Regulation of intracellular pH in the smooth muscle of guinea-pig ureter: Na^+ dependence

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1. Mechanisms involved in the regulation of intracellular pH (pH_i) in smooth muscle cells of guinea-pig ureter have been investigated using double-barrelled pH-sensitive microelectrodes in isolated strips of tissue.

2. Removal of CO_2–HCO_3^- from the superfusing solution caused a fall in the steady-state pH_i except in a few cells which had been excised from the animal for many hours (usually >24 h). The pH_i value was 7·22 ± 0·09 ($n = 89$; mean ± S.D. of an observation) in solution buffered with 5% CO_2–21 mM HCO_3^-, compared with 6·92 ± 0·24 ($n = 67$) in the nominal absence of CO_2–HCO_3^-. Recovery from experimentally induced acidosis was faster in the presence, rather than nominal absence, of CO_2–HCO_3^- (mean half-times of 2·7 ± 0·7 min, $n = 41$, and 4·6 ± 1·3 min, $n = 12$, respectively). These results suggest the presence of both HCO_3^--dependent and -independent mechanisms for the effective extrusion of acid equivalents.

3. Recovery from acidosis was dependent on external Na^+ (Na_o^+) in both the presence and nominal absence of CO_2–HCO_3^-, with an apparent half-maximal activation at approximately 4 and 20 mM Na_o^+, respectively. Removal of Na_o^+ in the steady state caused a fall in pH_i which proceeded at a faster rate in the presence rather than in the nominal absence of CO_2–HCO_3^-.

4. Amiloride (100 μM–1 mM) reversibly inhibited the recovery from acidosis and caused a fall in the steady-state pH_i when applied in the nominal absence of CO_2–HCO_3^-, but had no measurable effect on either the recovery from acidosis or steady-state pH_i in the presence of CO_2–HCO_3^-. These results suggest that Na^+–H^+ exchange was responsible for extrusion of acid equivalents in the nominal absence of CO_2 and HCO_3^-, but that it played little part under more physiological conditions.

5. Although Na^+–H^+ exchange appeared to be activated below a pH_i of about 7·2, it was incapable of maintaining a 'normal' pH_i in the nominal absence of CO_2–HCO_3^- in freshly excised cells, where values between 6·06 and 6·89 were recorded. Only in aged preparations, in which the intrinsic intracellular acid loading was substantially reduced (as judged from the rate of acidification on application of amiloride in the nominal absence of CO_2–HCO_3^-) did the steady-state value approximate to that observed in the presence of CO_2–HCO_3^-, at about 7·2.

Intracellular pH (pH_i) is a fundamentally important parameter to the physiology of all cells. Enzyme activity, and hence cell function, is critically dependent on pH and, conversely, changes in metabolic rate directly affect cellular pH. Therefore, mechanisms for regulation of pH_i are paramount and have been found to maintain a considerably more alkaline level than that predicted by a passive distribution of H^+ ions in the vast majority of cells (see Roos & Boron, 1981). However, unlike the universal nature of the Na^+ pump for active extrusion of Na^+ ions, the effective active extrusion of H^+ ions has proved to be mediated by several different mechanisms. Pioneering work in invertebrate preparations revealed a Na^+-, HCO_3^--, and Cl^--dependent mechanism (see Roos & Boron, 1981) but this has subsequently been only infrequently identified in vertebrate preparations, where an amiloride-sensitive Na^+–H^+ exchange has been universally found (see Aickin, 1986). It is therefore essential to characterize the mechanism or mechanisms involved in the regulation of pH_i in any given preparation

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in order to understand how changes in pH may affect other intracellular ions and vice versa. Smooth muscle is of particular interest because the small size of the cells, together with their high metabolic rate, would suggest that large and rapid changes in pH_i may occur. Consequently, cellular function may be modulated by pH_i. For example, changes in pH_i caused by accumulation of metabolites may contribute to autoregulation of the peripheral circulation (e.g. see Harder, 1982). Recently, it has also been suggested that the action of many pharmacological agents, including catecholamines (Owen, 1986) and angiotensin (Hatori, Fine, Nakamura, Cragoe & Aviv, 1987), may be mediated via alteration of pH_i.

Regulation of pH_i in mammalian smooth muscle has only recently been investigated, principally because of the technical problems imposed by the physical dimensions of the cells. Microelectrodes are difficult to use because of the sharpness required for successful impalement, whereas indirect methods, such as the distribution of weak acids (e.g. Gerstheimer, Mühleisen, Nehring & Kreye, 1987) or nuclear magnetic resonance (e.g. Dawson & Wray, 1985), are severely limited by their poor time resolution. Such problems have recently been circumvented with the development of intracellular fluorescent pH indicators and these have now been used by several groups in various smooth muscles. Nevertheless, the first indication of the nature of a mechanism which might be involved in the effective extrusion of acid equivalents in smooth muscle came indirectly from the finding of a substantial amiloride-sensitive fraction of Na^+ uptake (Smith & Brock, 1983); amiloride was assumed to have inhibited Na^+–H^+ exchange. Further indirect evidence for the involvement of Na^+–H^+ exchange in extrusion of acid equivalents came from the observation that amiloride slowed the decline in tension from a contracture induced by intracellular acidosis (Ighoroje & Spurway, 1985). Shortly afterwards, direct investigation of the regulation of pH_i in smooth muscle confirmed the role of Na^+–H^+ exchange with the demonstration that recovery from acidosis was Na^+ dependent (Aickin, 1985; Weissberg, Little, Cragoe & Bobik, 1987) and sensitive to the potent amiloride analogue EIPA (ethylisopropylamiloride; Weissberg *et al.* 1987). Virtually all of these experiments, however, were conducted in the nominal absence of the biological buffer CO_2–HCO_3^-.

Every subsequent investigation of the regulation of pH_i in smooth muscle has confirmed the Na^+ dependence and amiloride sensitivity of the recovery from acidosis in non-CO_2–HCO_3^- buffers, but there is no such consistency in the results obtained in the presence of CO_2–HCO_3^-. Some cells show an initially surprising steady-state alkalosis in the presence of CO_2–HCO_3^- (Aickin, 1984; Korbmacher, Helbig, Stahl & Wiederholt, 1988; Boyarsky, Ganz, Sterzel & Boron, 1988; Putnam & Grubbs, 1990) while others exhibit essentially the same steady-state pH_i in both the presence and nominal absence of CO_2–HCO_3^- (Aalkjær & Cragoe, 1988; Vigne, Breittmayer, Frelin & Lazdunski, 1988; Baro, Eisner, Raimbach & Wray, 1989). Furthermore, in some cells, the rate of recovery of pH_i from acidosis is accelerated by the presence of CO_2–HCO_3^- (Aalkjær & Cragoe, 1988; Boyarsky *et al.* 1988; Baro *et al.* 1989; Putnam, 1990) while in others the rate is unaffected by their presence or nominal absence (Korbmacher *et al.* 1988; Vigne *et al.* 1988).

With the exception of Aalkjær & Cragoe (1988), all of the work in the studies cited above was done in isolated or cultured cells. Although this approach allows acquisition of data that would be very much more difficult to obtain from smooth muscle cells *in situ*, it is open to question how representative of the normal physiological state the results are. In culture, smooth muscle loses at least one fundamental property, that of contractility. Therefore, I have used a double-barrelled microelectrode in freshly excised strips of smooth muscle. This method also has the clear advantage over fluorescent indicators of giving simultaneous measurement of membrane potential, which not only indicates the physiological state of the cell but also can provide invaluable information about the electrical coupling of transport processes. In this and the following paper (Aickin, 1994*a*), I have attempted to characterize the mechanisms responsible for regulation of pH_i in the smooth muscle cells of guinea-pig ureter. In this paper, I have demonstrated the presence of both HCO_3^--dependent and -independent mechanisms for the effective extrusion of acid equivalents and have investigated their Na^+ dependence and amiloride sensitivity. In the steady state in the nominal absence of CO_2–HCO_3^-, pH_i generally falls and recovery from acidosis is then brought about by Na^+–H^+ exchange. In the presence of CO_2–HCO_3^-, Na^+–H^+ exchange is apparently switched off and recovery from acidosis is then dominated by the Na^+- and HCO_3^--dependent mechanism. This mechanism has been characterized in the following paper as an electroneutral Na^+–HCO_3^- cotransport (Aickin, 1994*a*). This situation, however, does not hold for all freshly excised smooth muscles. In the third paper of this series (Aickin, 1994*b*), I describe results from similar experiments performed in the smooth muscle cells of guinea-pig femoral artery which indicate that Na^+–H^+ exchange dominates the recovery from acidosis in both the presence and nominal absence of CO_2–HCO_3^-.

Some of these results have been communicated to the Physiological Society (Aickin, 1985) and have been published in preliminary form (Aickin, 1988, 1989).

METHODS

These experiments were performed on isolated strips of smooth muscle from the central portion of the ureter of albino male guinea-pigs. The animals were stunned and bled before

the ureters were removed and maintained in modified Krebs solution, as given below. One of the ureters was usually stored in a refrigerator for use on the following day. Each ureter was bisected down its long axis, and a short length (approximately 5 mm) of endothelium peeled away. This section, together with approximately 5 mm at either end was then mounted, inner surface uppermost, with the complete thickness of the ureter wall in rotatable clamps. The preparation could then be well stretched over a silicone rubber wedge.

Double-barrelled microelectrodes (Aickin, 1981) were used to measure membrane potential (E_m) and pH_i simultaneously within a single cell. In brief, micropipettes, pulled from two heat-fused filamented glass capillaries, were rendered hydrophobic by treatment with dimethyltrimethylsilylamine vapour (Fluka, Gillingham, Dorset, UK) at 180 °C. The micropipettes were removed from the oven 2–4 days later and immediately backfilled, first with a proton cocktail (Ammann, Lanter, Steiner, Schulthess, Shijo & Simon, 1981; Fluka) in one barrel and then with a reference liquid ion exchanger (Thomas & Cohen, 1981) in the other. They were then usually left overnight under reduced pressure, to facilitate formation of uninterupted columns, before a citrate buffer (58 mM NaCl, 38 mM sodium citrate and 19 mM citric acid saturated with 100% CO_2) was placed above the proton cocktail and 500 mM KCl above the reference liquid ion exchanger. Electrical contact to varactor bridge diodes (model 311J; Analog Devices, Walton-on-Thames, Surrey, UK) was made via Ag–AgCl wires. Electrodes made using ion-selective ligands mostly suffer from interference and the response of these electrodes was therefore checked. Problems related to the CO_2 sensitivity of the proton cocktail were avoided by ensuring that the citrate buffer remained saturated with 100% CO_2. No pH sensitivity was found in response to ammonium salts (up to 7·5 mM) or trimethylamine (up to 5 mM) in either the presence or nominal absence of CO_2–HCO_3^-. However, the reference liquid ion exchanger showed an apparent 0–4 mV depolarization in the presence of an ammonium salt (up to 7·5 mM) and trimethylamine (up to 5 mM). No correction has been made to the intracellular recordings. Thus, both the depolarization and the alkalinization observed on application of these substances may be overestimated by up to 4 mV. The proton cocktail was found to show significant sensitivity to weak organic acids, but only in the presence of CO_2–HCO_3^-. Sodium butyrate (10 mM) added to the standard CO_2–HCO_3^--buffered solution caused an apparent alkaline shift of $0·20 \pm 0·05$ pH units (10 electrodes) but when added to nominally CO_2-free solution it caused, at most, an apparent 0·02 pH unit alkaline shift.

The modified Krebs solution had the following composition (mM): NaCl, 115; KCl, 5·9; $CaCl_2$, 2·5; $MgCl_2$, 1·2; $NaHCO_3$, 21; glucose, 11; Na_2HPO_4/NaH_2PO_4 at pH 7·35, 0·1; equilibrated with nominally 5% CO_2–95% O_2 at 35 °C. The pH of this solution varied between 7·36 and 7·47 with different gas cylinders (BOC, Guildford, UK). Nominally CO_2–HCO_3^--free solution was buffered with 21 mM NaHepes to the same pH as the CO_2-containing solution and was equilibrated with 100% O_2. Low-Na^+ and Na^+-free solutions were prepared by equimolar substitution with *N*-methyl-D-glucamine (NMDG) or Tris, where necessary the HCO_3^- salts (21 mM) being produced by prolonged bubbling with gas from the 5% CO_2 cylinder. Ammonium salts were added from 1 M stock solutions immediately prior to use. The drugs, amiloride (Sigma Chemical Co., Poole, Dorset, UK) and acetazolamide (Sigma), were added directly to the experimental solution immediately prior to use.

All values are given as means ± S.D. with the number of observations (n).

RESULTS

Modification of intracellular pH

Figure 1 illustrates the effects of standard procedures for the displacement of pH_i (for a full description see Thomas, 1984) in a smooth muscle cell of the guinea-pig ureter. Predictably, both removal of CO_2–HCO_3^- from the superfusing solution and addition of an ammonium salt caused immediate intracellular alkalosis, while readdition of CO_2–HCO_3^- and removal of the ammonium salt each caused an immediate intracellular acidosis. Each of these displacements, assumed to be due to the chemical consequences of the free passage of CO_2 or NH_3 across the cell membrane, was followed by a recovery. Recovery from acidosis induced by either method, recorded in CO_2–HCO_3^--buffered solution, could be described by a single exponential with a mean half-time of $2·7 \pm 0·7$ min ($n = 41$) and restored pH_i to the control level (i.e. the steady-state value previously recorded in CO_2–HCO_3^--buffered solution). This recovery must reflect the effective extrusion of acid equivalents against their electrochemical gradient and, in common with many earlier studies, has been used to investigate the nature of the mechanisms responsible. Notably, this recovery was accompanied by a transient hyperpolarization.

Recovery from intracellular alkalosis did not exhibit the same invariable characteristics as that from acidosis, but it should be stressed that, in this case, the recovery could result from a combination of passive leakage (either inwards of H^+, or in the case of exposure to an ammonium salt, NH_4^+, or outwards of OH^- or HCO_3^- ions), metabolic production of acid equivalents, and possibly effective inward transport of acid equivalents. The fall in pH_i following alkalosis on removal of CO_2–HCO_3^- from the superfusing solution was particularly variable, both in initial rate and extent. In the majority of cells, pH_i initially fell quite rapidly and stabilized at a considerably lower level than recorded in the presence of CO_2–HCO_3^-, but in some (11 of 60 cells) pH_i fell fairly slowly to stabilize at approximately the same level (see Fig. 8). This latter behaviour was more common in preparations which had been stored overnight at 7 °C (7 of 16 cells) but was also seen to develop over a period of several hours in fresh preparations (4 of 44 cells), as illustrated in Fig. 2. This behaviour was not affected by altering the buffering capacity of the solutions, either by reducing the NaHepes concentration in the nominally CO_2–HCO_3^--free solution from 21 to 5 mM or by adding 21 mM NaHepes to the CO_2–HCO_3^--buffered solution. The mean pH_i recorded in the nominal absence of CO_2–HCO_3^- was $6·92 \pm 0·24$ ($n = 67$, range 6·06–7·37) with a mean membrane potential

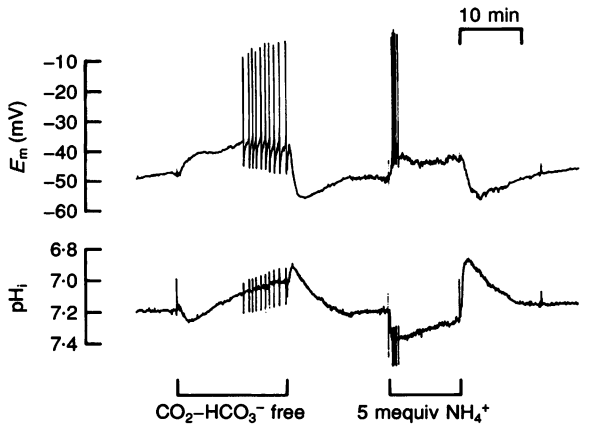

Figure 1. Pen recordings of the membrane potential (E_m) and intracellular pH (pH_i) in a smooth muscle cell of guinea-pig ureter, recorded using a double-barrelled microelectrode, illustrating the effects of cellular acid and alkaline loading by alteration of the external CO_2 level and by application and removal of an ammonium salt

E_m was recorded using the reference liquid ion exchanger and pH_i was recorded using the proton cocktail. The preparation was superfused with modified Krebs solution buffered with 5% CO_2–21 mM HCO_3^- except during the interval indicated when it was replaced with nominally CO_2–HCO_3^--free solution, equilibrated with 100% O_2 and buffered with 21 mM NaHepes. $(NH_4)_2SO_4$ (2·5 mM) was added to the modified Krebs solution during the 12 min interval indicated. Spontaneous action potentials are reduced in amplitude due to the low frequency response of the reference liquid ion exchanger and the pen recorder and appear in the pH_i trace due to inequality in the resistance of the two barrels of the microelectrode.

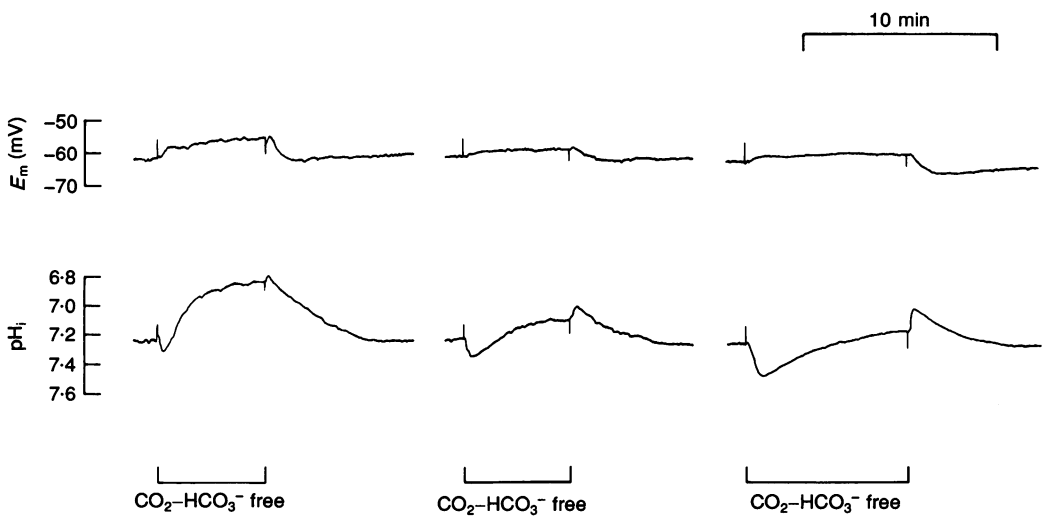

Figure 2. Pen recordings of parts of an experiment showing the effect of removal and readdition of CO_2–HCO_3^- to the superfusing solution in a single cell over a period of several hours

The first removal and readdition was made 1·5 h after impalement of the cell, about 3 h after the preparation had been excised from the animal. The second removal and readdition was performed after a further interval of 2·5 h and the third, after another 3 h. The preparation was maintained in modified Krebs solution buffered with 5% CO_2–21 mM HCO_3^- except during the intervals indicated when it was replaced with a solution equilibrated with 100% O_2, buffered to the same pH with 21 mM NaHepes.

(E_m) of $-49{\cdot}6 \pm 7{\cdot}6$ mV (range -38 to -60 mV) compared with a mean pH_i of $7{\cdot}22 \pm 0{\cdot}09$ ($n = 89$, range 7·03–7·45) at a mean E_m of $-57{\cdot}5 \pm 4{\cdot}3$ mV (range -40 to -65 mV) in the presence of 5% CO_2.

Effect of the nominal absence of CO_2–HCO_3^- on the recovery from intracellular acidosis

The low pH_i usually recorded in the nominal absence of CO_2–HCO_3^- may result from an increased rate of intracellular acid loading and/or from a decreased capacity for the effective extrusion of acid equivalents. Although observations in this and the following paper (Aickin, 1994*a*) suggest that the former may occur (see Discussion), the latter would also be involved if a HCO_3^--dependent mechanism contributed to the normal extrusion of acid equivalents. This possibility was therefore investigated, as illustrated in Fig. 3. Recovery from an acid load induced by application and removal of an ammonium salt was slower in the nominal absence of CO_2–HCO_3^- than in their presence. The mean half-time of the recovery was $4{\cdot}6 \pm 1{\cdot}3$ min ($n = 12$) compared with $2{\cdot}7 \pm 0{\cdot}7$ min ($n = 41$) in the presence of CO_2–HCO_3^-. In six cells where recoveries were directly compared, as in Fig. 3, the half-times were significantly different ($P = 0{\cdot}003$, Student's paired t test).

This result suggests the presence of a HCO_3^--dependent mechanism for the effective extrusion of acid equivalents and, since the cells still showed a fairly brisk recovery from acidosis in the nominal absence of CO_2–HCO_3^-, it also indicates the presence of a HCO_3^--independent process. Estimation of the relative contribution of each process, however, requires a knowledge of the intracellular buffering power.

Determination of intracellular buffering power

Measurement of intracellular buffering power, the capacity of the cellular constituents to resist a change in pH on application of an acid or alkali, is best made when an applied acid or alkaline load is not followed by any further change in pH_i. This condition was difficult to achieve on acidosis, since inhibition of the mechanisms responsible for effective acid extrusion resulted in a very low pH_i and unstable impalements, but was relatively simple to achieve on alkalosis. Addition of 5 mM trimethylamine, as shown in Fig. 4, caused an immediate intracellular alkalosis followed by little (in the presence of CO_2–HCO_3^-) or no (in the nominal absence of CO_2–HCO_3^-) recovery. Consequently, on removal of trimethylamine there was little or no overshoot in pH_i, in contrast to the marked overshoot observed on removal of an ammonium salt (e.g. Figs 1 and 3). Furthermore, the slow recovery from alkalosis observed in the presence but not nominal absence of CO_2–HCO_3^-, presumably reflecting HCO_3^- permeability or a HCO_3^--dependent transport process, could be inhibited by the presence of DIDS (4,4′-diisothiocyanatostilbene-2,2′ disulphonic acid). Thus, changes in pH_i observed on application and removal of trimethylamine seemed ideally suited for determination of the intracellular buffering power. It is clear from the experiment shown in Fig. 4 that the change in pH_i induced by application of a given alkaline load at a given

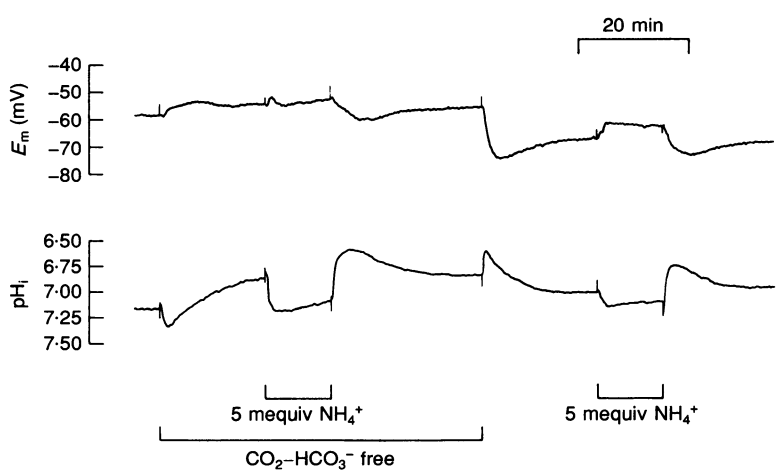

Figure 3. Pen recordings of an experiment to investigate the effect of the nominal absence of CO_2–HCO_3^- on the recovery of pH_i from an acid load induced by application and removal of 2·5 mM $(NH_4)_2SO_4$

The preparation was superfused with modified Krebs solution buffered with 5% CO_2–21 mM HCO_3^- except during the interval indicated when it was replaced with a solution equilibrated with 100% O_2, buffered to the same pH with 21 mM NaHepes.

pH_i was the same in the presence as in the nominal absence of CO_2. In other words, the intracellular buffering power was apparently unaffected by the presence or nominal absence of CO_2. The mean value calculated from application and removal of trimethylamine was $13{\cdot}4 \pm 4{\cdot}8$ mequiv H^+ (pH unit)$^{-1}$ l^{-1} ($n = 20$) in the presence of 5% CO_2 and $14{\cdot}1 \pm 4{\cdot}7$ mequiv H^+ (pH unit)$^{-1}$ l^{-1} ($n = 21$) in the nominal absence of CO_2.

Intracellular buffering power has also been calculated from experiments in which the recovery from alkalosis on application of an ammonium salt (and thus the overshoot on its removal) was inhibited by the presence of DIDS (see Fig. 9 of Aickin, 1994*a*) or by the prolonged absence of Cl_o^- (see Fig. 8 of Aickin, 1994*a*). Mean values obtained after equilibration with DIDS were $12{\cdot}7 \pm 3{\cdot}9$ mequiv H^+ (pH unit)$^{-1}$ l^{-1} ($n = 9$) in the presence of 5% CO_2 and $13{\cdot}6 \pm 3{\cdot}4$ mequiv H^+ (pH unit)$^{-1}$ l^{-1} ($n = 8$) in its nominal absence, and after prolonged exposure to Cl^--free solutions was $13{\cdot}8 \pm 3{\cdot}7$ mequiv H^+ (pH unit)$^{-1}$ l^{-1} ($n = 12$) in the presence of 5% CO_2. It should, however, be noted that the cellular buffering power is generally assumed to be increased in the presence of an extracellular CO_2–HCO_3^- buffer by 2·3 times $[HCO_3^-]_i$ (see Roos & Boron, 1981). Thus, if the CO_2–HCO_3^- system contributed to intracellular buffering as expected, the buffering power should be increased by about 35 mequiv H^+ (pH unit)$^{-1}$ l^{-1} at the normal steady-state pH_i. It is therefore very surprising that an increase of this magnitude was not detected.

The contribution of CO_2–HCO_3^- to intracellular buffering depends not only on there being an open system, conferred by the supposed free passage of CO_2 across cell membranes and by the essentially clamped external CO_2 level, but also on the rapid hydration and dehydration of CO_2. This reaction is catalysed by carbonic anhydrase in many cell types but apparently not in smooth muscle (e.g. Mühleisen & Kreye, 1985). It is notable that in cells where CO_2 contributes greatly to the intracellular buffering power and carbonic anhydrase is present, inhibition of carbonic anhydrase reduces the functional buffering power (Thomas, 1984). Therefore, it seemed possible that, if there was a lack of carbonic anhydrase in the ureter, slow equilibration of the CO_2 hydration reaction might be partly responsible for the apparent failure of CO_2–HCO_3^-

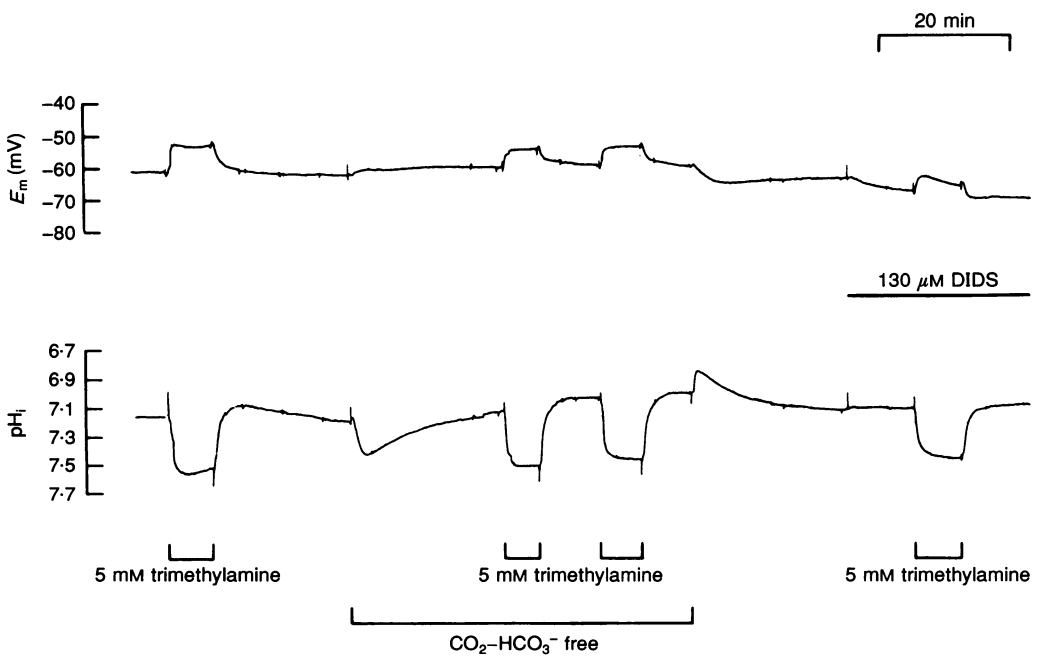

Figure 4. Pen recordings of an experiment to determine intracellular buffering power in the presence (5%) and nominal absence of CO_2

Trimethylamine (5 mM) was added to the superfusing solution during the intervals indicated and towards the end of the experiment 130 μM DIDS was added to the superfusing solution. Buffering power, calculated from the observed changes in pH_i, was 10·2, 10·1, 13·9 and 13·8 mequiv H^+ (pH unit)$^{-1}$ l^{-1} in the presence of 5% CO_2 and 12·0, 9·9, 11·6 and 10·6 mequiv H^+ (pH unit)$^{-1}$ l^{-1} in the nominal absence of CO_2–HCO_3^- (values given from left to right in each case). Note that if the CO_2–HCO_3^- system did contribute to intracellular buffering as expected theoretically, the buffering power should have been increased by about 48 mequiv H^+ (pH unit)$^{-1}$ l^{-1}. In other words, the changes observed in the presence of CO_2–HCO_3^- should have been about 6 times smaller. The preparation was superfused with modified Krebs solution buffered with 5% CO_2–21 mM HCO_3^- except during the interval indicated when it was replaced with a solution equilibrated with 100% O_2, buffered to the same pH with 21 mM NaHepes.

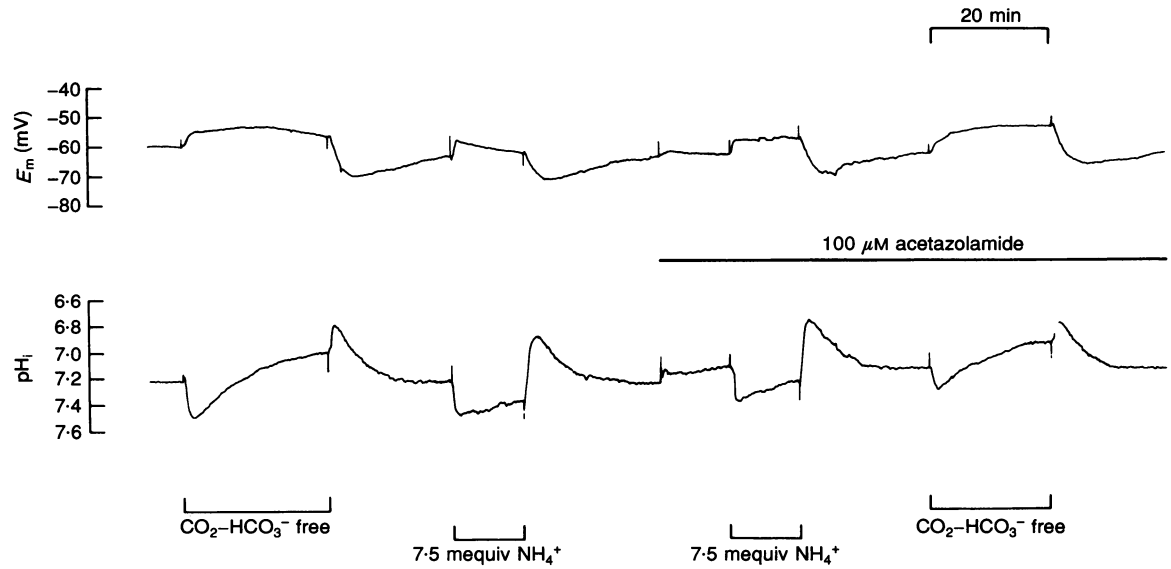

Figure 5. Pen recordings of an experiment to investigate the effect of the carbonic anhydrase inhibitor, acetazolamide on pH_i transients induced by alteration of the external CO_2 level and by application and removal of 3·75 mM $(NH_4)_2SO_4$

The preparation was superfused with modified Krebs solution buffered with 5% CO_2–21 mM HCO_3^- except during the intervals indicated when it was replaced with solution buffered to the same pH with 21 mM NaHepes, equilibrated with 100% O_2.

to contribute to cellular buffering. The presence of carbonic anhydrase was therefore tested by investigating the effect of acetazolamide. Application of 100 μM acetazolamide caused a small acidification, as shown in Fig. 5, which was reversible on removal of the drug (not illustrated). But the continued presence of acetazolamide had no effect on any transient changes in pH_i induced either by alteration of the CO_2 level or by application and removal of an ammonium salt. This would tend to confirm the absence of carbonic anhydrase in the smooth muscle of the ureter. Nevertheless, it is difficult to see how a lack of carbonic anhydrase could prevent contribution to buffering power over the long steady-state periods illustrated in Fig. 4 (up to 8 min).

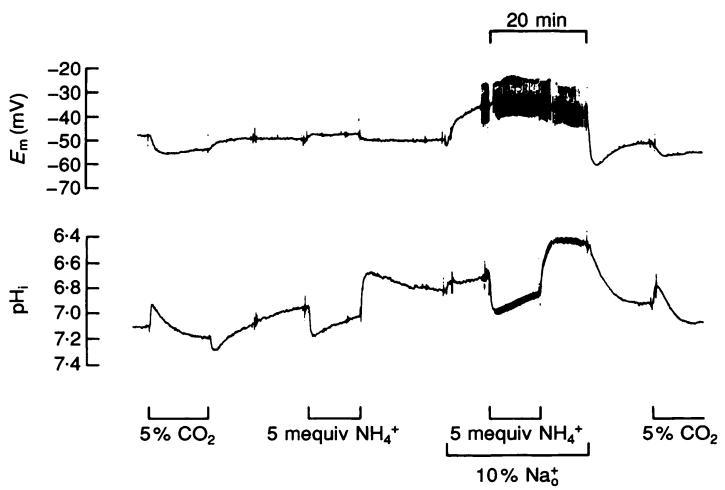

Figure 6. Pen recordings of an experiment to investigate the effect of 10% Na_o^+ (replaced by Tris) on the recovery of pH_i from an acid load induced by application and removal of 2·5 mM $(NH_4)_2SO_4$ in the nominal absence of CO_2–HCO_3^-

Note that recovery from acidosis induced by application of solution buffered with 5% CO_2–21 mM HCO_3^- was faster than the recovery from acidosis in the nominal absence of CO_2. The preparation was superfused with solution equilibrated with 100% O_2 and buffered with 21 mM NaHepes except where indicated otherwise.

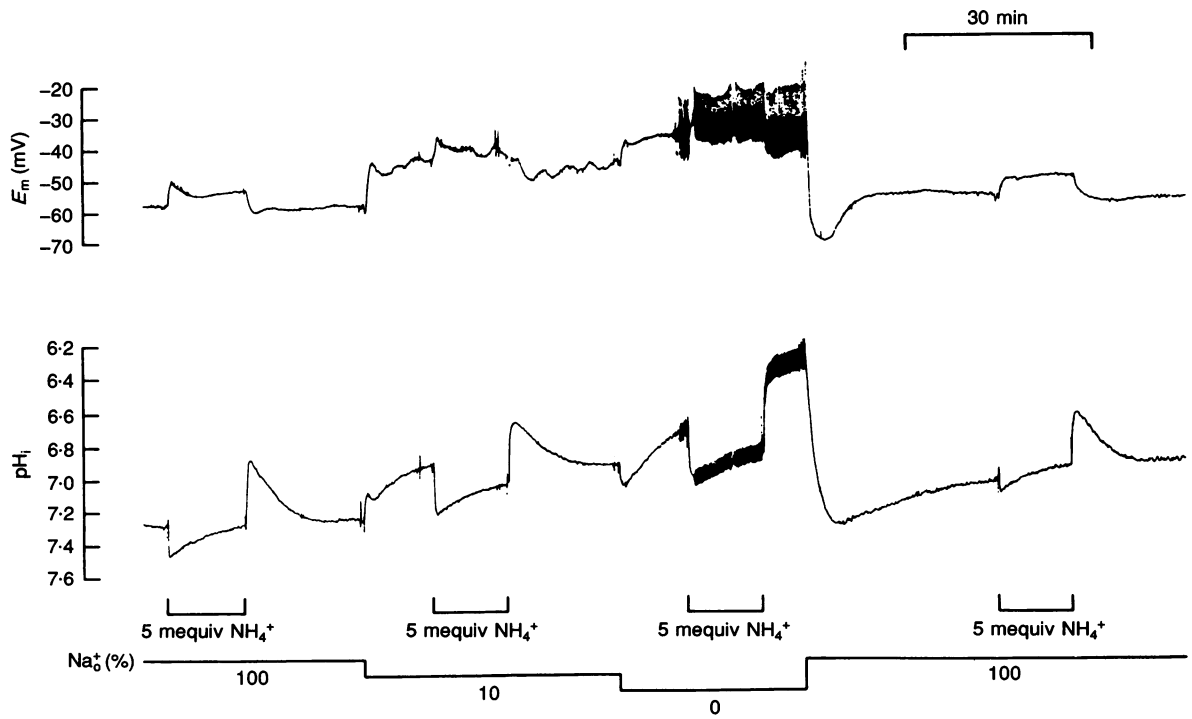

Figure 7. Pen recordings of an experiment showing the effect of 10% Na_o^+ and the absence of Na_o^+ (replaced by Tris) on the recovery from acidosis induced by application and removal of 2·5 mM $(NH_4)_2SO_4$ in the presence of 5% CO_2–21 mM HCO_3^-

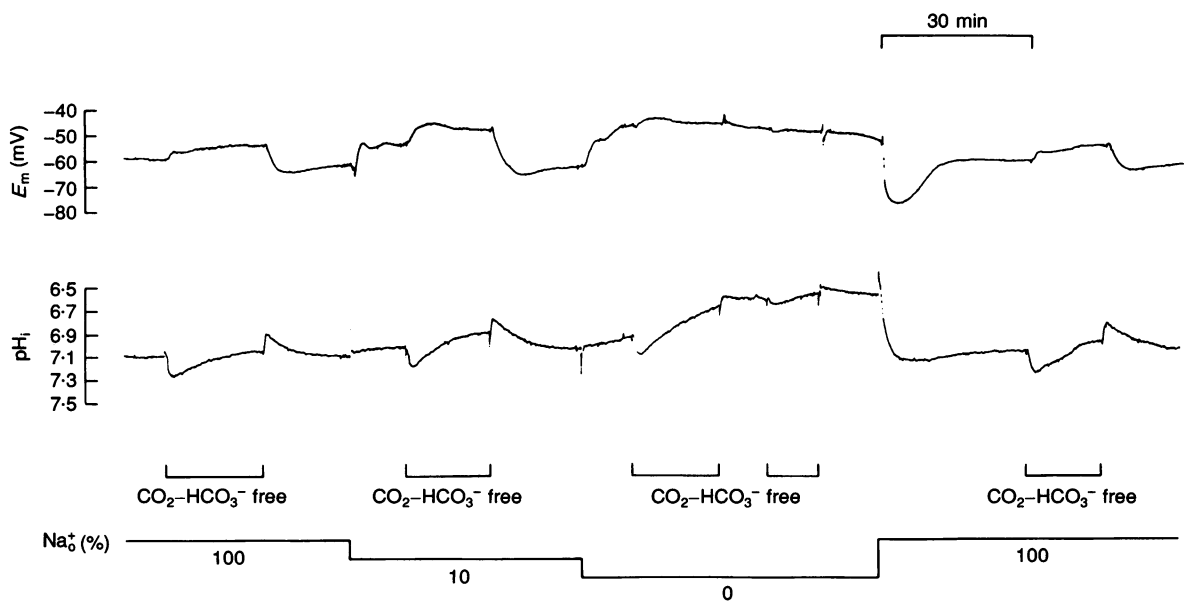

Figure 8. Pen recordings of an experiment to investigate the effect of 10% Na_o^+ and the absence of Na_o^+ (replaced by $NMDG^+$) on the pH_i transients induced by changing between solutions equilibrated with 5% CO_2, 95% O_2 and 100% O_2

The preparation was superfused with solution equilibrated with 5% CO_2 except during the intervals indicated otherwise.

The effect of reduction or removal of external Na^+ on the recovery from intracellular acidosis

Previous studies of the ionic mechanisms responsible for regulation of pH_i have almost universally demonstrated Na^+-dependent processes for the effective extrusion of acid equivalents (see Bock & Marsh, 1988). The Na^+ requirement of both the HCO_3^--independent and -dependent recoveries from acidosis was therefore tested. In the nominal absence of CO_2–HCO_3^-, recovery from an acid load induced by application and removal of an ammonium salt was substantially slowed when Na_o^+ was reduced to 10%, as shown in Fig. 6. Restoration of the normal Na_o^+ then accelerated the recovery to a greater rate than that observed under control conditions. The mean half-time for recovery was $26{\cdot}9 \pm 4{\cdot}6$ min ($n = 3$) in 10% Na_o^+ and $1{\cdot}6 \pm 0{\cdot}1$ min ($n = 3$) on reapplication of 100% Na_o^+ compared with $4{\cdot}6 \pm 0{\cdot}8$ min ($n = 3$, paired data) in the continual presence of 100% Na_o^+.

In CO_2–HCO_3^--buffered solutions, reduction of Na_o^+ to 10% had a much smaller effect on the rate of recovery from acidosis than it did in the nominal absence of CO_2–HCO_3^-. This is illustrated for application of an acid load both by addition and removal of an ammonium salt in the continuous presence of 5% CO_2 (Fig. 7) and by changing from nominally CO_2-free solution to one equilibrated with 5% CO_2 (Fig. 8). The mean half-time of the recoveries in 10% Na_o^+ was $4{\cdot}4 \pm 0{\cdot}9$ min ($n = 11$) compared with $2{\cdot}8 \pm 0{\cdot}6$ min ($n = 11$, paired data) in 100% Na_o^+. Recovery from acidosis in CO_2–HCO_3^--buffered solution could, nonetheless, be inhibited by complete removal of Na_o^+. As shown in Fig. 7, acidosis following removal of an ammonium salt in the absence of Na_o^+ was followed by a further slow fall in pH_i. However, when extremely low pH_i levels were achieved (below about 6·4), there was some Na^+-independent recovery of pH_i in the presence of CO_2–HCO_3^-, whereas there was none in the nominal absence of CO_2–HCO_3^- (see Fig. 8). In the absence of Na_o^+ and nominal absence of CO_2–HCO_3^-, pH_i continued to fall, even below a value of about 6·0 (see also Fig. 3 of Aickin, 1994*a*). Reapplication of Na_o^+ in the presence of CO_2–HCO_3^- caused an immediate and rapid recovery of pH_i (mean half-time of $1{\cdot}1 \pm 0{\cdot}5$ min, $n = 3$) with an

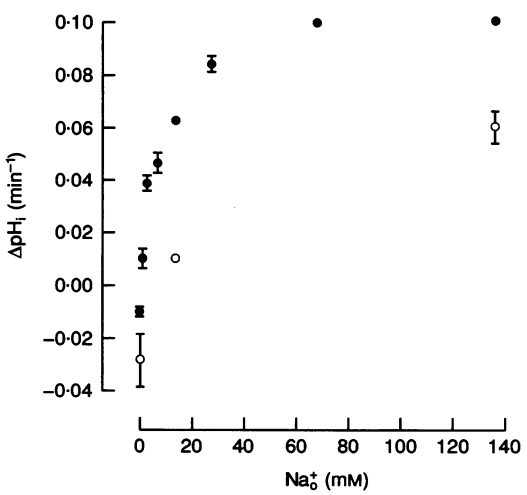

Figure 9. Relationship of the rate of change of pH_i to Na_o^+
Data in the presence (●) and nominal absence (○) of CO_2 were obtained from recoveries of pH_i following acidosis induced by application and removal of ammonium salts and have been normalized to the mean rate of change of pH_i in 100% Na_o^+ (136 mM) and 5% CO_2. They are given at 0·4 pH units acid displacement from the appropriate steady-state pH_i, i.e. that recorded in the experimental solution or, in the case of Na^+-free solutions, in 6·8 mM Na_o^+ with the same buffer system and Na^+ substitute. Data between 6·8 and 68 mM Na_o^+ were obtained from acid loads applied after pH_i had stabilized in the lowered Na_o^+ solution. This period will also have allowed equilibration of the transmembrane Na^+ gradient (Aickin, 1987). For Na_o^+ concentrations lower than 6·8 mM, acid loads were applied while pH_i was still falling (e.g. Fig. 7) but after the transmembrane Na^+ gradient should have stabilized. The rate of change of pH_i was calculated from the first differential ($-Ke^{-Kx}$) of the exponential describing the recovery ($y = e^{-Kx}$, where K is the rate constant of the recovery), except for the Na^+-free data where the rate was measured directly. It should be noted that this data does not represent net flux of acid equivalents, which would be derived from multiplication by the buffering power. Standard error is indicated by the vertical bars, except where it is within the size of the symbol; $n = 3$–11.

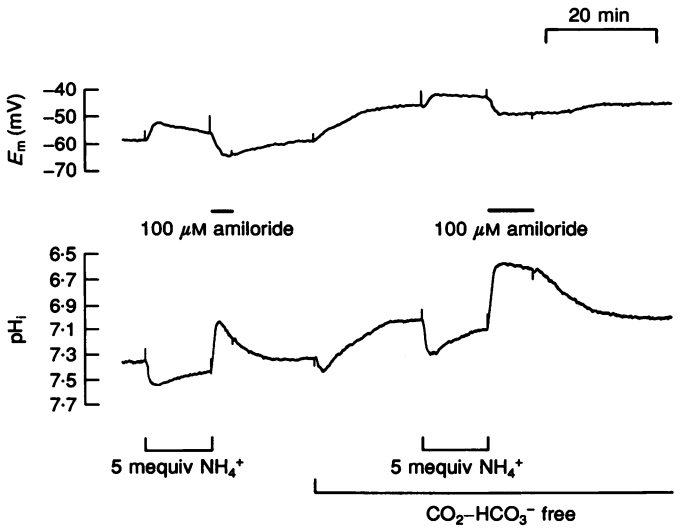

Figure 10. Pen recordings of an experiment showing the effect of 100 μM amiloride on the recovery from acidosis induced by application and removal of 2·5 mM $(NH_4)_2SO_4$ in the presence of 5% CO_2–21 mM HCO_3^- and in solution equilibrated with 100% O_2, buffered to the same pH with 21 mM NaHepes

increase of as much as 1 pH unit in 4 min. This recovery overshot the pH_i previously recorded under control conditions and was followed by a slow decline back to the control value. Both in the presence and nominal absence of CO_2–HCO_3^-, recovery of pH_i on reapplication of Na_o^+ was accompanied by a marked hyperpolarization (see Figs 6, 7 and 8).

It should be noted that reduction of Na_o^+ affected pH_i. Reduction from 100% always caused acidosis, although the extent was greater when Tris (Figs 6 and 7), rather than $NMDG^+$ (Fig. 8), was used as the Na^+ substitute (see also Deitmer & Schlue, 1988). Reduction from 10% Na_o^+ frequently caused a transient alkalosis (e.g. Fig. 7, see also Fig. 1 of Aickin, 1985). The cause of these changes, particularly the alkalosis, is not clear. Reversal of Na^+-dependent acid extrusion mechanisms may contribute to the acidosis, as also could reversal of Na^+–Ca^{2+} exchange via raising the intracellular Ca^{2+} concentration (Aickin, Brading & Walmsley, 1987; see also Meech & Thomas, 1977). But since the intracellular Na^+ stabilizes on reduction of Na_o^+ in under 10 min (Aickin, 1987), such reversals cannot account for the continuing acidosis observed in Na^+-free solutions (e.g. Figs 7 and 8). This would seem likely to reflect metabolic production of acid equivalents since, at least in the nominal absence of CO_2–HCO_3^-, pH_i fell well below the level predicted by a passive distribution of protons, i.e. pH_i continued to fall when any passive movement of acid equivalents would tend to raise pH_i.

Apparent Na^+ affinities of the HCO_3^--dependent and -independent mechanisms for effective extrusion of acid equivalents

Comparison of the effect of 10% Na_o^+ on the recovery from acidosis in the presence (Figs 7 and 8) and nominal absence (Fig. 6) of CO_2–HCO_3^- would suggest that the HCO_3^--

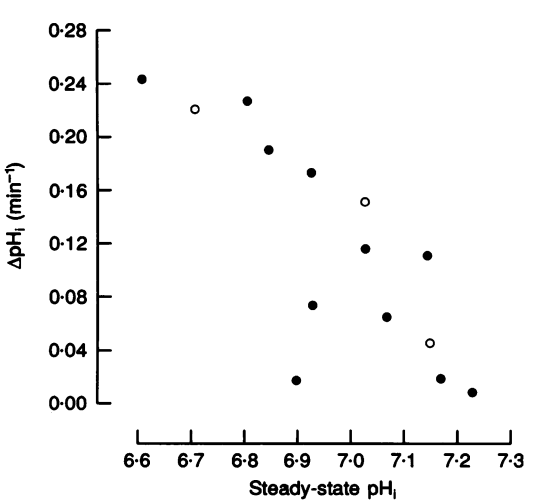

Figure 11. Relationship of the initial rate of fall of pH_i on application of amiloride (100 μM or 1 mM) to the steady-state pH_i in the nominal absence of CO_2–HCO_3^-

Each point represents the result of an individual application. Filled symbols depict data from eleven different cells while open symbols depict data obtained in a single cell over a period of several hours (see text).

dependent mechanism had a greater apparent affinity for Na_o^+ than the HCO_3^--independent mechanism. Indeed Na_o^+ had to be reduced to 1 or 2% in the presence of 5% CO_2 (e.g. see Fig. 1 of Aickin, 1985) to induce a similar degree of slowing of the pH_i recovery as observed on reduction to 10% in the nominal absence of CO_2–HCO_3^-. However, inhibition of the Na^+-dependent mechanisms by complete removal of Na_o^+ did not result in an essentially clamped pH_i following application of a moderate acid load, as it does in many other preparations (see Roos & Boron, 1981). The continued fall in pH_i observed in the absence of Na_o^+, at least to a pH_i of approximately 6·4, indicates that significant extrusion of acid equivalents would be required even to maintain a constant pH_i above about 6·4, and it should be noted that the fall in pH_i was faster in the nominal absence than in the presence of CO_2 (see Fig. 8). Figure 9 shows the relationship between the rate of change of pH_i and Na_o^+ in both the presence and nominal absence of CO_2 at 0·4 pH units acid displacement from the steady-state pH_i recorded under the appropriate conditions (see legend to Fig. 9). This would suggest an apparent half-maximal activation for the HCO_3^--independent mechanism of about 20 mM Na_o^+ and for the HCO_3^--dependent recovery of about 4 mM Na_o^+.

Effect of amiloride on the recovery from acidosis

The finding of a Na^+-dependent recovery from acidosis in the nominal absence of CO_2–HCO_3^- with an apparent half-maximal activation at about 20 mM Na^+ suggests that Na^+–H^+ exchange may be the underlying mechanism (see Aronson, 1985). I therefore tested the effect of amiloride, a well-established inhibitor of Na^+–H^+ exchange, in many preparations. Figure 10 shows that in the nominal absence of CO_2–HCO_3^-, 100 μM amiloride greatly slowed the recovery from acidosis (mean half-time 17·5 ± 5·8 min compared with 4·0 ± 1·0 min, $n = 5$, in the absence of the drug; paired data). This inhibition was reversible immediately on removal of the drug when recovery proceeded at the same rate as observed without prior drug treatment. Interestingly, amiloride did not cause as profound an inhibition of the recovery from intracellular acidosis as did removal of Na_o^+. Some recovery was observed at moderate acidosis (approximately pH 6·8) in the presence of amiloride, whereas pH_i continued to fall in the absence of Na_o^+ (see Fig. 8). This may reflect incomplete inhibition by amiloride or some residual activity of the HCO_3^--dependent mechanism in Hepes-buffered solutions due to metabolic production of CO_2 (see Aickin & Brading, 1984).

Application of amiloride in the steady state in the nominal absence of CO_2–HCO_3^- caused a fall in pH_i (see Fig. 4 of Aickin, 1989) at a rate dependent upon the steady-state pH_i, as illustrated in Fig. 11. Cells in which a relatively low pH_i was recorded in Hepes-buffered solution showed a rapid fall in pH_i on application of amiloride, whereas those in which pH_i in Hepes-buffered solution was close to that recorded in CO_2–HCO_3^--buffered solution

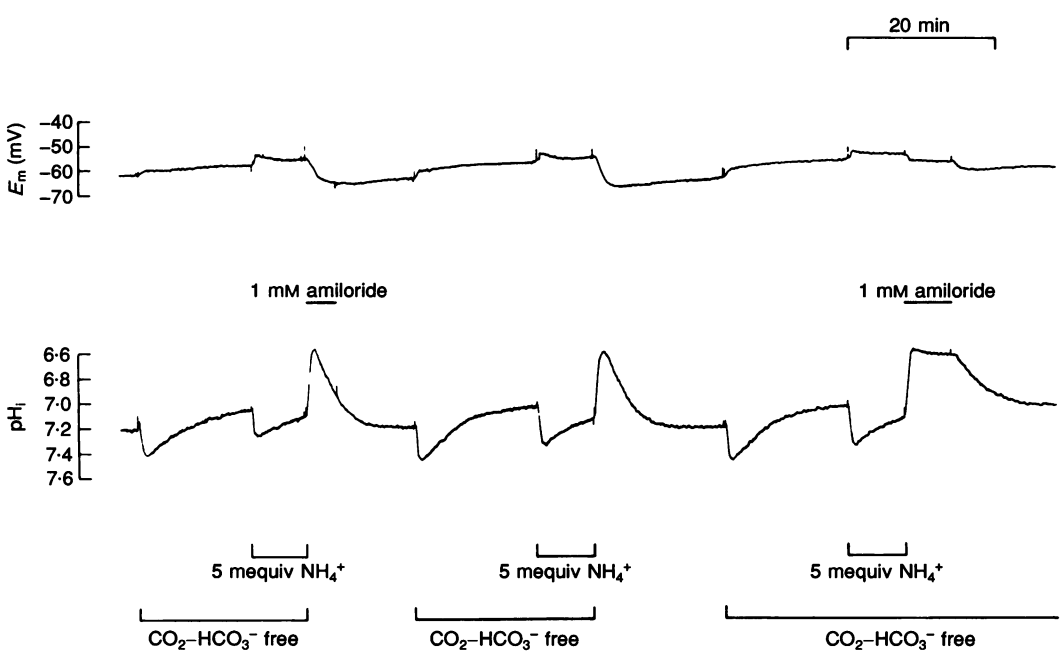

Figure 12. Pen recordings of an experiment to investigate the effect of 1 mM amiloride on the recovery from considerable acidosis in the presence of 5% CO_2–21 mM HCO_3^-

Acidosis was induced, after equilibration in nominally CO_2–HCO_3^--free solution, by application and removal of 2·5 mM $(NH_4)_2SO_4$ with simultaneous return to CO_2–HCO_3^--buffered solution on removal of the ammonium salt in the first two instances. Recovery from the third acidosis was recorded in the nominal absence of CO_2–HCO_3^-.

showed only a very slow fall. The relationship of the rate of fall of pH_i to the steady-state pH_i was particularly well demonstrated by the data obtained in a single cell (open symbols in Fig. 11) where the effect of removal of CO_2 from the superfusing solution and subsequent application of amiloride was repeatedly tested over a period of several hours (cf. Fig. 2). At first, pH_i fell to a very low level in the nominal absence of CO_2–HCO_3^- (6·71) and application of amiloride caused a dramatic fall in pH_i, but 3 and 7 h later, pH_i stabilized at progressively higher values (7·03 and 7·15, respectively) and application of amiloride had progressively smaller effects. On the assumption that application of amiloride in the nominal absence of CO_2–HCO_3^- leaves a largely unopposed intrinsic intracellular acid loading, this would suggest that the degree of intrinsic acid loading decreased with time from excision of the preparation from the animal. Consequently, this also suggests that, in the nominal absence of CO_2–HCO_3^-, pH_i is dictated by the degree of intracellular acid loading and that the capacity of the Na^+–H^+ exchanger alone is insufficient to maintain the 'normal' pH_i in a freshly excised preparation. Nevertheless, the data illustrated in Fig. 11 indicate that Na^+–H^+ exchange was stimulated by decreasing pH_i and probably reflect the activation of Na^+–H^+ exchange by internal H^+.

Although amiloride caused considerable inhibition of the recovery from acidosis in the nominal absence of CO_2–HCO_3^-, it had no discernable effect on the recovery from a similar experimental acid load in the presence of CO_2–HCO_3^-, as shown in Fig. 10. However, in this experiment and others like it (see Fig. 3 of Aickin, 1988 and Fig. 1 of Aickin, 1989), acidosis in the presence of CO_2–HCO_3^- decreased pH_i to a value similar to the steady-state pH_i recorded in the nominal absence of CO_2–HCO_3^-, i.e. to a pH_i at which Na^+–H^+ exchange no longer caused an alkalosis in the nominal absence of CO_2–HCO_3^-. I therefore investigated the effect of amiloride in the presence of CO_2–HCO_3^- at a sufficiently acidic pH_i for Na^+–H^+ exchange to cause an alkalosis in Hepes-buffered solution. In the experiment illustrated in Fig. 12, acid loading was induced by application of an ammonium salt in the nominal absence of CO_2–HCO_3^-, followed by removal of the ammonium salt together with simultaneous return to solution buffered with 5% CO_2–21 mM HCO_3^-. The pH_i fell to about 6·6, but 1 mM amiloride still had no discernable effect on the rate of recovery. Recovery from a similar pH_i in the nominal absence of CO_2–HCO_3^- was substantially inhibited by the presence of amiloride.

Since inhibition of the Na^+–H^+ exchanger by amiloride is through an essentially competitive interaction with Na^+ (Kinsella & Aronson, 1981; Ives, Yee & Warnock, 1983), another possible explanation for the failure of amiloride to inhibit the recovery from acidosis in the presence of CO_2–HCO_3^- is that the apparent affinity of the exchanger for Na^+ was increased (note that the apparent half-maximal activation for the recovery from acidosis in the presence of CO_2–HCO_3^- was about 4 mM compared with about 20 mM in the nominal absence of CO_2–HCO_3^-). However, reduction of Na_o^+ to 5% (Fig. 13) or even 2% (not shown) did not reveal any inhibition by amiloride in the presence of the CO_2–HCO_3^- buffer.

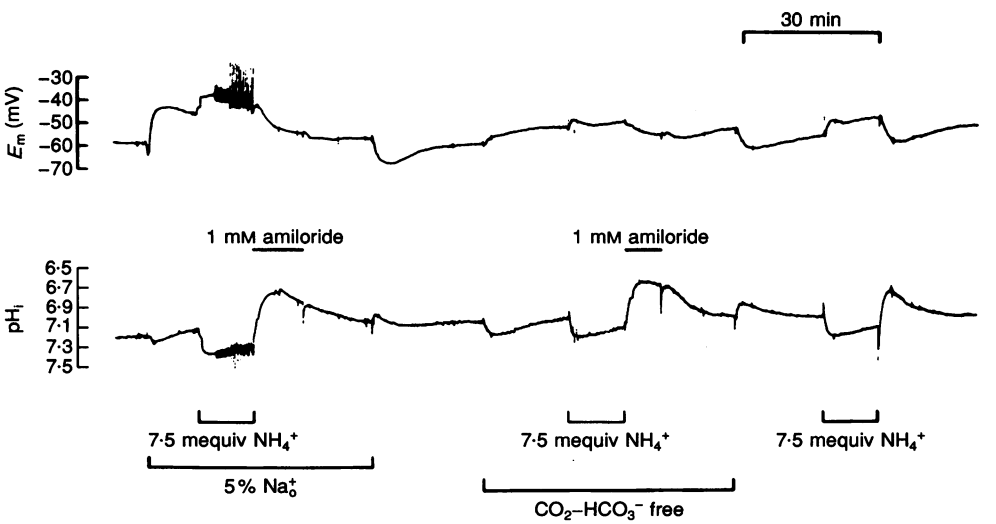

Figure 13. Pen recordings of an experiment to determine whether increased affinity for Na^+ and hence competition with amiloride was responsible for the failure of amiloride to inhibit recovery from acidosis in the presence of CO_2–HCO_3^-

Acid loading was induced by application and removal of 3·75 mM $(NH_4)_2SO_4$. Na^+ was replaced by $NMDG^+$. The preparation was superfused with modified Krebs solution equilibrated with 5% CO_2–21 mM HCO_3^- except during the interval indicated when it was replaced with solution buffered to the same pH with 21 mM NaHepes, equilibrated with 100% O_2.

DISCUSSION

These results demonstrate that recovery of pH_i from acidosis in the smooth muscle of guinea-pig ureter is almost entirely Na^+ dependent. Two mechanisms have been revealed by their different dependence on CO_2–HCO_3^-, apparent affinity for Na_o^+ and sensitivity to amiloride. These data indicate that Na^+–H^+ exchange is solely responsible for the recovery from an experimentally induced acid load in the nominal absence of CO_2–HCO_3^-. In this unphysiological condition, the pH_i of freshly excised cells falls to relatively low steady-state levels (6·06–6·89), although the Na^+–H^+ exchanger appears to be activated at about 7·2–7·3 (see Fig. 11). Only when the HCO_3^--dependent mechanism is operative in CO_2–HCO_3^--buffered solution, can the pH_i of freshly excised cells be maintained at the normal, relatively alkaline level (7·03–7·45), although under these conditions Na^+–H^+ exchange appears to play little part.

Na^+–H^+ exchange

The Na^+ dependence and amiloride sensitivity of the recovery from acidosis in the nominal absence of CO_2–HCO_3^- are characteristic of the operation of Na^+–H^+ exchange for H^+ efflux and add to the body of evidence for the presence of this exchanger in smooth muscle (see Introduction). More detailed analysis of the properties of the HCO_3^--independent recovery from acidosis also shows close similarities with the Na^+–H^+ exchanger so well characterized in a wide variety of plasma membranes (for review see Aronson, 1985). The apparent half-maximal activation of the HCO_3^--independent recovery at about 20 mM Na^+ compares well with that found in other preparations. In addition, the amiloride-sensitive mechanism in the ureter appears to have a steep activation with decreasing pH_i below about 7·2 (Fig. 11), closely matching the activation curve found in sheep heart Purkinje fibres (Kaila & Vaughan-Jones, 1987) and also in lymphocytes (Grinstein, Cohen & Rothstein, 1984). Finally, reduction of intracellular Na^+ would appear to stimulate the HCO_3^--independent recovery from acidosis, indicated by acceleration of pH_i recovery when 100% Na_o^+ was restored after a period in low Na_o^+ (when intracellular Na^+ would have been lowered; Aickin, 1987) over and above that observed when Na_o^+ was maintained at 100% (Fig. 6). This property has also been found for the Na^+–H^+ exchanger in lymphocytes (Grinstein *et al.* 1984).

Although Na^+–H^+ exchange is clearly capable of regulating pH_i to a level more alkaline than that predicted by a passive distribution of H^+ ions in the nominal absence of CO_2–HCO_3^- in the vast majority of cells tested, it does not necessarily follow that the exchanger forms an important part of the pH_i regulating mechanism under more physiological conditions. Even in the nominal absence of CO_2–HCO_3^-, this exchanger seems inadequate to maintain pH_i at its 'normal' level in freshly excised preparations. Despite apparent activation of the exchanger at pH_i values below about 7·2 (Fig. 11), pH_i in Hepes-buffered solution was always below 6·9 in freshly excised preparations and in some cells was even more acidic than predicted by a passive distribution of protons. Only many hours later, most noticeably after 24 h cold storage, did pH_i approximate to 7·2 in Hepes-buffered solutions. Deterioration of the preparation might be expected to: (1) decrease the capacity of active transport, (2) increase passive leaks and (3) decrease the metabolic rate. Only the last of these can explain the increase in steady-state pH_i with time. Furthermore, it is notable that the lower the steady-state pH_i in Hepes-buffered solution, the faster was the fall in pH_i on application of amiloride, i.e. the greater was the apparent rate of intrinsic intracellular acid loading. It is worth emphasizing that intracellular production of acid equivalents must be largely responsible for the acidosis observed when extrusion mechanisms were inhibited since, at least in the nominal absence of CO_2–HCO_3^-, pH_i continued to fall below the level predicted for a passive distribution of H^+ ions.

The failure of amiloride to affect the rate of pH_i recovery detectably, even from considerable acidosis, in CO_2–HCO_3^--buffered solutions suggests that the Na^+–H^+ exchanger plays little role under physiological conditions. At first sight, this result seems easy to explain. If the use of CO_2–HCO_3^--buffered solutions increased the intracellular buffering capacity as predicted on theoretical grounds, the effective rate of extrusion of acid equivalents could be up to 6 times greater in the presence than in the nominal absence of CO_2–HCO_3^-. Thus, the contribution from Na^+–H^+ exchange could be below the level of detection. However, determination of the intracellular buffering power very surprisingly revealed that it was the same in both the presence and nominal absence of CO_2–HCO_3^-. This result is difficult to rationalize. If the intracellular compartment behaved as a closed system, intracellular buffering would not be much affected by the presence of the CO_2–HCO_3^- buffer in the superfusing solution. But it would appear that CO_2 both enters and exits the intracellular space readily, judged from the speed of the initial changes in pH_i on alteration of the external CO_2 level, certainly rapidly enough for CO_2 to reach equilibrium across the sarcolemma during the time course of the changes used for estimation of cellular buffering. Thus, it would seem that the conditions for an open system should prevail. Likewise, although the apparent lack of carbonic anhydrase in this preparation, indicated by the lack of effect of prolonged exposure to acetazolamide (see also Mühleisen & Kreye, 1985), would decrease the contribution of the CO_2–HCO_3^- system to instantaneous buffering, it should have no effect on its contribution to the maintained alkaline loads used. Diffusional delays may complicate the pH_i transients recorded in this multicellular preparation and hence affect the calculated values of intracellular buffering power (see

Bountra, Powell & Vaughan-Jones, 1990). Such delays would tend to cause the values to be overestimated but it should be noted that particularly low values were obtained (12–14 mequiv H^+ (pH unit)$^{-1}$ l^{-1}). Slower equilibration of ammonia and trimethylamine in Hepes-buffered solution could perhaps be implicated (see Bountra *et al.* 1990), but this would have to be matched by a slow effective influx of acid equivalents such that after an initial change, pH_i was held constant. The records, however, provide no evidence for a difference in the rate of ammonia or trimethylamine entry, nor is there any obvious reason why the presence or nominal absence of CO_2–HCO_3^- should influence the permeation of these species.

Although I can offer no explanation of why CO_2–HCO_3^- does not apparently increase the intracellular buffering capacity as expected, it is notable that this finding has been made in other smooth muscle cells when the buffering power has been determined experimentally in both the presence and nominal absence of CO_2–HCO_3^-. Aalkjær & Hughes (1991) have reported values of 17 and 20 mequiv H^+ (pH unit)$^{-1}$ l^{-1} in the nominal absence and presence of 5% CO_2, respectively, in segments of rat mesenteric arteries, while Baro and co-workers (Baro *et al.* 1989) have given a preliminary report of a value around 14 mequiv H^+ (pH unit)$^{-1}$ l^{-1} in isolated vascular smooth muscle cells. Measurements in a smooth muscle-like cell line have given a mean value of 10 mequiv H^+ (pH unit)$^{-1}$ l^{-1} in the nominal absence of CO_2 (Putnam & Grubbs, 1990) while the published records in the presence of 5% CO_2 (Putnam, 1990) suggest a value of 11 mequiv H^+ (pH unit)$^{-1}$ l^{-1}. Significantly, all these results were obtained using a fluorescent indicator to measure pH_i, thus suggesting that this puzzling finding is not an artifact of the double-barrelled pH-sensitive microelectrode. It is also notable that estimation of buffering power in guinea-pig vas deferens, from changes in pH_i that were followed by minimal recovery on alteration of the CO_2 level from 3–7%, gave a mean value of 8·6 mequiv H^+ (pH unit)$^{-1}$ l^{-1} (Aickin, 1984). This value should have included the buffering capacity due to the CO_2–HCO_3^- system at 3% CO_2, at HCO_3^- concentrations up to 8 mM, which alone would have given a value of up to 18 mequiv H^+ (pH unit)$^{-1}$ l^{-1}. Finally, it should be noted that the observations of essentially equal rates of recovery from acidosis and effectiveness of inhibition by amiloride in both the presence and nominal absence of CO_2–HCO_3^- in the smooth muscle cells of the guinea-pig femoral artery (Aickin, 1994*b*) are consistent with the hypothesis that CO_2–HCO_3^- does not apparently contribute to intracellular buffering.

If the intracellular buffering power is not increased in the presence of CO_2–HCO_3^-, then the failure of amiloride to affect the rate of recovery from acidosis in the presence of CO_2 must be explained either by Na^+–H^+ exchange not being inhibited by amiloride in this condition, or by it being inoperative. An increase in the affinity of the exchanger for Na_o^+ (consistent with the lower Na^+ concentration for apparent half-maximal activation in the presence rather than nominal absence of CO_2–HCO_3^-) and hence decrease in the efficacy of amiloride was ruled out by the continued lack of effect of amiloride when Na_o^+ was reduced to 5 or even 2%. Certainly in other preparations Na^+–H^+ exchange remains sensitive to amiloride in CO_2–HCO_3^--buffered solution (see Aickin, 1986, 1992*b*). It would therefore seem more likely that Na^+–H^+ exchange was 'switched off' in the presence of CO_2–HCO_3^-.

Steady-state pH_i in the presence and nominal absence of CO_2–HCO_3^-

Given the presence of both HCO_3^--dependent and -independent mechanisms for the effective extrusion of acid equivalents, it is perhaps surprising that such different steady-state pH_i values are recorded in the presence and nominal absence of CO_2–HCO_3^- in freshly excised preparations. Steady-state acidosis in the nominal absence of CO_2–HCO_3^- was first reported in guinea-pig vas deferens (Aickin, 1984) and has since been found in other smooth muscles (e.g. Korbmacher *et al.* 1988; Boyarksy *et al.* 1988; Putnam & Grubbs, 1990) and glial cells (Kettenmann & Schlue, 1988; Deitmer & Schlue, 1989). Although now accepted as a familiar pattern of behaviour, its underlying cause has not been established.

The most likely explanation of this phenomenon is that the capacity of the HCO_3^--independent mechanism, the Na^+–H^+ exchanger, is by itself inadequate to counteract the rate of intrinsic intracellular acid loading. Certainly the exchanger is operative at the low steady-state pH_i recorded in the nominal absence of CO_2, as indicated by the fall in pH_i on application of amiloride. This rules out the possibility that a low threshold of the Na^+–H^+ exchanger could account for the low pH_i in the nominal absence of CO_2–HCO_3^-. Indeed the Na^+–H^+ exchanger appears to be geared to regulate pH_i close to the relatively alkaline level recorded in the presence of CO_2–HCO_3^-, as indicated by the approximation of steady-state values in the presence and nominal absence of CO_2–HCO_3^- in aged cells and by the negligible effect of amiloride when applied in the nominal absence of CO_2–HCO_3^- in these cells.

The fact that fresh cells could maintain a higher pH_i in the presence rather than nominal absence of CO_2–HCO_3^- could simply reflect operation of the additional HCO_3^--dependent mechanism, characterized in the following paper (Aickin, 1994*a*). The possibility that application of CO_2–HCO_3^- may stimulate the Na^+–H^+ exchanger, either by alteration of its kinetics or by insertion of more exchangers into the membrane, seems unlikely because of the decreased sensitivity to amiloride in the presence of CO_2–HCO_3^-. Indeed the failure of amiloride to affect the rate of pH_i recovery from acidosis in the presence of CO_2–HCO_3^- suggests that the HCO_3^--dependent mechanism was considerably more potent than Na^+–H^+ exchange. Recovery from acidosis occurred at the same

rate when the HCO_3^--dependent mechanism was operative (CO_2–HCO_3^--buffered solutions) whether or not the exchanger was functional (presence and absence of amiloride), whereas recovery was faster when both the exchanger and HCO_3^--dependent mechanism were functional (CO_2–HCO_3^--buffered solutions) rather than when the exchanger alone was operative (in the nominal absence of CO_2–HCO_3^-). Note that, if CO_2–HCO_3^- did add to the intracellular buffering capacity, the contribution of the HCO_3^--dependent mechanism would be even greater than is apparent from the recordings of pH_i. However, the rate of recovery from acidosis and the steady-state pH_i are not only dependent on the rate of effective acid extrusion but also on the rate of intrinsic intracellular acid loading. The latter was quite considerable in these experiments and appeared to be greater in the nominal absence of CO_2–HCO_3^- rather than in their presence. pH_i fell notably faster in the nominal absence rather than presence of CO_2–HCO_3^- after removal of Na_o^+ (until passive pH_i was reached; Figs 8 and 3 of Aickin, 1994*a*) and after removal of Cl_o^- (until normal pH_i was reached; Fig. 1 of Aickin, 1994*a*). Even if CO_2–HCO_3^- contributed to cellular buffering, as expected theoretically, these data would reflect up to 4 times greater flux of acid equivalents in the nominal absence of CO_2–HCO_3^-.

Finally, it is worth considering whether alteration of the extracellular buffering capacity and hence of the pH at the immediate surface of the preparation influences the pH_i transients and steady-state values in the presence and nominal absence of CO_2–HCO_3^- (see de Hemptinne, Morranne & Vanheel, 1987). Increased intracellular acid loading and hence acid extrusion in the nominal absence of CO_2–HCO_3^- may lead to a lower surface pH and hence contribute to the slower recovery and lower steady-state pH_i. If this were the case, decreasing the extracellular buffering (i.e. reducing the NaHepes concentration from 21 to 5 mM) should further decrease the rate of recovery from acidosis and the steady-state pH_i, but this was not observed. Indeed changes in surface pH do not appear to play a significant role in these experiments since increasing the buffering power of the CO_2-containing solution (by addition of 21 mM NaHepes) was also without effect on pH_i transients or steady-state values.

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