_o$ was reduced. Chapman & Miller (1972) show clearly that Na-free contractures can be prevented by superfusion with solution lacking Ca and with the addition of 1mM EGTA. Chapman (1974) in addition showed that a Na-free contracture can develop when the $[Ca]_o$ is as low as 10^{-5}M but when the $[Ca]_o$ was reduced to 10^{-7}M Ca the muscle relaxed. Miller & Moiscescu (1976) report that low Na contractures are prevented when $[Ca]_o$ is less than 5×10^{-8}M. Figure 33 demonstrates an experiment in which low Ca solutions ($<10^{-8}$M) were used to prevent large rises in $[Ca]_i$ when $[Na]_o$ was reduced. Exposing the cells to Na-free solution in the presence of normal $[Ca]_o$ brings about an intracellular acidification as was previously discussed. When the fibre was superfused with a Tyrode containing very low [Ca] there was little change

FIGURE 33

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


12 min
7.2
7.4
7.6
pHi
-30
-50
-70
-90
Em
(mV)
0 Na
0 Na
0 Ca, 1mM EGTA

in pH_i. (This is also demonstrated in Figure 34). The depolarization of approximately 30mV in low extracellular Ca is presumably due to an increase in cell membrane permeability. Under such conditions of very low $[Ca]_o$, exposure of the cells to Na-free Tyrode also produced no change in pH_i. The membrane potential becomes more negative during the Na-free exposure suggesting that much of the depolarization was in fact due to an increase in Na permeability. Experiments of this type suggest that much of the intracellular acidification produced by decreasing $[Na]_o$ is mediated by an increase in $[Ca]_i$, the rise in $[Ca]_i$ being brought about by the Na/Ca exchange mechanism promoting Ca influx when $[Na]_o$ is low. In order to check that the pH_i recovery from an acid load, which is apparently inhibited in low $[Na]_o$, was not due to changes in $[Ca]_i$, experiments like that shown in Figures 34, 35 were carried out. The depolarizations during the CO_2 exposures are due to $KHCO_3$ being used to buffer the CO_2. In Na-free (TMA substituted) normal $[Ca]_o$ Tyrode, the recovery is inhibited. When the same experiment is carried out in $< 10^{-8}$M Ca_o, complete Na removal again inhibits recovery although the slow acidification in Na-free solution is prevented. Similar results were obtained when K was used to completely substitute for Na (not illustrated). With 120mM Na present recovery occurs and in 14mM Na recovery proceeds but at a rate slowed by about 40% compared with that in 120mM Na. The experiments suggest that the apparent inhibition of recovery in low $[Na]_o$ is not simply due to rises in Ca_i.

Section 6 - FURTHER INTERACTIONS OF Ca AND H

The interaction of Ca and H that is apparent on removal of Na_o has been investigated further. When $[Na]_o$ is reduced during exposure of the fibre to 10^{-5}M strophanthidin (to inhibit the Na/K pump and so increase a^i_{Na}) then a large intracellular acidification is observed. Deitmer & Ellis (1980) suggested that this acidification could be brought about by (1) the Na/H exchange mechanism functioning in reverse thereby providing an exit route for Na and decreasing pH_i and/or (2) an increase in $[Ca]_i$ resulting from the reverse operation of the Na/Ca exchange i.e. Ca influx with Na efflux. This rise in $[Ca]_i$ would mediate a displacement of protons from buffer sites common to Ca and H. Two methods were used to differentiate between these two possibilities.

FIGURE 34

The effect on pH_i during reduction of Na_o in normal (2mM) or low (ca. 10^{-8}M) Ca_o. Solutions were normally HEPES buffered (100% O_2). When Na was removed it was replaced by 140mM TMA. During the periods indicated by the CO_2 bars, solutions were buffered with $NaHCO_3$ (95% O_2, 5% CO_2) and if Na was removed it was replaced with TMA (120mM) and $KHCO_3$ (20mM) producing some depolarization. When Ca was removed it was replaced by Mg (1mM) and EGTA (1mM).

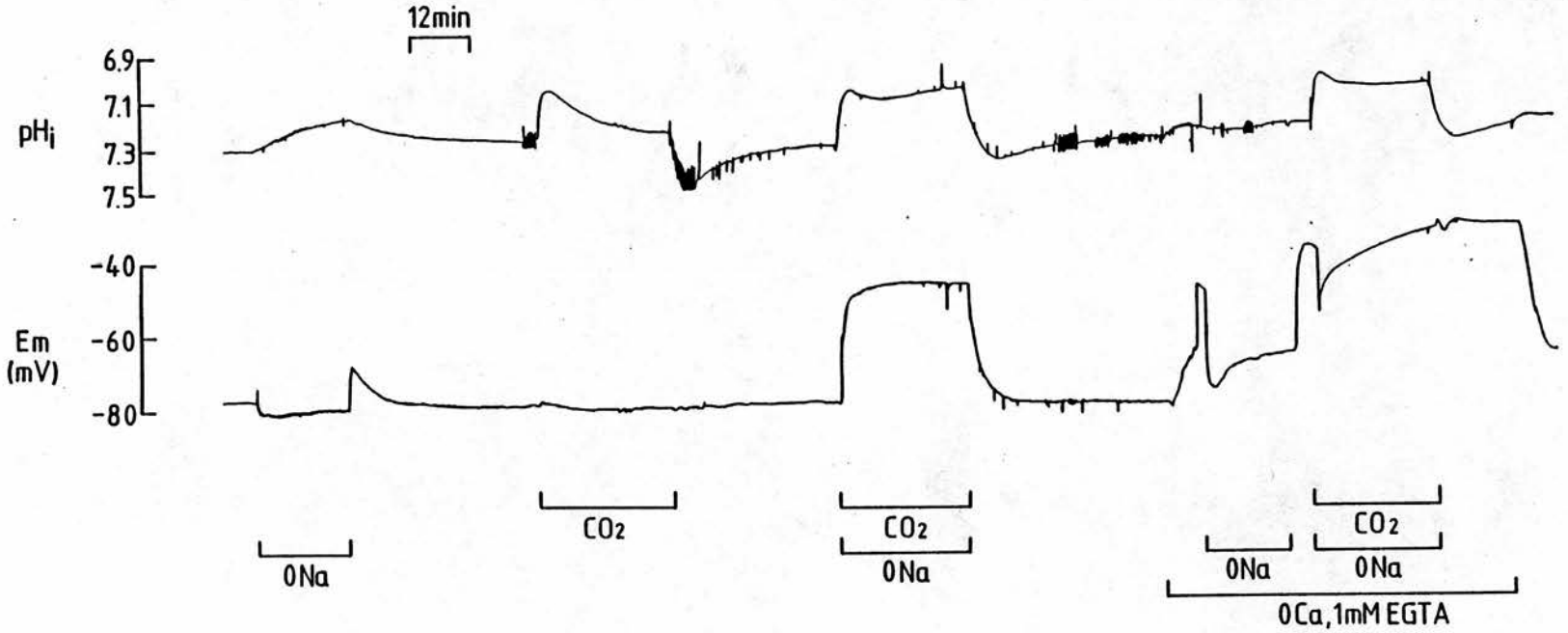
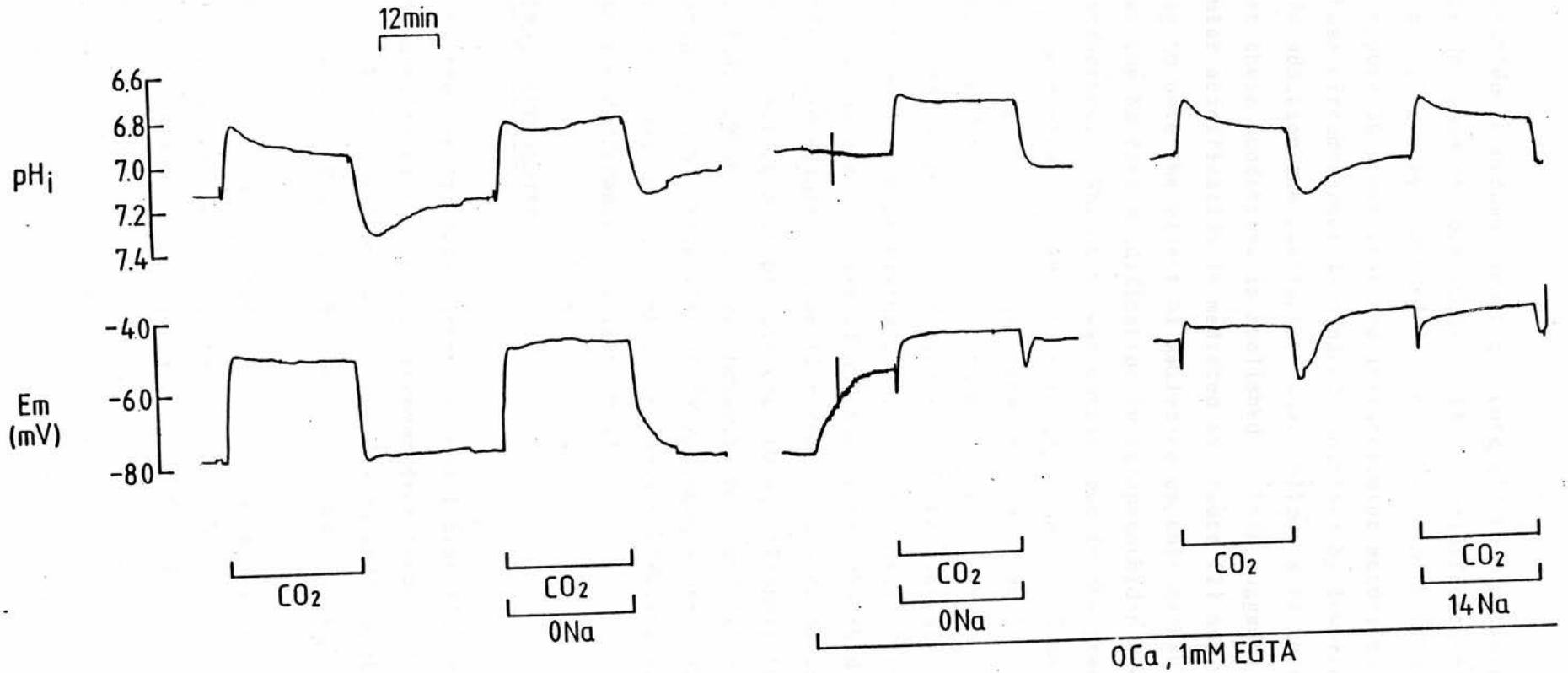

12min
6.9
7.1
7.3
7.5
pHi
-40
-60
-80
Em
(mV)
0Na
CO2
CO2
0Na
0Na
CO2
0Na
0Ca,1mM EGTA

FIGURE 35

The effect on pH_i recovery from CO_2-induced acid loading, of reduction of $[Na]_o$ in normal (2mM) or very low ($<10^{-8}$mM) Ca. 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.


12min
6.6
6.8
7.0
7.2
7.4
pHi
-40
-60
-80
Em
(mV)
CO2
CO2
0Na
CO2
0Na
CO2
CO2
14Na
0Ca, 1mM EGTA

If the acidification is brought about by route (1) we might expect that amiloride would decrease its magnitude. If the acidification is mediated via route (2) a very low [Ca] Tyrode buffered with EGTA should inhibit it. Figure 36 shows that the intracellular acidification observed in these circumstances is indeed inhibited by lowering $[Ca]_o$ to $<10^{-8}$M. In addition the contracture that follows a decrease in the $[Na]_o$ under these conditions is abolished. This suggests that the intracellular acidification is mediated via route (2) and it is now interesting to note the effect of amiloride on this mechanism. Figure 37 shows the Na-free acidification in strophanthidin and its associated contracture. When this was carried out in the presence of 1mM amiloride, the contracture and the rate of increase and magnitude of the pH_i change were reduced. While the effects of amiloride on pH_i changes could be due to an inhibition of Na_i/H_o (intracellular Na for extracellular H) exchange, 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 Na removal because contracture strength is proportional to a^i_{Na} (Eisner, Lederer & Vaughan-Jones, 1981, 1983b). I have, however, measured the rate of rise of a^i_{Na} during pump blockade with 10^{-5}M strophanthidin in the presence and absence of amiloride. 1mM amiloride may slow the rate of rise of a^i_{Na} but the differences are very small.

Section 7 - ALKALI RECOVERIES

Most of the results have centred around intracellular acidifications and the ability of the cell to recover from them. It is now appropriate to show an experiment which provides an additional piece of information on how a heart muscle cell recovers from an alkalinization.

Figure 38 shows an experiment where the acid loading (by NH_4Cl). is carried out during exposure to Na-free Tyrode substituted in the first instance by Li and in the last instance by K. The acid loading and recovery in normal Tyrode takes place in the middle of the trace. As already discussed in Section 2 there is complete inhibition of recovery from the acid load in Na-free (K substituted Tyrode). In Na-free (Li substituted Tyrode) the recovery is greatly slowed. There

FIGURE 36

The effect of very low Ca solutions on the intracellular acidification and contracture induced by removing Na_o during strophanthidin exposure.

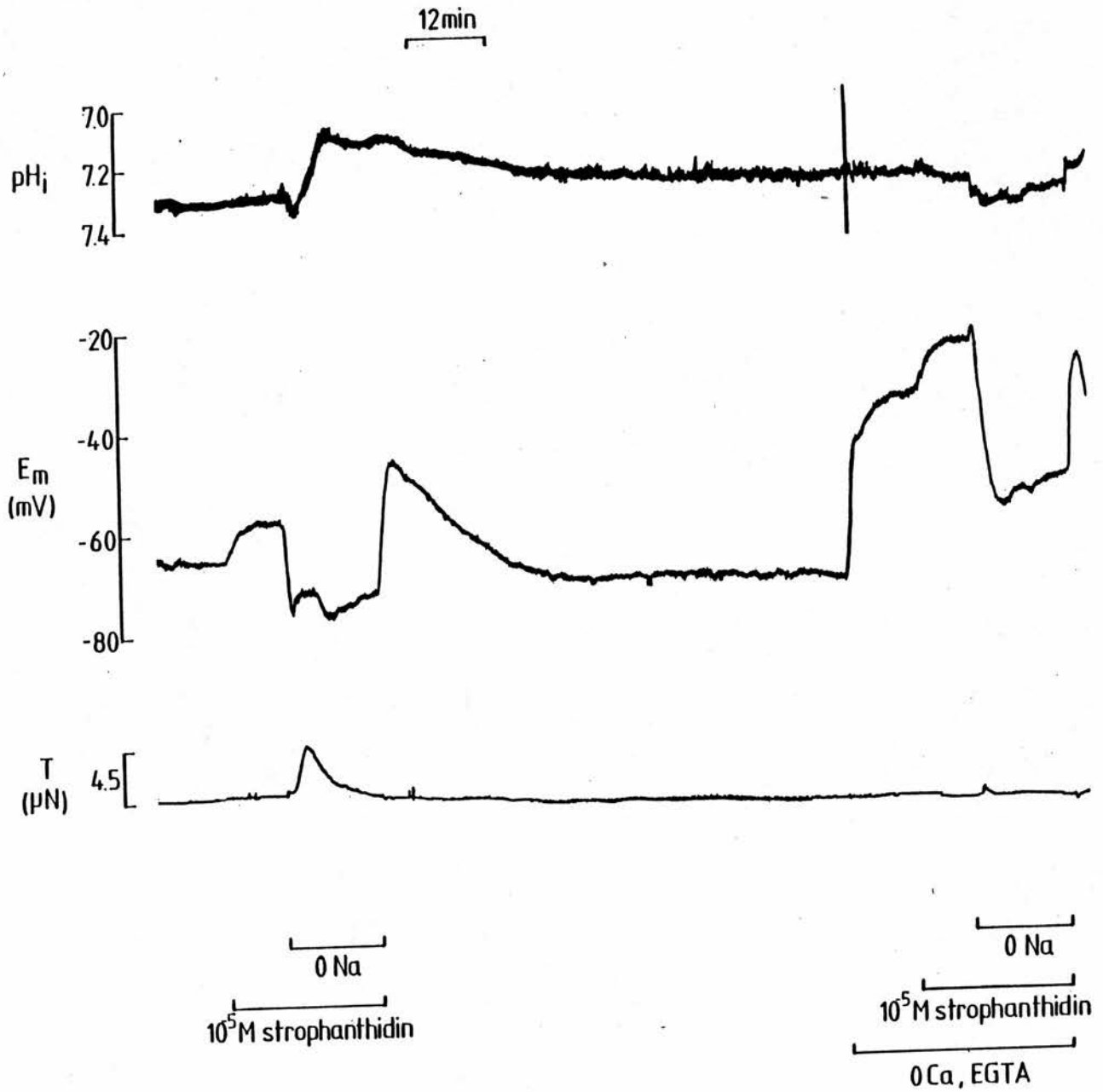

12min
7.0
pH$_i$
7.2
7.4
-20
-40
E$_m$
(mV)
-60
-80
T
(μN)
4.5
0 Na
10^{-5}M strophanthidin
0 Na
10^{-5}M strophanthidin
0 Ca, EGTA

FIGURE 37

The effect of amiloride on the contracture and the intracellular acidification produced by removal of Na_o during strophanthidin exposure. The Na was replaced by TMA. The break in the recording was for about 30 min.

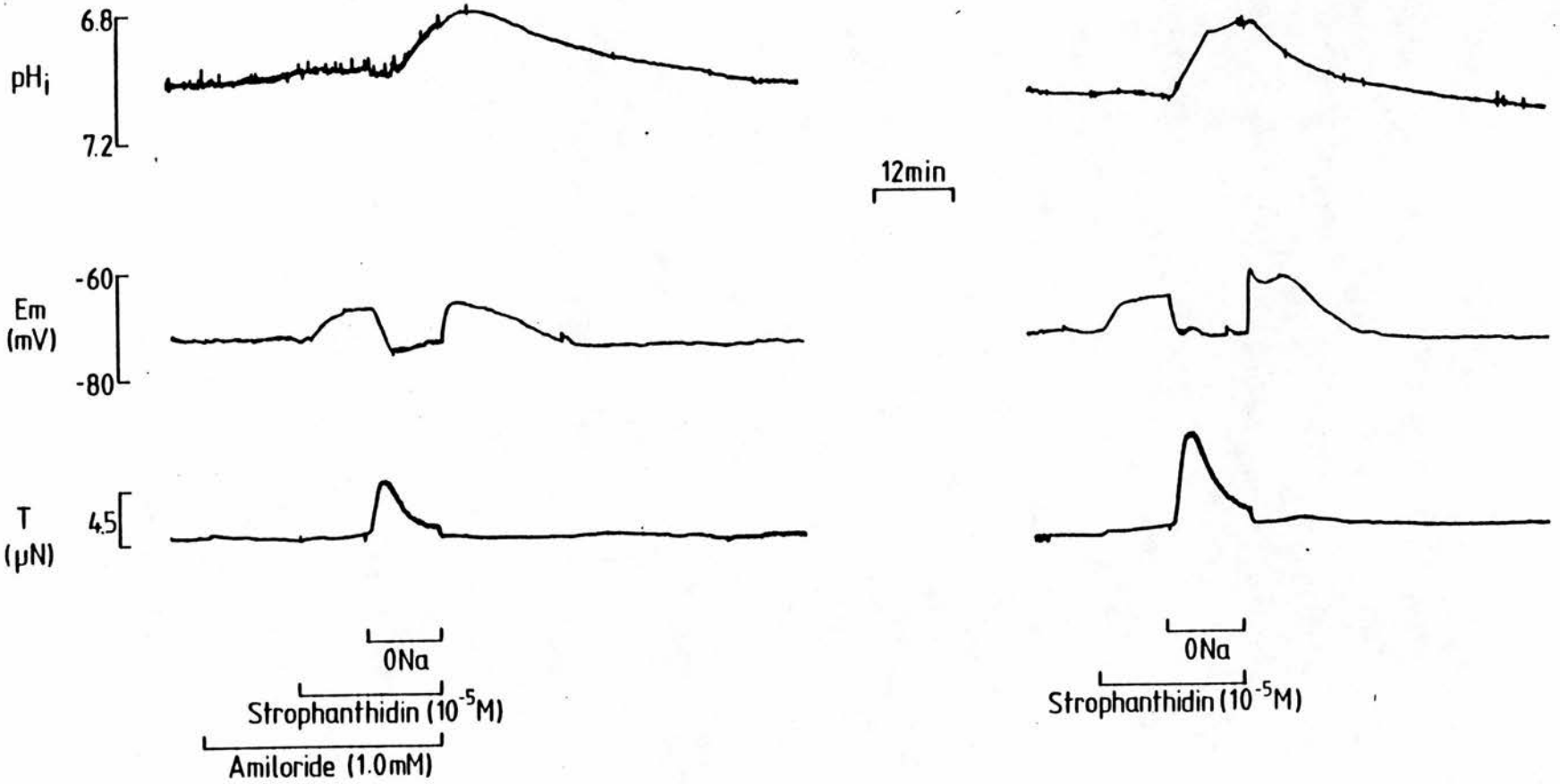
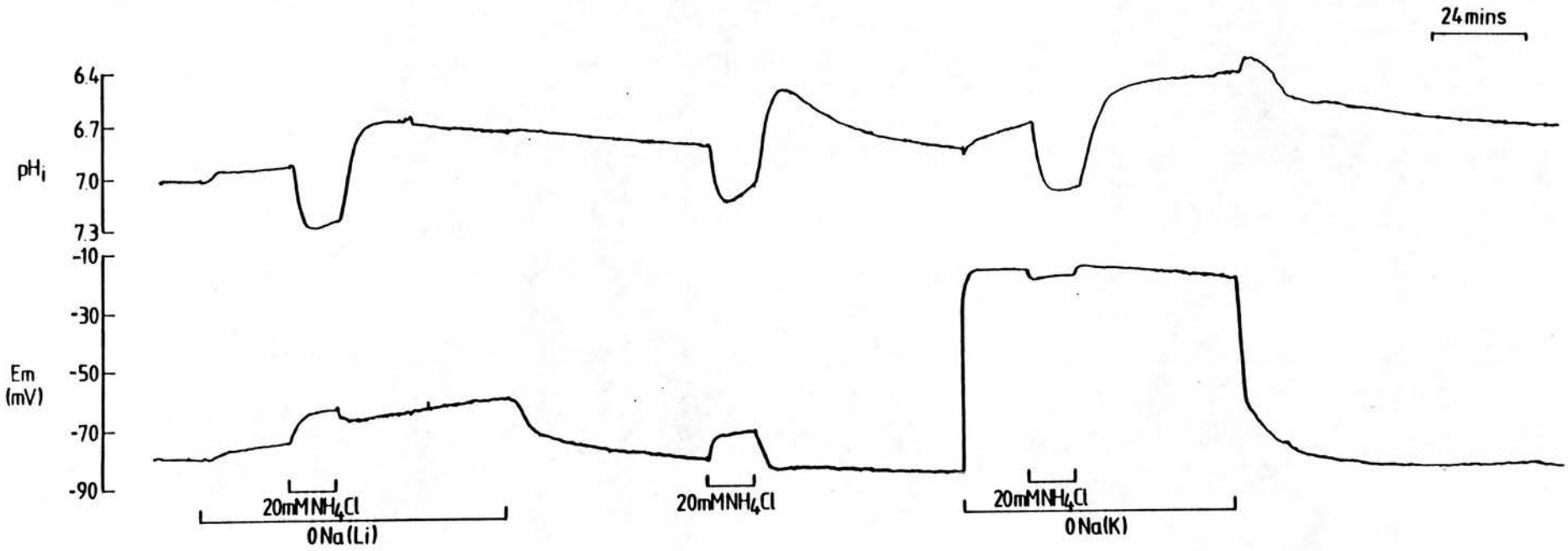

6.8
pH$_i$
7.2
12min
-60
Em
(mV)
-80
4.5
T
(μN)
0Na
Strophanthidin (10^{-5}M)
Amiloride (1.0mM)
0Na
Strophanthidin (10^{-5}M)

FIGURE 38

The effect of Na-free solutions on the pH_i recovery from NH_4Cl-induced alkalinizations. The first exposure of the fibre to 20mM NH_4Cl was made while the fibre was already in Na-free solution; the Na being replaced by Li. The second exposure to NH_4Cl was carried out in normal Tyrode. The third NH_4Cl exposure was in Na-free Tyrode where the Na was replaced with K.


24 mins
6.4
6.7
7.0
7.3
pHi
-10
-30
-50
-70
-90
Em
(mV)
20mMNH4Cl
0Na(Li)
20mMNH4Cl
20mMNH4Cl
0Na(K)

is a large intracellular acidification which occurs on removal of all Na_o (substituting with K) and a smaller acidification which occurs with a similar procedure but with Li substitution. However, the points to notice in this figure are the recoveries from the intracellular alkalinization <u>during</u> exposure to NH_4Cl. This recovery may be brought about by two components (see Section 1 and Discussion). Firstly, an influx of NH_4^+ (perhaps through K^+ channels) which dissociates producing H^+ and NH_3 and secondly a Cl^-/HCO_3^- exchange mechanism which provides a HCO_3^- efflux coupled to a Cl^- influx (Vaughan-Jones, 1981, 1982b). Under Na-free conditions, however, the recovery from the alkalinization is almost completely inhibited in K substituted Tyrode and partially inhibited in Li substituted Tyrode. This suggests that the recovery from an alkalinization is also Na dependent.

It is also useful to point out that as shown in Figure 23 temperature also decreases the recovery from alkalinization and subsequent acid loading. These points will be discussed in greater detail in the Discussion.

DISCUSSION

The acid loading techniques

A Na/H exchange

Na substitutes

The Na dependency of pH_i regulation

Ca and intracellular pH

Low temperature and a role for Cl/HCO_3 exchange

DISCUSSION

The average pH_i measured under steady-state conditions in this study (7.16) is almost an order of magnitude higher than that calculated if H^+ ions were in equilibrium across the cell membrane (6.2). The cell must therefore possess a mechanism for pH_i regulation whereby the intracellular accumulation of H^+ ions must be balanced by an energy-requiring extrusion of acid. The compensatory process must effectively "neutralize" the acidifying effects of at least two sources; (1) metabolism and (2) fluxes of ionised forms of weak acids and bases. Examples of the effects on pH_i of altered metabolism include the fall of pH_i produced by anoxia (Thomas, 1974) and metabolic inhibitors (Thomas, 1974; Boron & de Weer, 1976), and in muscle cells, prolonged contractile activity (Dawson et al, 1977). Fluxes of the ionised forms of weak acids and bases can also acidify the cell interior (see Results Section 1 and later Discussion). Following an acidification of the cell interior considerable acid can be neutralized by several reversible and rapidly responding mechanisms that serve to buffer acid loads so minimizing the pH_i decrease. These rapidly responding mechanisms probably include (1) chemical acid-base buffering, (2) the transfer of acid from the cytoplasm to cellular organelles and (3) cell utilisation of biochemically adaptable acid (e.g. aspartic, pyruvic and citric acid). All these mechanisms can reversibly consume H^+ but they can only be of limited capacity and can only offer an initial and partial buffering of the acid load. The "long term" restoration of pH_i must involve the extrusion, from the cell, of all the excess acid. As the extrusion proceeds, the intracellular buffering mechanisms (1) and (2) can yield H^+ ions that were previously buffered and can therefore be restored to their initial state.

The acid loading techniques

The method used to study the pH_i regulatory system in heart muscle was to acid load the cell and then measure the subsequent recovery from this acid load under different conditions. As already discussed, although the cell can bring into play several rapidly responding buffering mechanisms to minimise the pH_i decrease, the recovery must eventually represent a net active extrusion of protons from the

cell. I have already briefly discussed the three main methods of acid loading used in this study (see Results Section 1). Other methods have been used to change cell pH, e.g. intracellular injection of acid (Thomas, 1974; Meech & Thomas, 1980; Moody, 1981), and internal dialysis (Russell & Boron, 1976). However, these methods are unsuitable for the type of experiments involved in this study. It is now useful to describe more fully how the methods of treatment used give rise to an intracellular acid load.

Figure 8 shows an experiment in which a Purkinje fibre was first exposed to reduced pH_o. This causes a fall in pH_i which could be accounted for by a passive influx of H^+ ions across the cell membrane. The passive influx obviously overcomes any acid extrusion mechanism and the intracellular buffering capacity and so pH_i falls. pH_i will reach a new steady-state level in about 30 min. (Deitmer & Ellis, 1980). A full unit change of pH_o will not give rise to a unit change in pH_i. The ratio of ΔpH_o: ΔpH_i is 1:0.23 (Deitmer & Ellis, 1980). This is additional evidence that the distribution of H^+ ions across the cardiac cell membrane is not in equilibrium. When pH_o is raised back to its initial value pH_i returns to normal. This increase in pH_i when normal pH_o is restored could not simply be due to passive H^+ movement because there is always a large electrochemical gradient for H^+ entry.

The second acid load (Figure 8) is brought about by exposure to and subsequent removal of 20mM NH_4Cl at constant external pH. The following theoretical treatment regarding the influence of weak acids on pH_i is similar to that applied by Boron (1977) and Boron & de Weer (1976). On exposure of the cell to a solution containing NH_4^+ (and therefore, at physiological pH, some NH_3) the NH_3 will rapidly enter the cell and once inside will combine with available protons and establish a NH_4^+/NH_3 equilibrium governed by the law of mass action. The NH_3 combining with protons produces the initial intracellular alkalinization. The net influx of NH_3 will continue until its concentration on both sides of the membrane is identical. The magnitude of the alkalinization is mainly a function of the cell's intrinsic buffering power (van Slyke, 1922) and of the extracellular NH_3 concentration. When NH_3 has equilibrated then:

$$K_A \frac{[NH_4^+]_o}{[H^+]_o} = [NH_3]_o = [NH_3]_i = K_A \frac{[NH_4^+]_i}{[H^+]_i}$$

where K_A is the acid dissociation constant of ammonium (assumed to be identical inside and outside the cell). Consequently, when NH_3 is distributed evenly across the cell membrane, the equilibrium potentials for H^+ and NH_4^+ must be identical so:

$$E_H = \frac{RT}{F} \ln \frac{[H^+]_o}{[H^+]_i} = E_{NH_4} = \frac{RT}{F} \ln \frac{[NH_4^+]_o}{[NH_4^+]_i}$$

If these equilibrium potentials differ from the cell's membrane potential (E_m) there will be a driving force acting on these ions to move them across the membrane (provided they are permeable). In Figure 8, pH_i during exposure to 20mM NH_4Cl is approximately equal to pH_o and so $[NH_4^+]_i$ must also be approximately 20mM and $E_{NH_4^+}$ near to zero. However, E_m is close to -75mV hence a strong inward driving force exists for NH_4^+ and H^+. If the membrane were completely impermeable to NH_4^+ and H^+ removal of NH_4Cl from the bathing solution would reverse the above sequence and pH_i would return to its original value. However, the membranes of most cells do have a finite permeability to NH_4^+ (NH_4^+ perhaps enters the cells via K^+ channels. The narrow part of the K^+ channel is permeable to ions with crystal diameters from 2.66 Å (K^+) to 2.96 Å (Rb^+, NH_4^+) (Armstrong, 1974)). Boron & de Weer (1976) suggest P_{NH_4} may be about 6×10^{-8} cm/s compared with P_K of about 6.7×10^{-8}cm/s. Consequently, the driving force existing during the NH_4Cl exposure will tend to move NH_4^+ into the cell, which will upset the equilibrium:

$$NH_4^+ \rightleftharpoons NH_3 + H^+$$

produce NH_3, and acidify the cell. These movements of NH_4^+ thus bring about an acidification following the initial alkalinization. If these movements are interrupted by removing NH_4Cl from the bathing medium, then all NH_4^+ which entered the cell will rapidly exit as NH_3 after giving off a proton, presumably because NH_3 is much more permeant than NH_4^+. This extra source of protons (i.e. those produced

as a result of NH_4^+ ion entry rather than entry of NH_3) accounts for the fast pH_i change in the acid direction on removal of NH_4Cl from the bathing solution. Most of the NH_3 which entered will also exit as NH_3 and will not tend to produce a change in pH_i. Boron & de Weer (1976) thus propose that the size of the pH_i acidification on removal of NH_4Cl is therefore determined by (1) the intracellular buffering power (2) the permeability of the cell membrane to NH_4^+ ions (3) the magnitude of the electrochemical driving force for NH_4^+ (4) the $[NH_4^+]_o$ and (5) the length of time the cell is exposed to the NH_4Cl.

The pH_i decrease <u>during</u> the NH_4Cl exposure is always smaller than the size of the intracellular acidification on NH_4Cl removal. A reason for this is as follows. If NH_4^+ ions entered the cell and simply remained undissociated then pH_i would be unaffected. If NH_4^+ ions entered the cell and then completely left as NH_3 then the cell should be acidified to an extent dependent upon the amount of NH_4^+ that entered and the cell's intracellular (intrinsic) buffering power. This is what happens when NH_4Cl is injected into a cell bathed in ammonium-free solution (Thomas, 1974). In practice, following exposure to NH_4^+ the actual pH_i change lies someway between these two extremes. Only some fraction of the NH_4^+ dissociates and titrates the intracellular buffers in the acid direction. This is because the NH_3/NH_4^+ pair contributes significantly to intracellular buffering (Boron & de Weer, 1976a; Roos & Boron, 1981) so that the total cell buffering power is greater during the NH_4Cl exposure than after it. The equilibrium $NH_4^+ \rightleftharpoons NH_3 + H^+$ existing inside the cell during NH_4Cl exposure is disturbed as pH_i is lowered and shifts to the left. Since the acidification during NH_4^+ exposure and the acidification just after NH_4^+ removal are brought about by the same NH_4^+ influx, the ratio of the two should be inversely related to the ratio of total intracellular buffering values which exists during the NH_4Cl exposure and after NH_4Cl removal (Roos & Boron, 1981). This explains why the pH_i decrease during the NH_4Cl exposure is always smaller than the size of pH_i overshoot on NH_4Cl removal.

Figure 8 also shows the effect, on pH_i, of exposing the Purkinje fibre to Tyrode solution in equilibrium with 5% CO_2 for ~30 min. at constant pH_o. There is little doubt that the initial

acidification is due to CO_2 entering the cell very rapidly, becoming hydrated and forming carbonic acid which then dissociates, and acidifies the cell. The size of the pH_i change is determined by the intrinsic buffering power of the cell. When P_{CO_2} inside the cell equals that outside then from the equilibrium:

$$H^+ + HCO_3^- \rightleftharpoons H_2CO_3 \rightleftharpoons H_2O + CO_2$$

the relationship:

$$[HCO_3^-]_o \; [H^+]_o = K_D \; [H_2CO_3]_o = K_D \; [H_2CO_3]_i = [HCO_3^-]_i \; [H^+]_i$$

can be obtained where K_D is the apparent dissociation constant of carbonic acid. This means that when $P_{CO_{2_i}} = P_{CO_{2_o}}$ then the equilibrium potentials are also equal:

$$E_H = \frac{RT}{F} \ln \frac{[H^+]_o}{[H^+]_i} = E_{HCO_3} = \frac{RT}{F} \ln \frac{[HCO_3^-]_i}{[HCO_3^-]_o}$$

In a similar way to E_{NH_4} if E_{HCO_3} differs from E_m there will be an electrochemical driving force acting on the ions. Since many cells have a finite permeability to HCO_3^- one can expect movements of HCO_3^- to occur. HCO_3^- will tend to leave the cell. The decrease in $[HCO_3^-]_i$ will lower the pH_i and allow more CO_2 to enter. The net effect is to make the cell more acid. In practice the cell recovery from the acidification is quite rapid. This recovery is indicative of a pH_i regulatory mechanism. An "overshoot" is observed on returning to normal CO_2-free bathing solutions and Boron (1977) proposes that pH_i overshoots its initial value by an amount that is a measure of the net loss of protons which occured during the CO_2 exposure. Thus with CO_2 (1) the initial acidification is mainly determined by P_{CO_2} and the cell's intrinsic buffering power and (2) the size of the overshoot is determined by the intrinsic buffering power and by the number of protons extruded from the cell during the period of exposure to CO_2 (taking account of any passive HCO_3^- loss from the cell during this time).

A Na/H exchange

The fact that (1) pH_i is greatly out of equilibrium and (2) a pH_i recovery is obtained from all three types of acid loading is indicative of the existence of a pH_i regulatory mechanism. Such a system may involve proton extrusion or an equivalent mechanism. An equivalent mechanism could be for example an inwardly directed OH^- pump or HCO_3^- pump and these could not be easily distinguished from an active proton efflux pump. The word "pump" is used for convenience to indicate a system requiring energy to drive the relevant ion against its electrochemical gradient. Several arguments suggest that at least the second of these alternatives i.e. a HCO_3^- pump is unlikely. Firstly, as far as the recoveries from NH_4Cl or acid pH_o induced loads are concerned movement of HCO_3^- into cells is unlikely to have a large effect on pH_i because these solutions were HCO_3^- free and essentially CO_2-free. Deitmer & Ellis (1980) suggest that the tissue itself will produce some CO_2 and Moody (1981) assumes that nominally HCO_3^- free Ringer contains about 1mM HCO_3^- probably evolving from the preparation, so small effects due to HCO_3^- movements cannot be completely neglected but are probably very small. Secondly, as already discussed, passive HCO_3^- flux <u>into</u> the cell is energetically unfavourable. However, one mechanism for HCO_3^- entry is via a Cl^-/HCO_3^- exchange system which is coupled to the "downhill" inward energy gradient favouring Na entry. The electrical equilibrium is maintained by Cl^- efflux. The Cl^-/HCO_3^- exchange system can be inhibited by the disulphonic stilbene derivatives SITS and DIDS. (Cabantchik et al, 1978). However, I have found no evidence of an effect of SITS on recovery from any form of acid load but particularly from a CO_2 induced load (since during CO_2/HCO_3 exposure any Cl^-/HCO_3^- exchange would presumably be more active than in HCO_3^- free solution). Thomas (1976) finds that, following injection of acid into snail neurones in CO_2 Ringer, pH_i falls less and recovers faster than in CO_2-free Ringer and Boron et al, (1981) also find that in barnacle muscle HCO_3^- is important for normal pH_i recovery. The pH_i recovery system in cardiac muscle cells seems to be different from these other well described systems.

Experiments in this study, which will be discussed later in

more detail, suggest that it is movement of H^+ coupled with Na^+ that is important for pH_i recovery in cardiac muscle. Amiloride is thought to be an inhibitor of Na/H exchange (e.g. Bentley, 1969; Aickin & Thomas, 1977; Boron & Boulpaep, 1983a) and this drug can slow recovery from an acid load (e.g. Figure 22). In addition (as will also be discussed more fully later) the pH_i recovery is dependent upon the $[Na]_o$ and this would also suggest a link between Na and H. (e.g. Figure 27).

A plasma membrane or sarcolemmal Na/H exchange mechanism has been proposed for other types of cell (see Roos & Boron, 1981 for a comprehensive review and Introduction Section 4) such that the "downhill" electrochemical gradient for Na into the cell would supply the necessary energy for "uphill" extrusion of protons from the cell. Under these conditions, removal of Na from the extracellular fluid should inhibit this exchange process and the cell should lose the ability to regulate its pH_i by this route. The results in Sections 2 and 4 suggest that this may also be a mechanism for pH_i regulation in sheep heart Purkinje fibres as reduction of Na_o caused a decrease in pH_i and also inhibited pH_i recovery following acid loading.

There is a large amount of energy available from the Na entry gradient. The Na electrochemical gradient can be described by:

$$(E_m - E_{Na})\, zF$$

Similarly, the energy required for the transport of H^+ is:

$$(E_m - E_H)\, zF$$

If E_m = -70mV and assuming $[Na]_o$ = 140mM

$[Na]_i$ ~ 10mM (Ellis, 1977),

then E_{Na} = + 70mV. The energy thus available is $-\underline{140F}$ mV

If $[H]_o$ ~ 40nM

and $[H]_i$ ~ 69nM (this study)

then E_H = -14mV. The energy thus required is $-\underline{56F}$ mV

There is therefore a very adequate energy gradient for H^+ ion

extrusion. In fact the energy available is about 2.5 times the requirement. Theoretically, this would mean that the Na gradient is capable of transporting up to 2.5 H^+ ions per Na^+ ion moved on the carrier. The stoichiometry of the carrier remains as yet unestablished,but an exchange of more than one H^+ ion for one Na^+ ion would mean that the transport mechanism was electrogenic and since more positive charge would leave the cell than would enter then the operation would be hyperpolarizing. There is a lack of data regarding this possibility and no-one has succeeded in measuring an outward current attributable to a Na/H exchange during a pH_i recovery from an acid load. Kinsella & Aronson (1982) concluded that the stoichiometry of the Na/H exchange in rabbit renal microvillus membrane vesicles was 1:1. They were able to manipulate the intravesicular [Na] ($[Na]_i$) and intravesicular pH (pH_i) and they studied the net fluxes of Na while varying extravesicular pH (pH_o). They assumed that Na^+ leak pathways were negligible at intra and extravesicular Na concentrations ($[Na]_o$) of $\leq$ 1mM and at equilibrium:

$$\frac{[H^+]_i}{[H^+]_o} = \frac{[Na^+]_i}{[Na^+]_o}$$

With $[Na]_i$ = 1.0mM, $[Na]_o$ = 0.1mM and pH_i = 6.5 a pH_o of 6.5 ($[H]_i/[H]_o$ = 1.0) produced a net efflux of Na. When pH_o was 7.5 (i.e. $[H]_i/[H]_o$ = 10.0) there was little net flux of Na while at pH_o of 8.5 ($[H]_i/[H]_o$ = 100.0) a net influx of Na was found.

That Na/H exchange is electrogenic in Purkinje fibres remains a possibility but evidence from other tissues suggests it is electroneutral. If

$$\frac{[H^+]_i}{[H^+]_o} = \frac{[Na^+]_i}{[Na^+]_o}$$

then on removal of Na_o we can predict that $[H^+]_i$ must increase i.e. pH_i must fall and pH_i recovery from an acid load, if mediated by a Na/H exchange, should be inhibited. The next series of experiments were designed to investigate these theoretical predictions.

Na substitutes

It was shown that Li (when used as the replacement cation for Na) can allow pH_i to recover from an acid load. If all Na_o was substituted by Li then the recovery from an acid load, after an initial delay, proceeded generally at a comparable rate with that in normal Tyrode. Sodium channels of muscle are about as permeable to Li as they are to Na (Campbell, 1976) P_{ion}/P_{Na} 0.96:1.00. Boron et al (1981) reported that the rate of recovery of barnacle muscle from an acid load was reduced by 33% when Li was the Na substitute and recovery was completely abolished when Na was substituted by choline or by N-methyl-glucamine. Moody (1981) has reported that in crayfish Na-free (Li) Ringer was about 80 - 90% as effective in blocking pH_i recovery as Na-free (BDA) Ringer solution and Thomas (1977) found that Na replacement by Li inhibited pH_i recovery but less effectively than when Na was replaced by BDA. Kinsella & Aronson (1981) find that the Na/H exchanger in renal microvillus membrane vesicles has an affinity for Li but their technique was not sufficiently sensitive for them to be able to determine if the rate of Li^+/H^+ exchange was significantly slower than that of Na^+/H^+ exchange. Li, however, was found to be unable to substitute for Na in mouse skeletal muscle (Aickin & Thomas, 1977). Moolenaar et al (1981) find that Li can substitute for Na on a Na/H exchange in mouse neuroblastoma cells. Schwartz (1981), however, finds choline and Li equally effective in blocking a proposed Na/H exchange in rabbit proximal tubule. Although the evidence that Li can substitute for Na on a Na/H exchange seems equivocal it is not unreasonable that in this tissue Li can substitute for Na in promoting pH_i recovery and because of this other Na substitutes were tried.

Tris is often used as a Na substitute but even in relatively small quantities appears to cause pH_i changes probably due to its undissociated form entering the cells and chelating protons (Figure 12). Nahas (1962) suggests that as much as 30% of Tris can remain unionised at physiological pH and Casteels (1970) maintains that this fraction can easily penetrate the cells. For these reasons Li and Tris were not regularly used as Na substitutes in experiments investigating the Na dependency of pH_i regulation.

K and quaternary ammonium compounds were found to be suitable Na substitutes. pH_i recovery was inhibited in Tyrode solutions where Na was totally replaced by these ions. BDA has been used as a Na substitute by Thomas (1977) and by Moody (1981).

TMA has also been used as a Na substitute particularly in the work of Hille (1971). Campbell (1976) suggests that TMA is unlikely to enter cells through the Na channel since it has an extremely low permeability through such a channel $P_{ion}/P_{Na} < 0.008$. He finds that a solution of Na-free Ringer (TMA) cannot support inward current flowing through the Na channel.

It has been reported that amine derivatives of quaternary ammonium compounds can by themselves, cause pH_i changes (Zucker, 1981a). Zucker (1981b) reports that commercial TEA contains up to 5% triethylamine. Externally applied triethylamine caused an intracellular alkalinization. However, he reports that TMA chloride is free of triethylamine, the derivative likely to cause an alkali pH_i change. In this study when TMA was used as the Na substitute alkalinizations were never found.

In Na-free Tyrode (Na replaced by TMA, BDA or K) pH_i decreased. This is in contrast to the recent brief report of Coray and McGuigan (1983) who found pH_i <u>increased</u> when Na_o was removed (TMA replacement). Their measurements were carried out on ferret ventricular muscle using the pH neutral ligand liquid-ion exchange resin of Ammann et al (1981) and at room temperature (22 - 25°C). I have carried out similar experiments at room temperature with glass pH sensitive microelectrodes. In these experiments using sheep Purkinje fibres, one preparation showed a smaller acidification at 22°C than at 35°C in Na-free solution (TMA), while the other two preparations showed little change in pH_i at 22°C compared with the normal acidification at 35°C. In experiments carried out at 35°C using ferret papillary muscle Na-free solutions produced intracellular acidifications (see Figure 21). It can be concluded therefore, that temperature is important in the pH_i response to removal of Na_o. More effects of low temperature are discussed in the final section of the Discussion.

Since removal of Na_o causes an intracellular acidification and inhibits recovery from an acid load it was interesting to carry out experiments which further characterised the Na-dependency of pH_i regulation.

The Na dependency of pH_i regulation

When K was used as a Na substitute the accompanying depolarization induced small acid pH_i changes. This was also the finding of de Hemptinne (1981) who interpreted the pH_i change as being mediated by a release of Ca from internal stores. This may have given rise to the observations on the changes in steady-state pH_i occurring on reduction of Na (Figure 26). pH_i recovered to a new steady-state which became more acid as the $[Na]_o$ was reduced. It is interesting to note that Caldwell (1954) found little change in pH_i in crab muscle fibres upon depolarization and similarly Ellis & Thomas (1976a) found no change in pH_i in sheep heart Purkinje fibres on depolarizing by increasing $[K]_o$ (and decreasing $[Na]_o$). Abercrombie & Roos (1981) find that the pH_i, in frog semitendinosus muscle, decreases on depolarization (although this is followed by a recovery of pH in this tissue). It is notable that recovery time following an acid load in the depolarized Purkinje fibre ($[K]_o$~20mM) was very similar to that in the normal fibre ($[K]_o$ = 6mM). Thomas & Meech (1982) find a large increase in H^+ ion permeability on depolarization in snail neurones and find at very depolarized levels (0 to +15mV) that steady-state pH_i increases. They explain the potential dependence of pH_i at these very depolarized levels by suggesting that the membrane becomes very permeable to H^+ ions (and/or OH^-) so that their concentration inside the cell is simply determined by the external pH and the potential across the membrane.

Using TMA as the Na substitute allowed the membrane potential to be kept more constant throughout the experiments when changing $[Na]_o$. The data from these experiments using TMA suggests that $[Na]_o$ should be reduced to about 7mM for the rate of pH_i recovery to be halved. This would imply that the Na/H exchange in this tissue has a relatively high affinity for Na_o, requiring comparatively low amounts of Na_o for activation and is essentially saturated with respect to Na_o under physiological conditions. This is in contrast to

the Na/H exchange examined in skeletal muscle (Aickin & Thomas, 1977a,b) where only small reductions in $[Na]_o$ (11%) were required to inhibit the recovery from acid loading by 57%. Boron, McCormick & Roos (1981) found a K_m of 59mM for barnacle muscle so that reductions of $[Na]_o$ of 87% were required to inhibit the acid extrusion rate by 50%. Moolenaar et al (1981) find the Na/H exchange of mouse neuroblastoma cells is fully saturated with 40mM Na_o. Boron & Boulpaep (1983a) have recently found a K_m of ~5 -10mM Na in renal proximal tubule of the salamander. Kinsella & Aronson (1980) report a K_m for the Na/H exchange in rabbit renal microvillus vesicles of 5mM Na.

The pH_i recovery tended to be faster in 70mM Na than in 120mM Na. One possible explanation for this was that with 70mM Na_o there was sufficient extracellular Na to fully activate the exchange carrier (because of its low K_m for Na_o) but the reduction of a^i_{Na} in low $[Na]_o$ permits more H binding to the internal binding site of the carrier. This assumes that Na and H compete for the same internal binding sites. This prompted the investigation of how a^i_{Na} varied when $[Na]_o$ was reduced to very low levels. Assuming the Na energy gradient can be represented by:

$$E_m - E_{Na}$$

then the energy required for transport of H^+ is similar

$$E_m - E_H$$

equilibrium will be reached when $(E_m - E_{Na}) = (E_m - E_H)$

$$\text{or } E_H = E_{Na}$$

for a Na/H exchange mechanism with a stoichiometry of 1:1

Extrusion of H^+ ions will continue as long as the energy gradient for Na entry is great enough. Using the data from experiments of the type in Figure 30 the Na energy gradient only starts to fall to zero at values of extracellular Na of less than 3.5mM. These thermodynamic considerations support experiments of the type illustrated in Figure 20 where only 7mM Na was required to halt an acidification and promote a recovery.

There may be two components to the relationship of a^i_{Na} versus $[Na]_o$. A saturable component with an apparent K_m of ~3mM and a second component that is linear up to approximatly 120mM Na_o. The line fitted by linear regression and drawn through the points between 14 and 120mM has the equation:-

$$a^i_{Na} = 0.030[Na]_o + 1.43 \qquad \text{2}$$

I have plotted the data of Sheu & Fozzard (1982) which yields a similar equation:-

$$a^i_{Na} = 0.028[Na]_o + 2.21$$

The slopes of the lines are similar (Ellis & Deitmer (1978) observed a slope of 0.04) but Sheu & Fozzard and Ellis & Deitmer find larger values of a^i_{Na} at the same $[Na]_o$. This is presumably due to the present measurements being carried out on depolarized cells. Eisner et al (1981) find that depolarization decreased a^i_{Na}. They attributed this decreased a^i_{Na} to a reduction in the net passive Na influx at depolarized potentials. Evidence for this suggestion is shown in Figure 31 where the point at 140mM Na_o (i.e. measured when E_m was -70mV instead of -40mV) lies about 25% above the extrapolated line of equation 2.

Ca and intracellular pH

Care must be taken in interpreting the effect of reductions of $[Na]_o$ on pH_i to suggest or implicate a Na/H exchange mechanism. There could be two mechanisms bringing about the observed fall in pH_i and the inhibition of pH_i recovery from an acid load when Na_o is removed. Firstly, the Na/H exchanger may be inhibited and so $[H]_i$ will rise, and secondly on reduction of Na_o, Ca_i levels will rise and so consequently will a^i_H. The second mechanism requires further discussion. When Na_o is decreased there is a rise in $[Ca]_i$ and an accompanying contracture (Lüttgau & Niedergerke, 1958; Niedergerke, 1963) which are presumably brought about via the sarcolemmal Na/Ca exchanger (Reuter & Seitz, 1968; Baker et al, 1969; Glitsch et al, 1970). Rises in a^i_{Ca} on decreasing $[Na]_o$ have been noted by a number

of groups (e.g. Marban et al, 1980 and Lee et al, 1980 where Na was completely replaced by choline. Both groups used Ca selective microelectrodes).

Vaughan-Jones et al (1983) have shown that changes in Ca_i can alter pH_i, at least when the Na-K pump is inhibited, while Bers & Ellis (1982) and Ellis, Deitmer & Bers (1981) have found that changes in pH_i can alter intracellular Ca. There is disagreement as to the direction of this change i.e. whether an increase in pH_i leads to an increase in Ca_i (Weingart & Hess, 1981) or a decrease in Ca_i (Bers & Ellis, 1982) (see also Section 3, Introduction), but these phenomena are suggested to be due to H^+ and Ca^{2+} sharing common intracellular buffering sites. Meech & Thomas (1977) have previously suggested such an interaction in snail neurones where mitochondria are thought to provide the main site for this interaction. Fry & Poole-Wilson (1981) show results which provide more direct evidence for a coupled Ca/H exchange by mitochondria. When the pH of a medium suspending a preparation of rabbit cardiac mitochondria was abruptly changed from 7.2 to 6.8 by addition of HCl there was an efflux of about 6μM of Ca from the mitochondria.

If Ca_i were to rise under conditions of low Na_o the observations that (1) pH_i decreases in Na-free Tyrode and (2) pH_i recovery from an acid load is inhibited in low Na_o could be explained by the rise in Ca_i being preferentially buffered with a consequent rise in the proton level. It is difficult to differentiate between the two possibilities (viz. Na/H exchange inhibition or Na_o induced Ca_i and H_i rise) but one way is to use solutions of very low [Ca].

The experiments carried out using solutions of very low [Ca] were performed in order to prevent large rises of $[Ca]_i$ when the $[Na]_o$ was reduced. The results with very low Ca_o solutions confirm the suggestion that Na_o is an important regulator of pH_i in cardiac muscle. As $[Na]_o$ is reduced the ability of the cell to recover from a CO_2-induced acid load declines (Figures 34, 35). This is so even when large rises of $[Ca]_i$, which presumably occur on Na_o removal, have been prevented. Na is therefore important in order for pH_i to recover from an acid load. It seems that the acidification brought about by Na

removal alone (e.g. Figure 33) may be a secondary effect of an initial rise in Ca_i because the low Na_o acidification can be prevented by using very low Ca_o solutions. The effects of increases of $[Ca]_i$ may occur via an action on the mitochondria. It can now be seen that the pH_i regulatory mechanism is unusual.

If the Na/H exchange is continually operating in a mode which couples Na influx with H efflux then removal of Na_o while in very low Ca_o solutions should promote an acidification assuming, as on page 120, that:-

$$\frac{[H^+]_i}{[H^+]_o} = \frac{[Na^+]_i}{[Na^+]_o}$$

This is apparently not the case. Very little, if any, acidification is seen under such conditions (Figure 33). This would imply that either (1) net passive influx of H^+ ion is very small and/or (2) Na/H exchange is not the mechanism used by the cell to regulate steady-state pH_i and/or (3) the intracellular H^+ buffering mechanisms are more effective under these conditions.

Although estimates for the membrane permeability coefficient of H^+ ion result in fairly high values (Izutsu, 1972 $P_H = 10^{-3}$cm./sec.; de Hemptinne & Marranes, 1980 $P_H = 9.3 \times 10^{-3}$cm./sec.; Woodbury, 1971 $P_H \sim 500 \times P_{Cl} \sim 2.0 \times 10^{-3}$cm./sec. (assuming $P_{Cl} = 4 \times 10^{-6}$cm./sec. (Hodgkin & Horowicz, 1959))) , proton fluxes need not necessarily be as high since proton concentrations on either side of the membrane are of the order of 10^{-7}M.

If the drastic reduction in Ca_o also results in a reduction in Ca_i then the intracellular buffering capacity for H^+ ions may be increased since there may be less free Ca^{2+} to buffer under these conditions.

The only evidence in favour of steady-state pH_i regulation by Na/H exchange is the small acidification seen on application of 1mM amiloride. Amiloride (1mM) added to normal Tyrode produces falls in pH_i (Figure 17) although the rate of pH_i change is 33% of that

observed in Na-free solutions. The difference could be accounted for by an additional acidification brought about by the second mechanism. In other words, not only is the putative Na/H exchanger mechanism blocked but also the rise in Ca_i further acidifies the cytoplasm. This would seem to point to there being two mechanisms involved in pH_i regulation in heart muscle. Amiloride can slow but not fully inhibit pH_i recovery following acid loading. Amiloride may indicate the true part played by Na/H exchange in recovery from an acid load. The rest of the recovery may be due to internal sequestration of protons by mitochondria. In fact, Vághy (1979) suggests that in the normoxic myocardial cell, the proton-consuming mitochondrial oxidative phosphorylation is able to counterbalance the extramitochondrial proton-generating processes. Thus the apparent total inhibition seen by removing all Na_o could be due to an extra dumping of protons from the mitochondria which try to chelate the excess Ca_i. The small initial recovery occasionally observed in Na-free solutions (Figure 16) may be an internal chelation of protons. No small initial recoveries in Na-free solutions occurred after NH_4Cl-induced acid loads (see Figure 13) and this may indicate an additional effect of NH_4^+. Following exposure to NH_4Cl solutions there was a significantly slower rate of recovery from the acid loading than from the other two methods used to produce an acid load. If, after the NH_4Cl exposure, some of the NH_4^+ is slow to leave the cell (perhaps due to a slow release from an internal sequestration site) then NH_4^+ may 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 an affinity for NH_4^+ and can mediate Na^+/NH_4^+ exchange (Kinsella & Aronson, 1981). If the recovery from acid loads is also partially achieved by internal sequestering of excess protons then this system may also be inhibited by NH_4^+.

A secondary uptake mechanism may also partially explain the relatively high Q_{10} (2.65) for normal recovery in comparison with that calculated by Aickin & Thomas (1977b)for the component of pH_i recovery in mouse skeletal muscle that was due to Na/H exchange (1.4).

Steady-state pH_i may then be regulated by a mechanism involving mitochondria. Such a mechanism for heart Purkinje fibres is

related to the ideas regarding pH_i control proposed by Vaughan-Jones et al (1983) and could be seen to function in the following manner.

All metabolically produced and leak entry H^+ ions could be accumulated by the cardiac mitochondria which would couple the uptake of H^+ with the extrusion of Ca^{2+}. Ca release from mitochondria following acidification has been demonstrated by Fry & Poole-Wilson (1981). The Ca thus released could then exit from the cell either via a Na/Ca exchange mechanism or via a high affinity sarcolemmal Ca^{2+}-ATPase.

In barnacle muscle acidification induces a Ca^{2+} release which appears to originate from the SR (Lea & Ashley, 1981). H^+ may also be taken up by the SR which in turn may release Ca^{2+} ions which again may be extruded from the cell by various sarcolemmal Ca transport mechanisms.

Thus the control of steady-state pH_i may be mainly via a mechanism other than a sarcolemmal Na/H exchange. However, the results of the experiments with the very low Ca solutions support the idea that an acid load is counteracted by a sarcolemmal Na/H exchange. The rate of recovery under these low $[Ca]_o$ conditions seems to be governed by the $[Na]_o$ because increases in $[Ca]_i$ will probably be very small.

The low Ca experiments are not consistent with the possibility of a sarcolemmal Ca/H exchange regulating pH_i. The existence of such an exchange was tentatively proposed, at least for frog heart muscle cells by Chapman & Ellis (1975) and later substantiated by Chapman in 1980 although the authors did not propose that pH_i was regulated by such an exchange. Chapman (1980) observed further contractures on raising pH_o in Na-free fluid after the spontaneous relaxation of the initial contracture evoked by removing Na_o. These "alkalinity" contractures were affected by the $[Ca]_o$ and completely inhibited by a Ringer with no added Ca^{2+} but 0.2 - 1.0mM EGTA present. Chapman proposed that these contractures were due to an exchange of intracellular H^+ for extracellular Ca^{2+}. Such an exchange mechanism, if present in Purkinje fibres, does not seem to play a part in pH_i recovery

because recovery still takes place in very low Ca solutions of the type which inhibited the Chapman alkalinity contracture. Further evidence of Ca involvement in changing pH_i comes from experiments using strophanthidin. The intracellular acidification seen on removal of Na during strophanthidin exposure can be prevented if the $[Ca]_o$ is very low (i.e. $<10^{-8}M$). This illustrates the important interaction of Ca and H in cardiac muscle (Ellis, Deitmer & Bers, 1981; Fry & Poole-Wilson, 1981; Bers & Ellis, 1982; Eisner et al, 1983a; Vaughan-Jones et al, 1983) where rises in $[Ca]_i$ can mediate acid pH_i changes through Ca and H sharing similar buffering systems. The actions of amiloride on the effects of Na_o removal during strophanthidin exposure (Figure 37) are particularly interesting. Amiloride appeared to have only small effects on the pH_i changes on reduction of $[Na]_o$. This would be consistent with most of the pH_i changes being due to perturbations of Na/Ca 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 a brief report by Siegl et al, (1983), amiloride can inhibit Na/Ca exchange. Amiloride may also inhibit Na, K, ATPase (Soltoft & Mandell, 1983). Inhibition of the contracture produced by reduction of $[Na]_o$ could however be mediated by amiloride decreasing the membrane Na permeability. A slower rate of rise of a^i_{Na} during strophanthidin blockade would result in a smaller contracture on Na_o removal (see Eisner et al, 1981a, 1983b). Measurements of the rate of rise of a^i_{Na} in strophanthidin in the presence and absence of amiloride (1mM) however indicated only small differences.

It has been proposed that H^+ ions are extruded from the cell via the Na/K pump (Woodbury, 1965). In this model Na and H were envisaged to compete for sites on the carrier although Woodbury had to propose that the carrier's affinity for H was many thousand times larger than that for Na. Ellis & Thomas (1976) and Aickin & Thomas (1977), however, find no effect of ouabain (applied at a concentration sufficient to fully inhibit the Na/K pump) on the pH_i recovery from an acid load in the presence of a normal $[Na]_o$.

As in previous work examining pH_i regulation in cardiac muscle (Deitmer & Ellis, 1980; Vaughan-Jones, 1982a, b) I have been unable to find a Cl^-/HCO_3^- exchange contribution to pH_i recovery from an acid load. No effect of the stilbene derivative, SITS, was found on the pH_i recovery from any form of acid loading but particularly that from CO_2-induced acid loading. It seems that Cl/HCO_3 exchange may only be of importance when the cell recovers from an alkalosis (see Vaughan-Jones, 1979b, 1981, 1982a, b) or perhaps when pH_i <6.8 (Vanheel, 1982). Vaughan-Jones (1979b,1982b) concludes that in addition to counteracting alkali loads, a possible major function of a Cl/HCO_3 exchange in heart cells is to maintain a high intracellular Cl^-. His previous work (Vaughan-Jones, 1979) demonstrated that a^i_{Cl} was between 13 - 20mM, a level which is 2 - 4 times higher than that expected if Cl^- was distributed passively across the cell membrane. The reasons why heart muscle cells should require such a high level of intracellular chloride are, however, unknown.

In this connection it is interesting to describe how the results in Section 7 may fit into this model. Under Na-free conditions the recovery from the alkalinization (seen during exposure to NH_4Cl) is inhibited. When Na was replaced by K it is probable, from earlier discussion, that the acid loading (by NH_4^+ influx) would be depressed (as the NH_4^+ influx relies on the negative E_m) so the "recovery" would also be depressed. However, depression of the "recovery" was also seen using Li as the substitute. This points to Na also being important for Cl/HCO_3 exchange function. Vaughan-Jones has shown that this recovery from alkalinization is SITS sensitive (Vaughan-Jones, 1981) and that the recovery is also associated with a rise in a^i_{Cl}. This suggests that the recovery is due to a HCO_3^- efflux (so leaving free H^+ ions in the cell) which is coupled to an inward movement of Cl^-. This exchange may therefore be Na dependent in a manner similar to that proposed by Thomas (1977) for snail neurone. In this model the "downhill" entry gradient for Na provides the energy driving the Cl/HCO_3 exchanger.

The Cl/HCO_3 exchange seems to have a high Q_{10}. Aickin &

Thomas have calculated the Q_{10} of the exchange in skeletal muscle to be 6.9. However, in Purkinje fibres the Q_{10} of the Cl/HCO_3 exchange is probably not as high as this. Vaughan-Jones (1979b) finds that the Q_{10} in this preparation is about 2.6. In this regard it is noteworthy that, as shown in Figure 23, low temperature also decreases the recovery from alkalinization quite apart from its effect on the subsequent recovery from the acid loading. However, some of the "recovery" from alkalinization is also due to an influx of NH_4^+ during this time as this is what causes the acid load. The depolarization, presumably associated with the NH_4^+ influx, is also less.

The effect of temperature on pH_i is interesting. The way temperature effects pH (in general) depends upon the nature of the solution and more precisely the buffer constituents. This is because temperature affects the pK value of the buffer in a way predicted from the van't Hoff equation.

$$\frac{\delta \log K}{\delta T} = \frac{\Delta H^o}{2.303\ RT}$$

where R is the gas constant and ΔH^o is the change in heat content for the dissociation of 1 mole of the acid or base in the standard state. Plots of -log K (pK) as a function of temperature are roughly parabolic in form and for many weak electrolytes have a minimum value in the range 0 to 60°C. (Bates, 1954). In general pK values for weaker acids decrease as temperature decreases and for weak bases pK_o usually decreases with increased temperature (Bates, 1954). Bates states that in general the pH of alkaline solutions usually falls with rising temperature whereas that of acid solutions usually increases. Roos & Boron (1981) review the experimental evidence for the effect of temperature on pH_i. Evidence indicates that there is an inverse relationship between temperature and pH_i (Hinke & McLaughlin, 1967; Aickin & Thomas, 1977a, b; Saborowski et al, 1973; Malan et al, 1976). In this study steady-state pH_i increased as temperature was decreased by an average of 0.21 pH units (n = 4).

Aickin & Thomas (1977a) point out that the change of pH_i on changing temperature may be due to either (1) a change in the

collective pK of the intracellular buffers or (2) to the maintenance of a constant alkaline deviation from neutral pH rather than a change in an absolute number of H^+ ions. They measured pH_i to increase by 0.16 units for a change of temperature between 28 and 37°C.

It is clear that there will be a vast number of cell processes which will be affected by temperature changes. The rates of reactions in most mammalian cells decrease with temperature and metabolic effects may explain the differences in pH_i response to removal of Na_o at 37° and 22°C.

CONCLUSIONS

CONCLUSIONS

As was summarised in the introduction many cells appear to control their intracellular pH by an anion Cl^-/HCO_3^- exchange mechanism which is often coupled with the movement of Na into the cell. Some cells also appear to possess a Na/H exchange mechanism sometimes in addition to the anion exchange. From this study it can be concluded that cardiac muscle cells control their pH_i in a different manner. Some of these differences could be accounted for by adaptations specifically designed for the specialised function of a cardiac cell. The Cl^-/HCO_3^- exchange mechanism seems to be used only for a pH_i recovery from an alkalinization and may normally function to maintain a high a^i_{Cl} (Vaughan-Jones, 1982b). The Na/H exchange may play a role in promoting a pH_i recovery from an acid load. The recovery, if mediated in this way, requires only small amounts of Na and is sensitive to amiloride.

Steady-state pH_i may not, however, be controlled by the Na/H exchange. The only evidence in favour of Na/H exchange contributing towards the maintainance of an alkali pH_i, which is not in accordance with an equilibrium distribution of H^+ ions is the small, slow intracellular acidification observed in the presence of amiloride. Steady-state pH_i may be controlled by internal proton seqestering mechanisms. The mitochondria may be involved in this process.

Evidence for a Ca/H interaction at the mitochondria is growing. In cardiac muscle, as outlined in the Introduction, Ca^{2+} and H^+ ions share many similar types of behaviour and it is their repective sarcoplasmic concentrations upon which the contractility of heart muscle depends. It is possible that mitochondria provide the cardiac muscle cell with a method for maintaining contractility.

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APPENDIX

[*From the Proceedings of the Physiological Society*, 12–13 *November* 1982
Journal of Physiology, **336**, 69–70*P*]

The dependence of intracellular pH regulation on extracellular Na in sheep heart Purkinje fibres

By D. Ellis and K. T. MacLeod. *Department of Physiology, University Medical School, Teviot Place, Edinburgh EH8 9AG*

Intracellular pH (pH_i) regulation in cardiac muscle may involve an exchange of intracellular H^+ for extracellular Na^+, (Na_o) (Deitmer & Ellis, 1980). We present further evidence for this suggestion. pH_i was measured in sheep heart Purkinje fibres using recessed-tip pH-sensitive micro-electrodes. The fibres were acid loaded by (1) exposure to CO_2-containing solutions, (2) the addition and removal of NH_4Cl, or (3) changing extracellular pH. pH_i recovery from acid loading was measured in solutions of various $[Na]_o$, e.g. Fig. 1. The normal pH_i recovery in the presence of CO_2 (at end of recording) is inhibited if $[Na]_o$ is reduced (other exposures to CO_2). Inhibition is

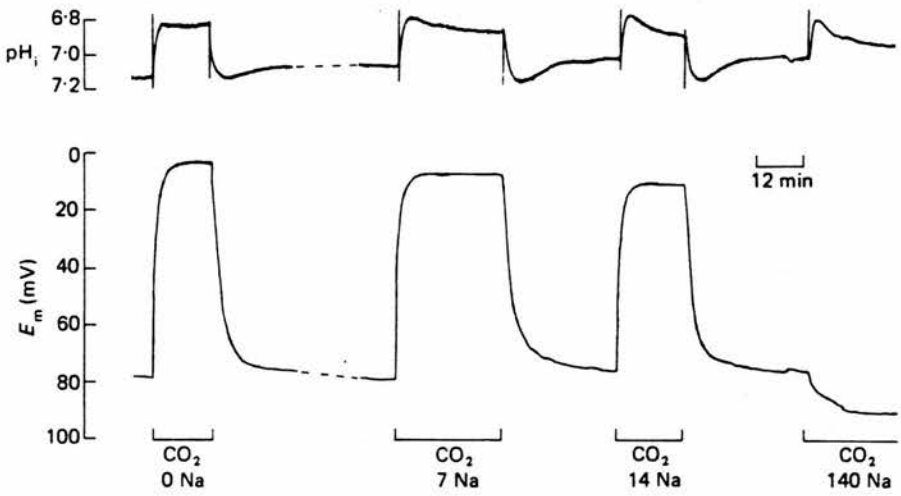

Fig. 1. Effect of $[Na]_o$ on pH_i recovery from acid loading. The preparation was superfused throughout with modified Tyrode solutions ($pH_o = 7{\cdot}35 \pm 0{\cdot}05$). Solutions were normally HEPES buffered (5 mM) and gassed with 100 % O_2 except during periods indicated by the bars (5 % CO_2; 95 % O_2, bicarbonate buffer), When $[Na]_o$ was reduced it was replaced by K. Dotted lines indicate a recording break of 15 min. Temperature 35 °C.

not dependent upon K-induced depolarization; other Na replacers produce similar results but Li can partially substitute for Na. Preliminary results suggest that less than 14 mM-Na is required to produce a half-maximal effect.

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Ca-dependent effects of extracellular Na removal on intracellular pH in isolated sheep heart Purkinje fibres

By D. Ellis and K. T. MacLeod. *Department of Physiology, University Medical School, Teviot Place, Edinburgh EH8 9AG*

Evidence has been presented (e.g. Ellis & MacLeod, 1983) that extracellular Na (Na_o) is involved in the control of intracellular cardiac pH (pH_i). For example, the recovery of pH_i after acid loading is inhibited by decreasing Na_o. Vaughan-Jones, Lederer & Eisner (1983) have shown, however, that changes in Ca_i can alter pH_i at least when the Na/K pump is inhibited. Decreasing $[Na]_o$ could cause a rise in $[Ca]_i$ (Reuter & Seitz, 1968) and thus a secondary change in pH_i. In the present experiments, low Ca_o solutions were used to prevent a large increase in $[Ca]_i$ while reducing $[Na]_o$. Fig. 1 shows that removal of Na_o decreased pH_i and inhibited pH_i recovery from an acid load (exposure to CO_2). Part of this inhibition was probably due to a rise in $[Ca]_i$ because in 10^{-8} M-Ca_o the low Na_o acidification is prevented and there is partial recovery from the CO_2-induced acid loading. However, Na may play a more direct role in pH_i regulation as pH_i does not recover fully in low $[Na]_o$ even in the absence of large rises in $[Ca]_i$.

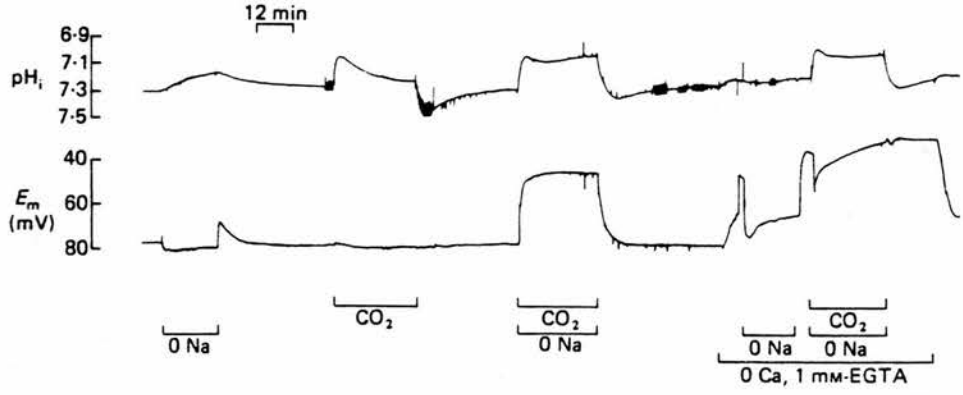

Fig. 1. Ion-sensitive glass micro-electrode measurement of pH_i during reduction of Na_o in normal (2 mM) or low (*ca.* 10^{-8} M) Ca_o. Solutions were normally HEPES-buffered (100% O_2). When Na was removed it was replaced by 140 mM-tetramethylammonium (TMA). During periods indicated by the CO_2 bars, solutions were buffered with $NaHCO_3$ (95% O_2, 5% CO_2) and if Na was removed it was replaced with TMA (120 mM) and $KHCO_3$ (20 mM) producing some depolarization. When Ca was removed it was replaced by Mg (1 mM) and EGTA (1 mM). $pH_o = 7{\cdot}4 \pm 0{\cdot}1$. Temp. = 35 °C.

Supported by the M.R.C. K.T.M. is an M.R.C. scholar.

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