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pH DEPENDENCE OF INTRINSIC H^+ BUFFERING POWER IN THE SHEEP CARDIAC PURKINJE FIBRE

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SUMMARY

1. Intrinsic, intracellular H^+ buffering power (β) was estimated in the isolated sheep cardiac Purkinje fibre at various values of intracellular pH (pH_i) in the range 6·2–7·5 and for various values of extracellular pH (pH_o) in the range 6·5–8·5. Buffering power was calculated from the fall of pH_i (recorded with an intracellular pH-selective microelectrode) induced by addition and removal of extracellular, permeant weak acids and bases (NH_4Cl, trimethylamine chloride, sodium propionate). Experiments were performed under conditions nominally free of CO_2–HCO_3.

2. β was estimated firstly following acid loads induced by NH_4Cl removal (10–20 mM) under conditions where Na^+–H^+ exchange was operational (i.e. in Na^+-containing Tyrode solution). At constant pH_i, the value of β appeared to double (from a control level of 39·7 mM) as pH_o was increased from 7·5 to 8·5. Notably, raising pH_o in this range greatly accelerated pH_i recovery from an intracellular acid load, indicating stimulation of acid extrusion. It is likely that this stimulation results in an overestimation of β because it blunts the intracellular acid load. The apparent elevation of β at high pH_o may therefore be an artifact.

3. Estimates of β were compared (NH_4Cl removal) before and after inhibiting Na^+–H^+ exchange in Na^+-free solution or with amiloride (1 mM). The acid load was larger and in many (but not all) cases the apparent value of β decreased after inhibition of acid extrusion. This indicates that, if Na^+–H^+ exchange is operational, it can result in an overestimate of β. In amiloride, β was $26·6 \pm 1·4$ mM ($n = 8$) at a mean pH_i of $6·84 \pm 0·03$.

4. Small stepwise reductions of external NH_4Cl (from 40 to 0 mM), in the presence of Na^+-free solution plus 5 mM-$BaCl_2$ at constant pH_o, resulted in small stepwise reductions of pH_i ($\sim 0·1$ units). When these were used to calculate β, we observed that β increased roughly linearly as pH_i became more acid. For a pH_i of 7·2, $\beta \sim 20$ mM.

5. An almost identical relationship between β and pH_i was found when using the method of sodium propionate addition (10–50 mM); amiloride (1 mM) was present and pH_i was manipulated to various test levels by changing pH_o. This confirms that β varies inversely with pH_i and also that it is independent of pH_o. We conclude that the apparent variation of β with pH_o observed earlier (paragraph 2) was indeed an artifact.

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6. An inverse, linear relationship between β and pH_i was also observed when using the method of addition and removal of trimethylamine chloride (0 to 20 mM) in the pH_i range 6·3–7·4 (Na^+-free solution). Comparison of the propionate and trimethylamine method (with 1 mM-amiloride in normal Tyrode solution) in the same fibre resulted in similar values of β confirming that the values are independent of the method used to determine them.

7. We conclude that, at pH_i 7·2, $\beta \sim 20$ mM, a value somewhat lower than previous estimates in this tissue. The value of β is independent of pH_o. In the range of pH_i 6·2–7·5, β varies inversely with pH_i, doubling for a 1·0 unit fall of pH_i. We also conclude that accurate determination of β can only be made if acid extrusion is minimized by inhibition of Na^+–H^+ exchange. The importance of these observations in relation to quantitative analyses of pH_i regulation is discussed.

INTRODUCTION

Intracellular pH (pH_i) in cardiac muscle is stabilized by means of transport mechanisms that move acid (or its ionic equivalent) across the sarcolemma (Deitmer & Ellis, 1980; Piwnica-Worms, Jacob, Horres & Lieberman, 1985; see Vaughan-Jones, 1988*a* for review). In addition physico-chemical buffering of H^+ at intracellular sites provides a more immediate protection against large swings in pH_i. Although some buffering is mediated by intracellular bicarbonate/CO_2, much is independent of this and is presumed to represent the intrinsic H^+ buffering capacity of intracellular proteins. A recent comparison of the intrinsic buffering power (β) of ventricular cardiac muscle and of isolated ventricular myocytes (Bountra, Powell & Vaughan-Jones, 1990) led to the conclusion that its value was 15–20 mM per pH unit which was considerably lower than previous estimates (e.g. Clancy & Brown, 1966; Ellis & Thomas, 1976; ~ 70 mM). In the present work, we use pH-selective microelectrodes to estimate β in the sheep cardiac Purkinje fibre. This is done by measuring the change of pH_i in response to application of various external weak acids and bases (NH_4Cl, sodium propionate and trimethylamine chloride). We conclude that the value of β in the Purkinje fibre is about 20 mM at a pH_i of 7·2 which is, again, somewhat lower than previous estimates (Ellis & Thomas, 1976; Vanheel, de Hemptinne & Leusen, 1986; 35–50 mM). More importantly, we find that β is not constant over the physiological range of pH_i. Its value rises roughly linearly as pH_i falls from 7·5 to 6·3, with a doubling of β occurring for a 1·0 unit fall of pH_i. In contrast, we find β to be independent of changes in extracellular pH. Finally, we show that estimates of acid equivalent fluxes are influenced considerably by selection of a pH_i-dependent and lower value of β than has been used hitherto.

A preliminary account of this work has been published (Vaughan-Jones & Wu, 1989).

METHODS

General methods. These are similar to those described previously (e.g. Bountra, Kaila & Vaughan-Jones, 1988). Briefly, fresh sheep hearts were obtained from a local abattoir. Thin (core diameter 100–250 μm), free-running Purkinje fibres were dissected from the endocardial wall of either ventricle, they were shortened to about 2 mm (crushing the cut ends with fine forceps) and pinned in the experimental chamber with one end attached to a force transducer (Akers; AE801). Tyrode

solution (see below) was pumped through the chamber (volume $\sim 100\ \mu l$) at a rate of $\sim 1{\cdot}0$ ml min^{-1} using a peristaltic roller-pump, while a servo-controlled impeller-motor extracted waste solution from the chamber at the same rate (Cannell & Lederer, 1986). Temperature was maintained at $37{\cdot}0 \pm 0{\cdot}5$ °C by means of a heater coil wrapped around the input-tube to the chamber and linked, via servo-control, to a thermistor bead inserted into the chamber.

Solutions. Basic solution was a modified Tyrode solution of composition (mM): NaCl, 140; KCl, 4·5; $CaCl_2$, 2·5; $MgCl_2$, 1·0; glucose, 11; HEPES, 30; pH adjusted to 7·4 at 37 °C using 4 M-NaOH. For solutions of pH 6·5, HEPES was substituted by 30 mM-PIPES (piperazine-*N*,*N*-bis-2 ethanesulphonic acid; pK_a 6·5). For solutions of pH 8·0 and 8·5, no substitution was performed, 4 M-NaOH titration being continued to the required pH (pH checked immediately before each experiment). Ammonium chloride, sodium propionate and trimethylamine chloride were added directly to solutions without osmotic compensation if their final concentration was $< 5{\cdot}0$ mM. For final concentrations $\geqslant 5{\cdot}0$ mM an equivalent concentration of NaCl (or of *N*-methyl-D-glucamine chloride, see below) was omitted in order to maintain isotonicity. The pH of these weak acid/base solutions was re-adjusted to pH 7·4 at 37 °C. For Na^+-free solutions, all NaCl was replaced by *N*-methyl-D-glucamine chloride and the final pH adjusted to 7·4 by using 5 M-HCl. Amiloride (Sigma Chemicals) was added, as a solid, to solutions shortly before use.

Microelectrodes. Single-barrelled H^+-selective microelectrodes were prepared as described previously (Vaughan-Jones, 1988*b*). Briefly, micropipettes are pulled from non-filamented, borosilicate glass tubing (o.d. 1·5 mm, i.d. 1·0 mm, Clarke Electromedical), laid horizontally on an aluminium tray in an open Petri dish and baked in an oven (180 °C) for *at least* 12 h. This pre-baking is, in our experience, essential for producing final electrodes with long-term stability and H^+ sensitivity. A 100 μl drop of fresh dimethyl trimethyl silylamine (Fluka chemicals) is then placed in a small glass container (the broken top of a standard glass ampoule) and transferred to the Petri dish in the oven. The dish is covered and baking continued for a further 12–24 h. These 'blanks' are then dry-bevelled (Kaila & Voipio, 1985) at 45 deg, to a tip size of just $< 1{\cdot}0\ \mu m$ (an equivalent microelectrode filled with 3 M-KCl would have an electrical resistance of ~ 4 MΩ). Pipettes are back-filled with 100 mM-NaCl + 50 mM-NaHEPES (pH 7·4 at 20 °C), solution being forced to the tip by means of positive pressure (~ 3–5 bar) applied from an ordinary bottled-air cylinder (British Oxygen). The pipettes are then dipped in H^+-sensor (Fluka, code 95291) and negative pressure applied from a 50 ml hand syringe (syringe held mechanically at maximum extension during suction period) in order to draw up a short column (Vaughan-Jones & Kaila, 1986) of sensor ($\sim 100\ \mu m$). Completed electrodes were calibrated at 37 °C in Tyrode solution, pH 7·4 (HEPES) and 6·4 (PIPES). The voltage signals from the electrodes were monitored using a varactor bridge diode (Analogue Devices, 311J). Acceptable electrodes displayed a sensitivity of 56–61 mV $(pH\ unit)^{-1}$.

Conventional microelectrodes (filamented borosilicate glass, 1·5 mm o.d., 1·0 mm i.d.) were similarly dry-bevelled and back-filled with 3 M-KCl (4–5 MΩ). A blunt 3 M-KCl-filled microelectrode was also used as a bath-reference electrode. In addition, the chamber was earthed via a Ag–AgCl wire. For further electrical arrangements, see Vaughan-Jones (1988*b*). Intracellular pH (pH_i) was recorded as the difference between an intracellular pH microelectrode and an intracellular conventional microelectrode; microelectrodes were impaled with a longitudinal spacing of about 30–100 μm.

Calculation of intrinsic H^+ buffering power (β). The method of estimating β using application of external, permeant weak acids or bases is described in detail by Roos & Boron (1981). In the present work we used application and removal of NH_4Cl or trimethylamine chloride (weak base method) and application of sodium propionate (weak acid method). These agents freely permeate the cell membrane in their uncharged form (i.e. NH_3, trimethylamine and propionic acid) but they exist mainly in ionized form once inside the cell. The ionization of a weak base absorbs H^+ ions while that of a weak acid liberates H^+ ions. Both types of agent can therefore be used to induce changes of pH_i which can then be used to estimate β:

$$\beta\ (\text{mM}) = \frac{[H^+]_i}{\Delta pH_i}, \tag{1}$$

where $[H^+]_i$ (mM) is the concentration of acid or alkali (per litre of cytoplasm) introduced into the cell and ΔpH_i is the resulting change of pH_i. $[H^+]_i$ is assumed to equal (i) the intracellular concentration of NH_4^+ or trimethylamine (TMA) ions at the moment of their *removal* from the

external solution (this assumes that each millimole of intracellular ionized base that exits from the cell does so as uncharged NH_3 or TMA thus leaving behind an equal number of H^+ ions), (ii) the intracellular concentration of propionate ion ($[propionate^-]_i$) at peak acidosis induced by addition of external sodium propionate (this assumes that each millimole of weak acid that enters the cell does so in the form of uncharged propionic acid which then dissociates into one millimole of propionate ions and an equal number of H^+ ions). As an example, for the case of NH_4Cl removal, $[NH_4^+]_i$ at the moment of removal is given by: $[NH_4^+]_i = [NH_4^+]_o\ 10^{(pH_o - pH_i)}$ where $[NH_4^+]_o$ is calculated from the total concentration of external NH_4Cl (C) and its pK using a rearrangement of the Henderson–Hasselbalch equation: $[NH_4^+]_o = C/[10^{(pH_o - pK)} + 1]$. By a similar procedure one may calculate $[propionate^-]_i$ following sodium propionate addition (see Szatkowski & Thomas, 1989; Bountra *et al.* 1990).

The above methods for determining β rely upon the assumptions that (i) there is rapid equilibration of the uncharged form of the weak acid/base, (ii) pK_a values inside the cell are identical to those in extracellular solution; the pK_a values used in the present calculations were 9·02 (NH_4Cl; Aickin & Thomas, 1977); 4·87, (sodium propionate; Szatkowski & Thomas, 1989); 9·80 (TMA-Cl; Szatkowski & Thomas, 1989), (iii) solubility coefficients are identical outside and inside the cell (see Roos & Boron, 1981), (iv) significant transmembrane movements of NH_4^+, $[TMA]H^+$ or propionate ions do not occur during the rapid, acid-loading phase; this point is addressed further in the Discussion, (v) during the acid-loading phase, significant transmembrane pumping of acid equivalents does not occur, e.g. via Na^+–H^+ exchange; this point is addressed in the Results section.

RESULTS

Estimates of β can be distorted by transmembrane acid extrusion

Figure 1*A* shows estimates of β using the NH_4Cl-withdrawal technique. As with all other experiments in the present work, the perfusing solution was nominally free of CO_2–HCO_3 in order to remove the CO_2 contribution to intracellular buffering power. Intrinsic β was estimated from the internal acidosis induced by removing 20 mM-NH_4Cl (see Methods for details). Note that the acidosis is not sustained. This is because a fall of pH_i stimulates acid extrusion via Na^+–H^+ exchange (e.g. Deitmer & Ellis, 1980) so that pH_i recovers.

The problem with the above technique is that acid extrusion during the loading period may blunt the acidosis thus leading to an overestimate of β (Roos & Boron, 1981; Bountra *et al.* 1990). In order to reduce errors due to acid extrusion, we have extrapolated the pH_i recovery back to a point where it intersects the line defining the maximum initial rate of acid loading (see first acid loading shown in Fig. 1*A*). A similar extrapolation method has been used in previous work (Thomas, 1976; Aickin & Thomas, 1977; Aickin, 1984; Vanheel *et al.* 1986; Bountra *et al.* 1990). In Fig. 1, the apparent value of β calculated in this way is listed above each acidosis. For convenience we have indicated (at arrow) the pH_i measured at 50% of peak acidosis. We show later that β varies with pH_i, so that the pH_i values arrowed in Fig. 1 will represent roughly the *mean* pH_i at which β was determined. It is apparent that, although mean pH_i, is reasonably constant (6·70–6·93), β varies greatly among the six determinations (range 38–76 mM) and appears to increase as *extracellular* pH is made more alkaline.

Figure 1*B* shows data averaged from six experiments similar to Fig. 1*A*. In all cases, β apparently increased steeply with pH_o, a doubling of β occurring as pH_o increased from 7·5 to 8·5. A similar phenomenon has been reported by Aickin (1984) for the mouse, vas deferens smooth muscle. As pointed out by Aickin, it is unlikely that intracellular intrinsic buffering would be influenced by extracellular pH. It is

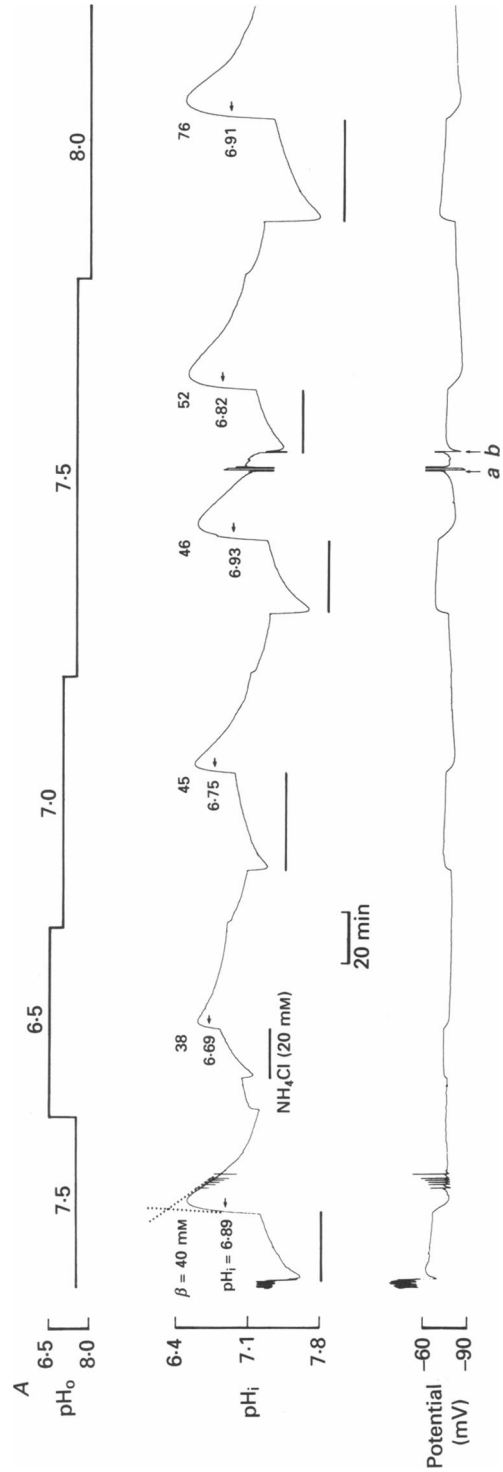


Fig. 1. In the presence of acid extrusion, β appears to increase at high pH_o. *A*, experimental protocol. Traces show (top) intracellular pH (intracellular, ion-selective microelectrode) and (bottom) membrane potential (conventional microelectrode, 3 M-KCl-filled). Bars under pH_i trace indicate periods of perfusion of NH_4Cl (20 mM). Changes of extracellular pH are indicated at the top of the figure. Note that changing pH_o produces a change of steady-state pH_i that is complete within 15–20 min. All solutions buffered with 20 mM-HEPES except for pH_o 6·5, which was buffered with 20 mM-PIPES (see Methods). The acid load following NH_4Cl removal was estimated using the back-extrapolation method as illustrated in the first acid loading of Fig. 1*A*. For details, see text. The horizontal arrows indicate the pH_i at 50 % acid loading. The vertical arrows (*a* and *b*) indicate transient disturbances of the conventional membrane-potential-recording microelectrode.

more likely that sarcolemmal acid extrusion, which is stimulated at the more alkaline values of pH_o (Vanheel *et al.* 1986; Vaughan-Jones & Wu, 1989), blunts the intracellular acid load thus leading to an overestimate of β. Consistent with this idea is the acceleration in the rate of pH_i recovery seen in Fig. 1*A* at high pH_o.

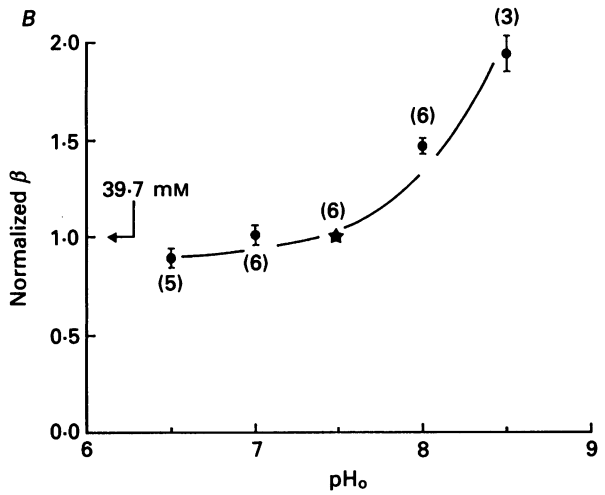


Fig. 1*B*. Apparent intrinsic buffering power plotted as a function of pH_o. Data averaged from six fibres, similar to that shown in *A*. For each experiment, data has been normalized to the value of β (star in graph) estimated at pH_o 7·47 (mean value of β at pH_o 7·5 = 39·72 ± 2·44 mM). Number of fibres indicated next to each point. Line fitted by eye.

Figure 2 shows an experiment where β was estimated (again from the acid load following NH_4Cl removal) under conditions where acid extrusion had been minimized. This was achieved by inhibiting Na^+–H^+ exchange in two ways (i) with Na^+-free solution (Na^+ substituted by *N*-methyl-D-glucamine) and (ii) with 1 mM-amiloride (Deitmer & Ellis, 1980). Figure 2 shows that either method greatly reduced the rate of pH_i recovery and, when this happened, the peak acidosis was increased, i.e. pH_i fell to a lower value than in control conditions. Nevertheless Fig. 2 shows that when the back-extrapolation procedure was applied then similar peak acid loads and similar values of β were obtained (β was 28, 32 and 29 mM respectively). This would suggest that, although acid extrusion normally blunts the acid load, back-extrapolation makes adequate correction. This does not always appear to be the case, however. Figure 3 presents data from eight experiments where β was estimated (20 mM-NH_4Cl removal) first in control conditions and then after addition of 1 mM-amiloride. In both cases, the estimation was performed at a similar mean pH_i. In all cases, back-extrapolation was employed to estimate β. In Fig. 3 the mean value of β was apparently reduced from 34·1 ± 3·2 to 26·6 ± 1·4 mM (n = 8) after addition of amiloride. Closer inspection of the individual results in Fig. 3 also reveals that, when the initial control value of β appeared to be rather high, i.e. > 35 mM then inhibiting acid extrusion with amiloride resulted in a significant fall in the estimated value of

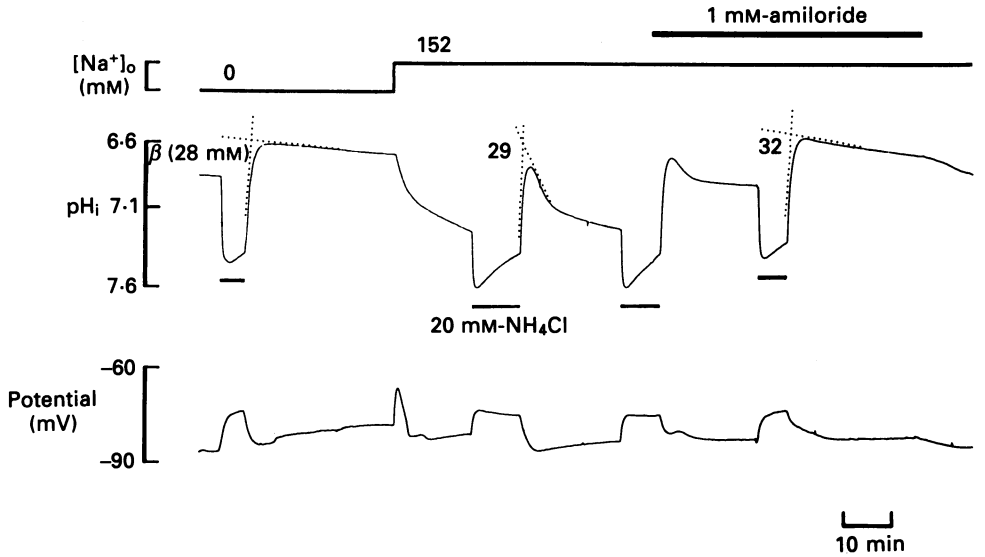


Fig. 2. Effect of inhibiting Na^+–H^+ exchange (Na^+-free solution or 1 mM-amiloride) upon apparent value of β. Traces show (top) pH_i and (bottom) membrane potential. Bars under pH_i trace indicate period of NH_4Cl perfusion (20 mM). Period in Na^+-free solution (Na^+ substituted by *N*-methyl-D-glucamine) indicated at top of figure, as is the period of administration of amiloride. Dotted lines over the pH_i trace indicate estimates of the peak acid load made using the back-extrapolation technique. Note that, in this experiment, although the actual measured acid load is smallest in normal Tyrode solution (at centre of figure), the peak acid loads estimated using back-extrapolation are similar in all three illustrated cases, as are the resulting estimates of β, listed beside the pH_i trace.

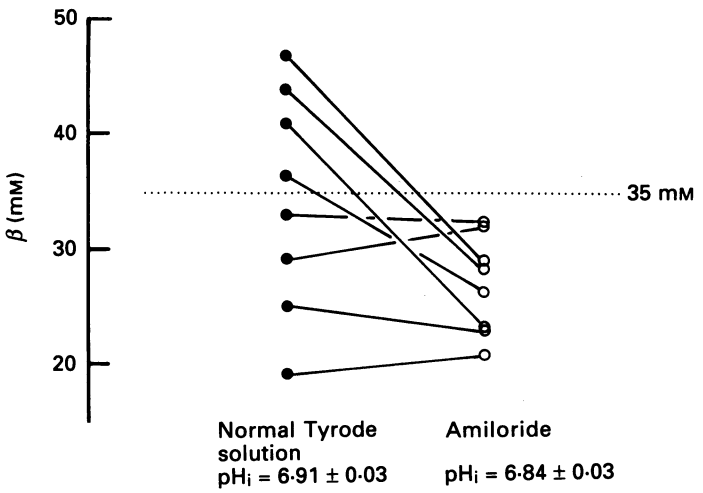


Fig. 3. Inhibition of acid extrusion by amiloride can reduce apparent β. Note that for any individual experiment, when the initial level of β was > 35 mM, its value was significantly reduced by amiloride whereas when initial β was < 35 mM its value was not greatly reduced. β was estimated from the acid load following NH_4Cl removal ± 1 mM-amiloride, as illustrated in Fig. 2.

β. In those control cases where β was initially estimated to be < 35 mM, then the value of β remained essentially unchanged in amiloride. The simplest interpretation of this result is that the larger control values of β are spuriously high due to

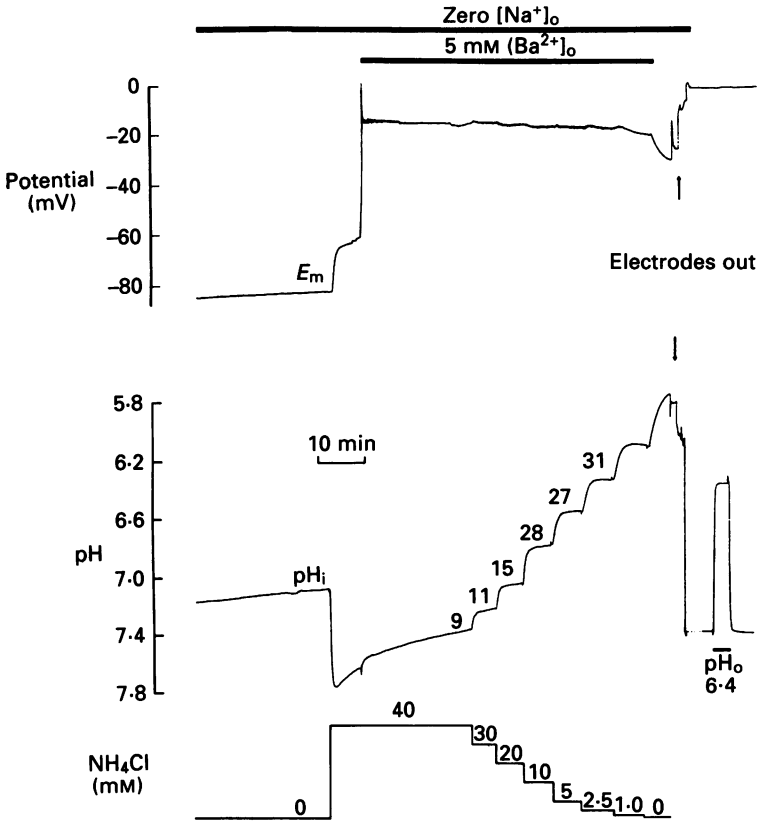


Fig. 4. Experiment to determine pH_i dependence of β using NH_4Cl removal. The fibre was superfused with Na^+-free Tyrode solution (Na^+ substituted by *N*-methyl-D-glucamine) for 30 min before start of records shown here. Traces show (top) membrane potential and (bottom) pH_i. Period of perfusion of 5 mM-$BaCl_2$ shown at top of figure. External NH_4Cl was added and then removed as indicated at the bottom of the figure. Also shown is an extracellular calibration of the pH microelectrode obtained at the end of the experiment. Calculated values of β, for each stepwise acid load, are shown (mM) next to the pH_i trace. pH_o 7·40 throughout experiment.

inadequate correction for acid extrusion but that the lower control values of β are closer to the *true* value of β.

The data shown in this section therefore suggest that back-extrapolation of pH_i recovery may, in some cases, make inadequate correction for acid extrusion. In the remainder of the present work, we have therefore investigated β under conditions where acid extrusion is reduced in Na^+-free solution or in the presence of amiloride.

Variation of β with pH_i: estimated by NH_4Cl removal

Figure 4 shows an experiment where a graded intracellular acid load was induced by means of a graded reduction in the extracellular concentration of NH_4Cl. The advantage of this approach is that it permits many estimates of β in a single

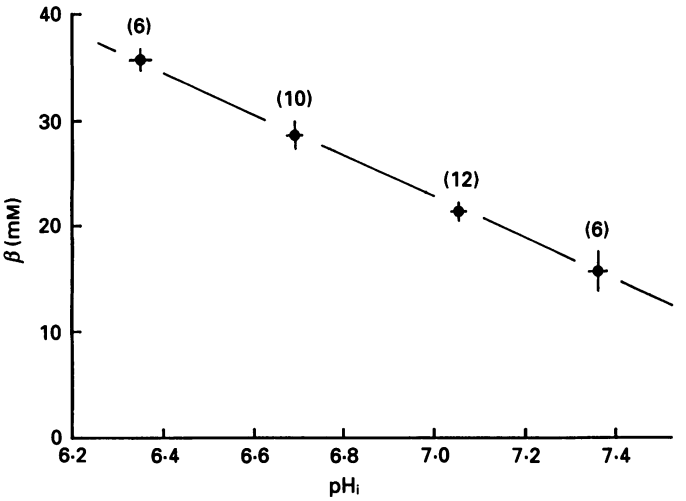


Fig. 5. β increases as pH_i becomes more acid. Data pooled from six fibres (thirty-four determinations of β); experiment similar to that shown in Fig. 4. The fall of pH_i following each external NH_4Cl reduction was used to estimate β. Each value of β is plotted *versus* pH_i (pH_i was taken as the mid-point of each stepwise acid load following NH_4Cl reduction; see Fig. 4). Data have been collected and averaged over four ranges of pH_i (> 6·1–6·5; > 6·5–6·9; > 6·9–7·3; > 7·3–7·7). Line is fitted by least-squares linear regression (correlation coefficient, 0·998) and drawn according to the equation: $\beta = -19{\cdot}6\ pH_i + 160$.

preparation over a wide range of pH_i. Sodium-free solution was used throughout in order to inhibit acid extrusion via Na^+–H^+ exchange. For this reason, initial pH_i was drifting slowly in an acidifying direction. The fibre was then exposed to 40 mM-NH_4Cl in order to permit accumulation of intracellular NH_4^+ ions. Shortly after this, the cell was exposed to 5 mM $[Ba^{2+}]_o$. This was applied in order to reduce movement of NH_4^+ ions through K^+ channels during the subsequent incremental acid-loading phase (any NH_4^+ ion exit following removal of external $[NH_4^+]$ would attenuate the intracellular acid load and would lead to overestimates of β; see Discussion). As a result of the Ba^{2+} application, the membrane potential depolarized to about −15 mV. The fibre was then left for some minutes until pH_i had begun to stabilize whereupon external NH_4Cl was reduced in steps from 40 mM to zero. This produced a stepwise fall of pH_i which settled within about one minute. Each fall of pH_i could be used to estimate β: one observes that the values of β *increase* as pH_i becomes more acid (in Fig. 4, β increases from 9 to 31 mM in the range of pH_i 7·32–6·2). Figure 5 pools data obtained from six experiments similar to that of Fig. 4 (a total of thirty-four determinations of β). Two features are worthy of note: (i) the mean values of β are reasonably low, i.e. < 35 mM; (ii) β is inversely related

to pH_i, with a doubling of β occurring for a 1·0 unit fall of pH_i. The relationship can be described by the empirical equation:

$$\beta = -19{\cdot}6\ pH_i + 160, \tag{3}$$

(linear regression analysis, correlation coefficient, 0·998). For $pH_i = 7{\cdot}2$ (i.e. 'normal' pH_i), $\beta = \sim 20$ mM.

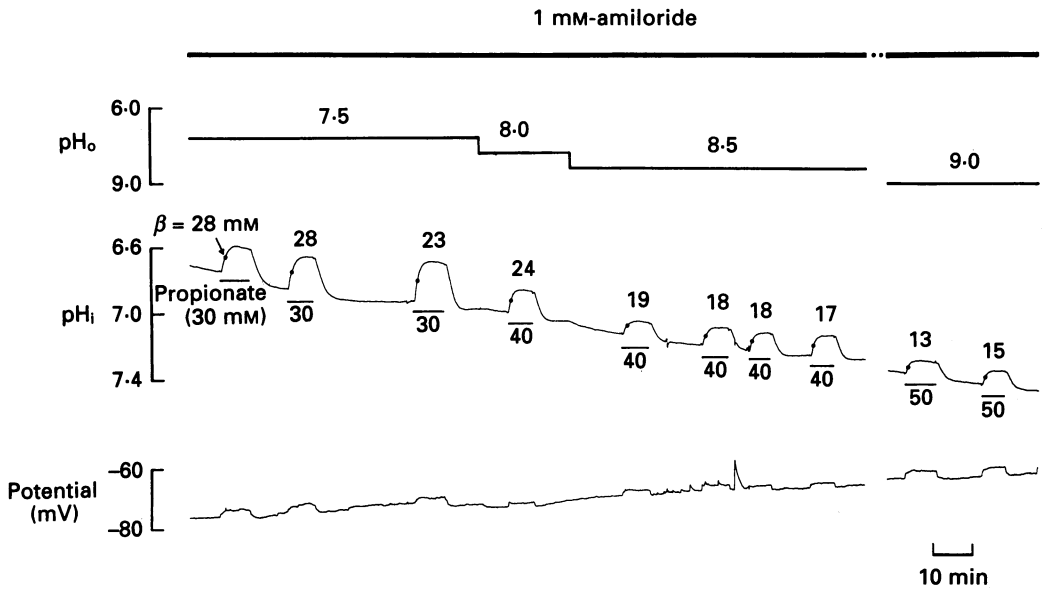


Fig. 6. The pH_i dependence of β determined using propionate addition. The value of β was estimated from the acid load induced by adding external sodium propionate (30–50 mM as indicated at the bars). Traces show (upper) pH_i and (lower) membrane potential. At the beginning of the traces, pH_i was acid because the fibre had been pre-exposed to an acid solution (pH_o 6·5; not shown) for 90 min. The pH_o was then raised to 7·5, 8·0, 8·5 and 9·0 (indicated at top of figure) in order to produce a slow rise of pH_i, onto which the propionate determinations of β could be superimposed; calculated values of β are indicated above the pH_i trace. The filled circles (pH_i trace) indicate pH_i at 50% acid loading during propionate addition: taken as the mean pH_i for each β determination. Gap in traces near to end of figure represents a time interval of 50 min. Amiloride (1 mM) present throughout experiment, to inhibit Na^+–H^+ exchange.

Variation of β with pH_i: estimated by propionate addition

An alternative method of acid loading, other than by NH_4Cl removal, is to *add* a weak acid to the external solution. Figure 6 shows an experiment where external sodium propionate was added, leading to a fall of pH_i which was used to estimate β (see Methods). Amiloride (1 mM) was present throughout in order to minimize acid extrusion. In order to increase the range of pH_i over which β could be measured, pH_i was varied by altering pH_o from 7·5 to 8·0, 8·5 and 9·0. This produced a slow rise of pH_i throughout the experiment onto which several experimental estimates of β were then superimposed. One observes a gradual fall in the value of β as pH_i rises

throughout the experiment. Data pooled from six such experiments are plotted in Fig. 7. Once again, β increases as pH_i becomes more acidic. The relationship is roughly linear and can be described empirically by the equation: β (normalized) = $-0{\cdot}73\ pH_i + 6{\cdot}14$. The mean value of β at pH_i 7·1 (i.e. when β (normalized) = 1·0) was

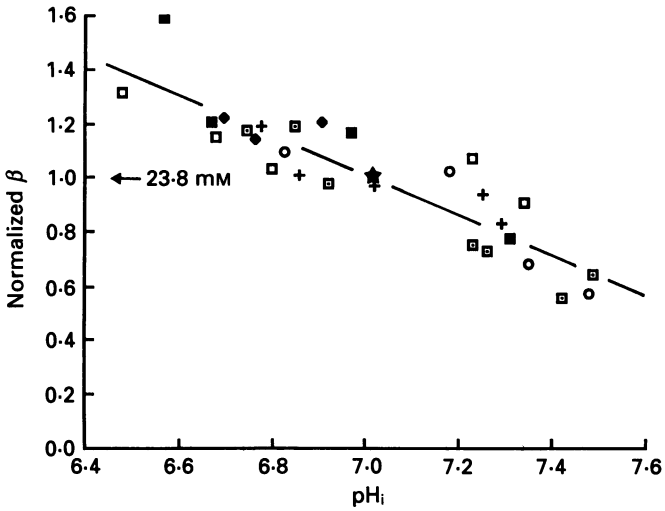


Fig. 7. pH_i dependence of β: propionate method. Data pooled from six experiments similar to that shown in Fig. 6. Different symbols denote different fibres. In each experiment, data are normalized relative to β determined at $pH_i = 7{\cdot}01$ (★). Mean value of β at this pH_i was $23{\cdot}8 \pm 3{\cdot}1$ mM ($n = 6$). β increases roughly linearly as pH_i becomes more acidic. Line (fitted by least-squares linear regression, correlation coefficient 0·80) drawn according to the equation: β (normalized) = $-0{\cdot}732$, $pH_i + 6{\cdot}140$.

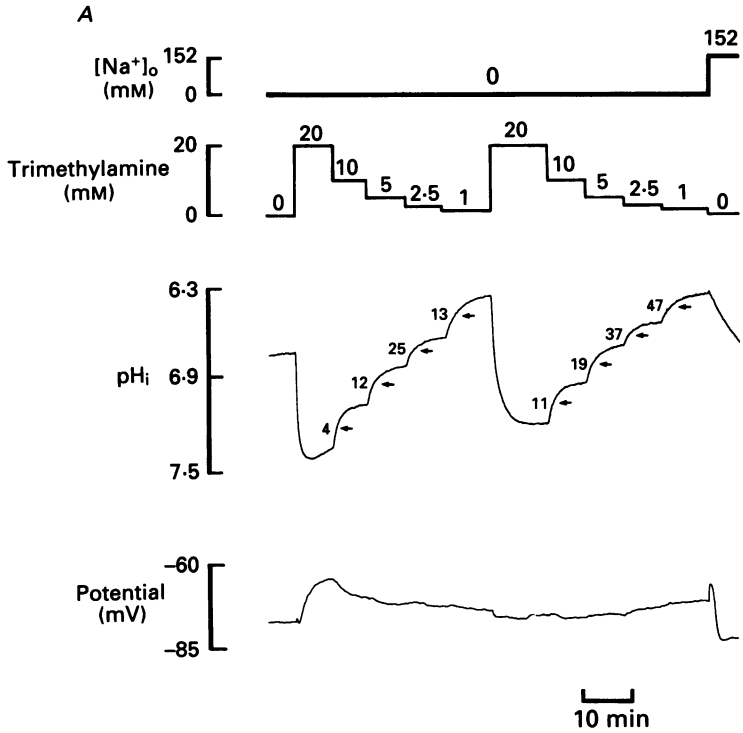
$23{\cdot}8 \pm 3{\cdot}1$ mM ($n = 6$). Using this mean value of β, the equation of the line of best fit in Fig. 7 would be equivalent to:

$$\beta(\text{mM}) = -17{\cdot}4\ pH_i + 146. \quad (4)$$

This equation is very similar to that derived in Fig. 5 and confirms that the variation of β with pH_i is not an artifact of the experimental method of acid loading. Furthermore, the present relationship, determined using propionate addition, was made under conditions where pH_o was varied whereas the previous one (NH_4 removal, Figs 4 and 5) was made under conditions of constant pH_o (7·40). The same result using the two procedures therefore indicates that, at a given pH_i, β is independent of pH_o. This confirms that the apparent increase of β with *extracellular* pH which was observed in Fig. 1 was indeed an artifact caused by stimulation of acid extrusion at high pH_o.

Variation of β with pH_i: estimated by trimethylamine addition or removal

A variety of weak acids and bases were tested recently (Szatkowski & Thomas, 1989) for their suitability in estimating β in molluscan neurones. Two favoured



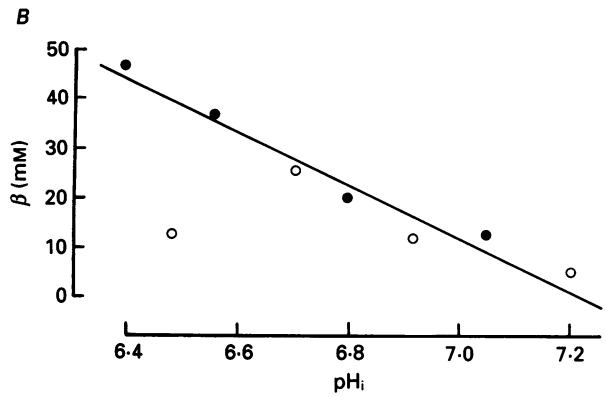


Fig. 8. The pH_i dependence of β determined using trimethylamine removal. *A*, experimental protocol. fibre exposed to Na^+-free solution (for 28 min before start of traces). Addition and removal of TMA-Cl is indicated at top of figure. Traces show (upper) pH_i and (lower) membrane potential. Calculated values of β (mM) are shown beside each stepwise acid load following TMA-Cl reduction. Arrows indicate mean pH_i for each β determination (50% acid load point). pH_o 7·40 throughout experiment. *B*, values for β determined in *A* plotted *versus* pH_i. Line drawn by least-squares, linear regression through all points except that measured at pH_i, 6·48. ○, from first TMA-Cl sequence; ●, from second TMA-Cl sequence shown in *A*.

agents were propionate (weak acid) and trimethylamine (weak base). We therefore concluded our study of β in the Purkinje fibre by testing trimethylamine and comparing it directly with propionate.

Figure 8*A* shows an experiment where 20 mM-external trimethylamine chloride (TMA-Cl) was added and then reduced in a stepwise fashion (Na^+-free solution). This produced a pH_i response similar to that observed with stepwise NH_4Cl withdrawal except that pH_i took much longer to settle following TMA-Cl withdrawal (4–5 min compared with $\sim$ 1 min for NH_4Cl withdrawal). The calculated values of β are plotted *versus* pH_i in Fig. 8*B*. Once again β rises with a fall of pH_i although, in this experiment, the dependence upon pH_i appears steeper than the average slope seen in the previous determinations (Figs 5 and 7). Finally we compared propionate addition with TMA removal in the same fibre. This is shown in Fig. 9. In the first part of the experiment, pH_o was reduced to 6·5 and amiloride (1 mM) added to inhibit acid extrusion. The pH_i therefore acidified slowly throughout this part of the experiment (up to the vertical dotted line). Onto this slow acidosis were superimposed five reversible additions of TMA-Cl and the corresponding *alkaline* loads were used to estimate β. During the second part of the experiment, pH_o was raised to 7·5 and then 8·5 in order to raise pH_i slowly. During this rise of pH_i, several reversible additions of sodium propionate were made and β calculated from the corresponding *falls* of pH_i. Figure 9*B* plots β measured using these two methods, as a function of pH_i. Both procedures produced similar values of β (also comparable to those illustrated in Figs 4–8) confirming that the values of β are independent of the methods used to determine them.

We therefore conclude that, providing acid extrusion is inhibited, three different methods of determining β (NH_4Cl withdrawal, TMA-Cl withdrawal or addition, sodium propionate addition) produce similar values of β and these values vary inversely with pH_i.

Effect of β upon estimates of net acid extrusion

The value chosen for β can significantly influence calculations of net acid extrusion. This is illustrated in Fig. 10 which shows two graphs where net acid extrusion has been plotted as a function of pH_i, the data being taken from the last two pH_i recoveries shown in Fig. 1*A*. The graphs in Fig. 10 should therefore describe the pH_i dependence of the acid extrusion mechanism. Figure 10*A* indicates acid extrusion when pH_o was 7·5 (data from the penultimate pH_i recovery in Fig. 1*A*) and Fig. 10*B* indicates extrusion for $pH_o = 8{\cdot}0$ (the final pH_i recovery in Fig. 1*A*). In each case extrusion has been calculated from the equation:

$$\text{net acid extrusion} = \beta\, d(pH_i)/dt.$$

For the data shown by filled symbols, the chosen value of β is indicated above the pH_i recovery shown in Fig. 1*A* (i.e. the *apparent* value of β, determined by back-extrapolation and thus corrected inadequately for the effects of acid extrusion). For the data shown by open symbols the value of β was estimated using eqn (3). In both Fig. 10*A* and *B*, the activation curves for acid extrusion *versus* pH_i are significantly steeper and the overall magnitude of the acid fluxes larger (●) if the *apparent* β is chosen. Based upon the evidence of the present work, we now conclude that these

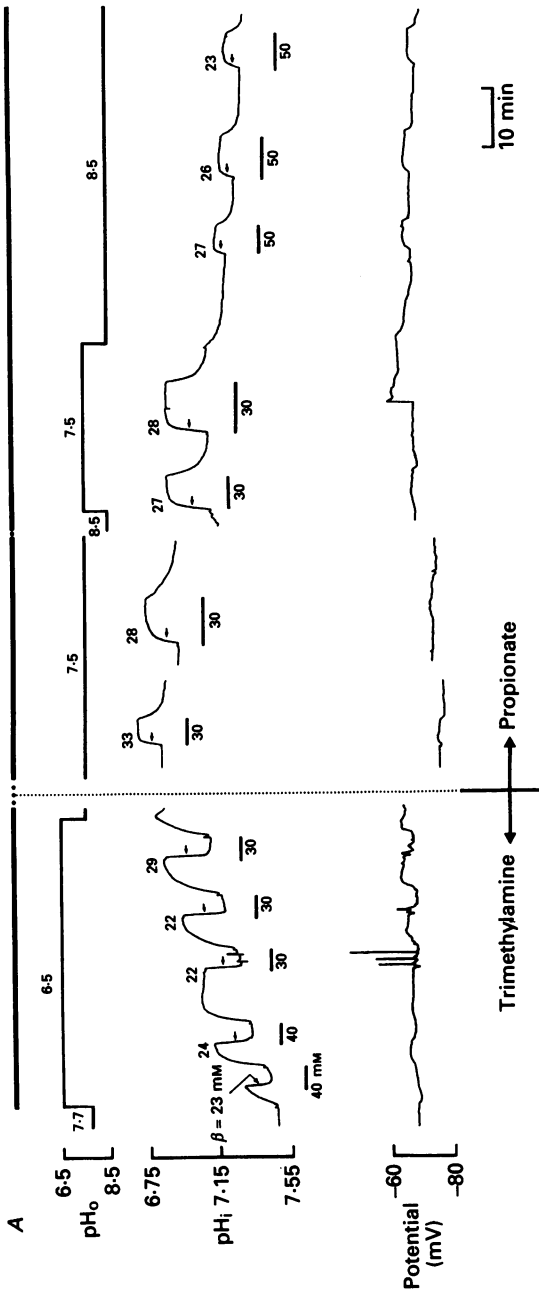


Fig. 9.4. For legend see facing page.

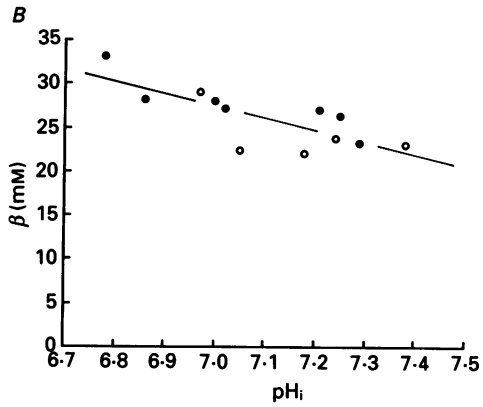


Fig. 9. The pH_i dependence of β: direct comparison, in same fibre, of propionate addition and trimethylamine addition. *A*, experimental protocol. Traces show pH_i (upper) and membrane potential (lower). Changes of pH_o are indicated at the top of the figure as is the period of application of amiloride (1 mM). Trimethylamine chloride or sodium propionate (30–50 mM) were added reversibly at various intervals as indicated by the short bars. Arrows indicate mean pH_i during TMA-induced alkaline load or propionate-induced acid load. Calculated values of β (mM) are displayed above the pH_i trace. Gaps in traces denote time intervals of, respectively: 15 min (at vertical dotted line); 20 min and 50 min. *B*, graph of β plotted as a function of pH_i (data taken from *A*). Symbols show β determined by propionate addition (●) and by TMA addition (○). Line fitted by least-squares linear regression. Note that both methods produce comparable values of β.

latter activation curves are quantitatively incorrect. A lower value of β should be selected (using eqn (3)) and this, plus its variation with pH_i results in the lower curves illustrated in Fig. 10*A* and *B*. Quantitative analyses of pH_i regulation are therefore critically dependent upon the selection of an appropriate value of β.

Finally, inspection of the lower curves in Fig. 10 indicates that, at a given pH_i, elevating pH_o from 7·5 to 8·0 significantly increases net acid extrusion, in agreement with previous observations on the pH_o dependence of Na^+–H^+ exchange in cardiac tissue and in other cell types (Aronson, 1985; Vanheel *et al.* 1986; Vaughan-Jones, Wu & Bountra, 1989).

DISCUSSION

The present work shows that the intrinsic H^+ buffering power, β, of the sheep cardiac Purkinje fibre is about 20 mM when pH_i is at its 'normal' level of about 7·2. The value of β is independent of pH_o but dependent on pH_i. There is a roughly linear increase of β as pH_i falls, a doubling of β occurring for a 1·0 unit decrease in pH_i. Two aspects of these results will be considered, firstly, the absolute magnitude of β and, secondly, the variation of β with pH_i.

The magnitude of intrinsic H^+ buffering power

The present estimate of β (20 mM, at pH_i 7·2) is somewhat lower than earlier estimates in the Purkinje fibre (Ellis & Thomas, 1976, 35 mM; Vanheel *et al.* 1986, 30–50 mM). There are at least two possible reasons for this discrepancy.

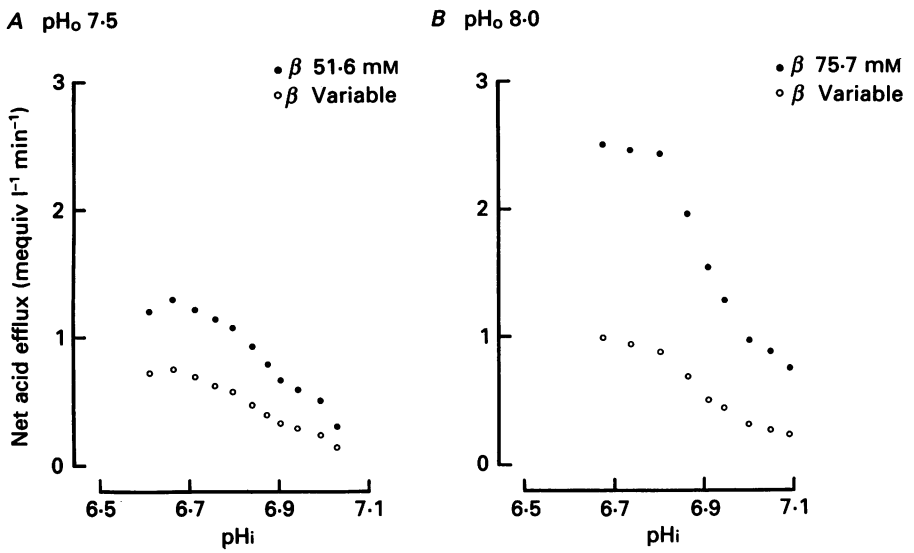


Fig. 10. Influence of value chosen for β upon the pH_i dependence of net acid extrusion. Net acid efflux (efflux = $\beta\, d(pH_i)/dt$) during pH_i recovery from intracellular acidosis (induced by NH_4Cl removal; 20 mM) has been plotted *versus* pH_i for the case where pH_o is 7·5 (*A*) and 8·0 (*B*). Data from last two pH_i recoveries of Fig. 1*A*. In both *A* and *B* the chosen value of β was either that determined directly in Fig. 1*A* (●) or that determined using text eqn (3) (○, labelled 'variable' in *A* and *B*). Note that, in both graphs, the choice of β greatly influences the calculated dependence of acid efflux upon pH_i. Note also that, at a given pH_i, acid efflux is increased by raising pH_o.

(a) We find that β is about 20 mM at pH_i 7·2 but rises to about 40 mM at pH_i 6·2. In order to detect this change in buffering it is necessary to estimate β following small acid loads which decrease pH_i by 0·1–0·2 units. In previous work (Ellis & Thomas, 1976; Vanheel *et al.* 1986), larger acid loads were applied (pH_i changes of 0·5–1·0 units) and so the value of β would have been averaged over the more acidic range of pH_i encompassed by the acid load. As a result, the averaged values of β would automatically be higher than 20 mM.

(b) Previous estimates of β in the Purkinje fibre were made without taking the precaution of inhibiting acid extrusion (Ellis & Thomas, 1976; Vanheel *et al.* 1986). The present work shows that this is not always a problem (cf. Figs 2 and 3) providing the pH_i recovery is extrapolated back to some point close to the moment of initial acid loading. Vanheel *et al.* (1986) have also concluded that such back-extrapolation is acceptable. None the less, the present work shows that, in some experiments, back-extrapolation is inadequate (Fig. 3) so that, at best, the procedure leads to an upper limit for the value of β: it is unlikely to provide a truly reliable measurement. It is far more preferable to inhibit acid extrusion and then apply only small acid loads in order to detect the absolute value of β and its dependence upon pH_i.

Estimates of intrinsic buffering power (β) in other cardiac tissues will also be subject to the same methodological restrictions. Many estimates of β in myocardial tissue have been in the range 45–75 mM (Clancy & Brown, 1966; Ellis & Thomas,

1976; Elliott, 1987). Bountra *et al.* (1990) have recently demonstrated that previous estimates of β in isolated multicellular ventricular preparations have been erroneously high: adding amiloride causes the apparent value of β to fall from ~ 70 to ~ 30 mM. This overestimate of β is much larger than observed here in the Purkinje fibre (cf. Figs 2 and 3) and is probably related to the fact that acid extrusion from ventricular tissue is faster than from the Purkinje fibre, most especially in relation to the speed at which the initial acid load can be imposed. Bountra *et al.* (1990) estimate that the time taken for acid loading of ventricular muscle (NH_4Cl removal or CO_2 addition) is comparable to the time taken for acid extrusion so that a major fraction of the acid load is extruded before peak acidosis has been achieved (thus resulting in a large overestimate of β). Figure 2 of the present work shows that this is much less of a problem in the Purkinje fibre: acid loading (in Na^+-free solution or in the presence of amiloride) is complete within 2 min whereas pH_i recovery in normal Tyrode solution is much slower and is not complete until 15–20 min after peak acidosis. In the present work, in a total of seven fibres, the half-time for acid loading (removal 20 mM-NH_4Cl) in Na^+-free solution was $1{\cdot}06 \pm 0{\cdot}12$ min and in 1 mM-amiloride, it was $0{\cdot}94 \pm 0{\cdot}12$ min ($n = 9$). Vanheel *et al.* (1984, 1986) have reported much slower rates of acid loading in the Purkinje fibre (half-times of 5–7 min) which could severely influence their estimates of β. We do not know the reason for such slow loading but it is possible that their selected preparations were of much larger diameter and less well perfused than our own.

When acid extrusion is inhibited, then values of β estimated in all isolated cardiac preparations and also in single isolated ventricular myocytes appear to be fairly similar: Purkinje fibre, 20–30 mM in pH_i range 7·2–6·6, present work; guinea-pig ventricular muscle, 33 mM although suggested to be even lower, Bountra *et al.* (1990); guinea-pig ventricular myocyte, 15–20 mM, Bountra *et al.* (1990); rat ventricular myocyte, 25 mM, Eisner, Nichols, O'Neill, Smith & Valdeolmillos (1989). This suggests that the intrinsic buffering power among various cardiac tissues is more uniform and significantly lower than previously believed.

It is of interest to compare the above measurements of β with previous estimates made for whole mammalian hearts using nuclear magnetic resonance (NMR) P_i spectroscopy where values of 13–20 μg H^+ per g wet weight of tissue have been reported (Wolfe, Gilbert, Brindle & Radda, 1988). The problem with the NMR method is that the distribution of water between intracellular and extracellular compartments must be known before an estimate of the concentration of cytoplasmic H^+ buffers can be made. If we assume, that about 50% of wet weight is cytoplasmic water (Wolfe *et al.* 1988), then the NMR measurement of β becomes 26–40 mM. Since the NMR estimate was made for the ischaemic heart when pH_i is reduced (hence β would be higher), the value compares favourably with our own measurements made at low pH_i in the Purkinje fibre.

There is one other possible methodological error in estimating β. If NH_4^+ or trimethylamine ions leave the cell upon NH_4Cl withdrawal or propionate ions enter upon sodium propionate addition, there will be a reduction in the intracellular acid load (Roos & Boron, 1981) and, consequently, an overestimate of β. For this reason we applied Ba^{2+} to the external solution when estimating β from NH_4Cl withdrawal (Fig. 4) since Ba^{2+} is known to inhibit K^+ channels which are probably the major transmembrane route for NH_4^+ ions (Boron, 1977). No attempt was made in the present work to limit propionate ion entry but, again, this is likely to have been small. Transmembrane movement

of propionate could occur via Cl^- channels but since, in the steady state, Cl^- conductance is so low ($P_{Cl} \sim 10^{-8}$ cm s^{-1}, Carmeliet & Verdonck, 1977; Vaughan-Jones, 1979) this is unlikely to represent a major route. Alternatively there may be propionate ion entry via the anion-exchange system. However, since $[Cl^-]_o$ was 155 mM, it seems unlikely that propionate entry, at least in competition with external Cl^-, would be significant over the one or two minutes required for acid loading (see Fig. 6), especially given the reasonably high affinity of the anion-exchange system for $[Cl^-]_o$ (Vaughan-Jones, 1986). In general, the good agreement between β calculated using three different methods, suggests that errors are not large.

Variation of β with pH_i

The increase of β with intracellular acidosis may represent an important defence mechanism against acid overload in the heart. As pointed out by Szatkowski & Thomas (1989), in an open system, the buffering power of CO_2–HCO_3 is equivalent to 2·3 $[HCO_3^-]$. At a constant P_{CO_2}, $[HCO_3^-]_i$ will increase exponentially with increases in pH_i (Henderson–Hasselbalch equation). Thus, in the presence of physiological amounts of CO_2, the CO_2–HCO_3 system will dominate total intracellular buffering power at alkaline values of pH_i (when $[HCO_3^-]_i$ is high) but will make much smaller contributions at more acid values of pH_i (when $[HCO_3^-]_i$ is low). An increase in the intrinsic, 'non-CO_2' buffering power at more acidic pH_i values would therefore help to offset the loss of CO_2–HCO_3 contributions to total cellular buffering power.

When estimating β at a given pH_i one is, in effect, measuring the slope of the acid–base titration curve of the intracellular cytoplasmic compartment. The variation of β with pH_i therefore indicates that the buffer slope is not constant, but increases as pH_i falls, i.e. the titration is a non-linear one. The fact that β appears to be a linear function of pH_i may, however, be fortuitous. If a larger range of pH_i were examined the relationship might appear curvilinear. One should note that the buffering power of a compound rises steeply as pH approaches the pK_a of the compound. The rise of β at low pH_i therefore suggests an average pK_a for intrinsic cellular buffers significantly lower than physiological pH.

An increase of intrinsic β with a fall of pH_i has been suggested recently by Wolfe *et al.* (1988) for the ischaemic myocardium. They propose that β increases steeply at $pH_i < 6{\cdot}4$, consistent with an intracellular buffer system of $pK_a \sim 5{\cdot}0$. At $pH_i > 6{\cdot}4$, β may vary by much less but inspection of their Fig. 4 indicates a doubling of β over the pH_i range 7·4–6·4 which is similar to our own findings in the Purkinje fibre. A qualitatively similar variation of β with pH_i has been reported for the giant barnacle muscle fibre (Boron, 1977), the snail neurone (Szatkowski & Thomas, 1988) and the rat renal mesangial cell (Boyarsky, Ganz, Sterzel & Boron, 1988). It is therefore puzzling that an *opposite* dependence of β upon pH_i (i.e. β increases with *increasing* pH_i) has been proposed for rat brain synaptosomes (Jean, Frelin, Vigne, Barbry & Lazdunski, 1985) where high values for β (up to 48 mM) were reported at a pH_i of $\sim 7{\cdot}4$.

The increase of β with acidosis in the mesangial cell (Boyarsky *et al.* 1988) resembles our own data for the Purkinje fibre to the extent that β doubles for a 1·0 unit fall of pH_i (7·4 to 6·4). Absolute values for β in the mesangial cell, however, are notably much lower (~ 5 mM at pH_i 7·2), more in line with the preferred value (~ 9 mM) selected by Aickin (1984) for the mouse vas deferens and by Szatkowski & Thomas (1989) for the molluscan neurone. Although we have measured similarly low

values in some cardiac Purkinje fibres (e.g. Figs 4 and 8), nevertheless, *on average*, β appears to be at least twice this value. When combined with contributions from CO_2–HCO_3 (5% CO_2; $[HCO_3^-]_i$, 12 mM at pH_i of 7·2) the overall intracellular buffering power of the normal cardiac cell would therefore be 20 (non-CO_2) + 27·6 (CO_2; open system) = 47·6 mM. Confirmation, however, is still required that the CO_2 contribution can indeed be estimated by assuming an open system. Aickin, for example, has proposed virtually no CO_2 contribution to total β in isolated guinea-pig vas-deferens (Aickin, 1988).

Finally, we should consider what chemical moieties may be contributing to intrinsic buffering in the Purkinje fibre. As argued recently by Bountra *et al.* (1990), the bulk of H^+ buffering must come from H^+ titratable groups on intracellular proteins. House, Miller & O'Dowd (1989) report that imidazole levels are ~ 10 mM in rat ventricular tissue and since these can have pK_a values within the physiological range of pH_i, they could contribute significantly to β. House *et al.* (1989) report higher imidazole levels in atrial tissue. It would be of interest, therefore, to measure β in atrial tissue to see if it is higher than in ventricle.

Value of β is important for analysis of pH_i regulation

The present work suggests that previous estimates of β in the Purkinje fibre (35–50 mM) are only appropriate when pH_i is < 6·6. Hence, when analysing pH_i recovery from an acid load, if these values for β are employed then the computed net acid efflux will be overestimated at the more alkaline values of pH_i. As shown in Fig. 10, this sort of error distorts the overall shape of the activation curve for acid efflux *versus* pH_i. There are obvious reasons for wishing to minimize such distortion, not least of which is the fact that the shape and steepness of the pH_i activation curve is frequently used to distinguish among possible molecular models for acid extrusion (see for example Aronson, 1985). The present analysis of the pH_i dependence of β is therefore essential for any quantitative analysis of pH_i regulation in the heart.

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