Regulation of intracellular pH in the smooth muscle of guinea-pig ureter: HCO_3^- dependence

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1. HCO_3^--dependent mechanisms involved in the regulation of intracellular pH (pH_i) were characterized using double-barrelled pH-sensitive microelectrodes in smooth muscle cells of the isolated guinea-pig ureter.

2. Removal of external Cl^- in the presence of CO_2–HCO_3^- caused a transient alkalosis, consistent with the presence of Cl^-–HCO_3^- exchange, before pH_i slowly recovered. Recovery from acidosis in the presence of CO_2–HCO_3^- was not affected, at a time when intracellullar Cl^- would have been maximally depleted, indicating that a counter transport of Cl^- and HCO_3^- was not involved. The recovery was also not affected by amiloride, indicating that Na^+–H^+ exchange was not involved.

3. A transient hyperpolarization was associated with the recovery from acidosis in the presence of CO_2–HCO_3^-, consistent with rheogenic coupling of Na^+–HCO_3^- cotransport. However, depolarization caused by elevation of the extracellular potassium (K_o^+) concentration, which should favour inward transport by the rheogenic mechanism, caused a fall in pH_i and decreased the rate of recovery from acidosis. Furthermore, ouabain abolished the transient hyperpolarization without affecting the recovery of pH_i. It is concluded that Na^+–HCO_3^- cotransport in the ureter is electroneutral.

4. Recovery from acidosis in the presence of CO_2–HCO_3^- was insensitive to DIDS even after prolonged pre-equilibriation and extreme acidosis. The results suggest that Na^+–HCO_3^- cotransport in the ureter is insensitive to DIDS and that Cl^-–HCO_3^- exchange does not reverse to contribute to the extrusion of acid equivalents. A HCO_3^- conductance may account for the Na^+-independent, HCO_3^--dependent recovery from extreme acidosis.

5. Recovery from experimentally induced alkalosis was inhibited by Cl^--free conditions and by DIDS, indicating that Cl^-–HCO_3^- exchange was involved.

6. It is concluded that pH_i in the smooth muscle of guinea-pig ureter is controlled by three transport mechanisms. By far the most important is an electroneutral Na^+–HCO_3^- cotransporter. Na^+–H^+ exchange appears to play little role in the presence of the physiological buffer. Both of these mechanisms extrude acid equivalents and so protect the cell against its fairly substantial intrinsic intracellular acid loading. Cl^-–HCO_3^- exchange, on the other hand, is stimulated by intracellular alkalosis to transport acid equivalents into the cell and so restore a more normal pH_i.

The results presented in the preceding paper (Aickin, 1994*a*) indicate the presence of two Na^+-dependent mechanisms capable of effectively extruding acid equivalents in the smooth muscle of guinea-pig ureter: one HCO_3^- dependent and the other HCO_3^- independent. However, despite the presence of the HCO_3^--independent mechanism, shown to be the amiloride-sensitive Na^+–H^+ exchanger, cells in freshly isolated strips of ureter were unable to maintain a 'normal' steady-state intracellullar pH (pH_i) in the nominal absence of CO_2–HCO_3^-, pH_i values as low as 6·06 being recorded (lower than the value predicted by passive distribution of protons). Only in aged cells, mainly in strips of tissue that had been isolated from the animal for more than 24 h, did the steady-state pH_i in the nominal absence of CO_2–HCO_3^- approximate to that recorded in the presence of the physiological buffer, up to a value of

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7·37. This, together with the fact that inhibition of the Na^+–H^+ exchanger by application of amiloride had no measurable effect on the recovery from experimentally induced acidosis in the presence of CO_2–HCO_3^-, suggests that the HCO_3^--dependent mechanism is by far the more important process for the regulation of pH_i.

Na^+ and HCO_3^- dependence are characteristic of both the Na^+-dependent Cl^-–HCO_3^- exchanger (Thomas, 1977; Boron, McCormick & Roos, 1981; Boron & Russell, 1983) and the more recently identified Na^+–HCO_3^- cotransporter (Boron & Boulpaep, 1983), which has also been implicated in the effective extrusion of acid equivalents (e.g. Deitmer & Schlue, 1989). In this paper, I have differentiated between these mechanisms by investigation of the dependence of the recovery from acidosis on Cl^-. The results indicate that the latter mechanism is present in the smooth muscle of guinea-pig ureter and that it is electroneutral. Further investigation of the HCO_3^- dependence of the regulation of pH_i has also revealed that Cl^-–HCO_3^- exchange is involved in the recovery from intracellular alkalosis and that a HCO_3^- conductance may be involved in the recovery from extreme acidosis.

Some of these results have been published in preliminary form (Aickin, 1988, 1989).

METHODS

The methods used were the same as described in the preceding paper (Aickin, 1994*a*). Briefly, experiments were performed on isolated strips of smooth muscle of guinea-pig ureter using double-barrelled pH-sensitive microeleoctrodes. These electrodes were made from two heat-fused filamented capillaries and were rendered hydrophobic by treatment with dimethyltrimethylsilylamine vapour at 180 °C. Their pH sensitivity was conferred by a proton cocktail (Ammann, Lanter, Steiner, Schulthess, Shijo & Simon, 1981; Fluka, Gillingham, Dorset, UK) in one barrel, while membrane voltage was recorded with a reference liquid ion exchanger in the other (Thomas & Cohen, 1981). The preparation was maintained in modified Krebs solution of the following composition (mM): NaCl, 115; KCl, 5·9; $CaCl_2$, 2·5; $MgCl_2$, 1·2; $NaHCO_3$, 21; Na_2HPO_4/NaH_2PO_4 at pH 7·35, 0·1; glucose, 11; equilibrated with nominally 5% CO_2–95% O_2 at 35 °C. The pH of this solution varied between 7·35 and 7·43 with different gas cylinders (BOC, Guildford, UK). Nominally CO_2–HCO_3^--free solution was equilibrated with 100% O_2 and buffered with 21 mM NaHepes to the same pH as the CO_2–HCO_3^--buffered solution. Cl^--free solutions were prepared using the following salts (mM): sodium glucuronate, 115; potassium gluconate, 5·9; calcium gluconate, 12·5; $MgSO_4$, 6. The concentration of the divalent cations was increased in the Cl^--free solutions to compensate for binding by the organic anions. The drugs, amiloride (Sigma Chemical Co., Poole, Dorset, UK), DIDS (4,4′-diisothiocyanostilbene-2,2′-disulphonic acid; Calbiochem, San Diego, CA, USA and Sigma), and ouabain (strophanthin-G, BDH (Merck), Lutterworth, UK) 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

The effect of removal of Cl^- on the recovery from acidosis in the presence of CO_2–HCO_3^-

In common with many other smooth muscles (see Casteels, 1981), intracellular Cl^- concentration is considerably higher than predicted by a passive distribution in guinea-pig ureter (Aickin & Vermuë, 1983). Passive permeability to Cl^- is relatively low and removal of extracellular chloride (Cl_o^-) results in a fairly slow loss of intracellular Cl^- (half-time of 6·7 min in the presence of 3% CO_2), at least in part due to efflux via Cl^-–HCO_3^- exchange (Aickin & Vermuë, 1983). The recording of pH_i in Fig. 1 supports the involvement of Cl^-–HCO_3^- exchange in this efflux by the alkalinization (increase in intracellular HCO_3^-, 0·46 ± 0·14 pH units, $n = 6$) recorded following the removal of Cl_o^- (see also Vaughan-Jones, 1979; Aickin & Brading, 1984). Unlike in the smooth muscle of guinea-pig vas deferens (Aickin & Brading, 1984) and in the cardiac Purkinje fibre (Vaughan-Jones, 1979), however, this alkalinization was not maintained throughout the exposure to Cl^--free solution. Before the expected completion of loss of intracellular Cl^-, pH_i began to fall and slowly returned to the level previously recorded in the presence of Cl_o^-. This fall was greatly accelerated by removal of CO_2 from the superfusing solution, after the initial alkalinization expected from the loss of intracellular HCO_3^- and CO_2 (see Fig. 1), but pH_i still stabilized at the same value as recorded in nominally CO_2–HCO_3^--free solution before the removal of Cl_o^-.

When pH_i had stabilized in Cl^--free solution (about 1 h after removal of Cl_o^- in Fig. 1), intracellular Cl^- would have been maximally depleted (intracellular Cl^- activity reached minimal values in 40 min in the presence of 3% CO_2; Aickin & Vermuë, 1983) but recovery from acidosis proceeded at the same rate as recorded before removal of Cl_o^- and this remained the case with repeated testing (see Fig. 1). Because Cl^--sensitive microelectrodes suffer from interference from other anions, it may be argued that intracellular Cl^- concentration did not fall to zero in Cl^--free solution and that sufficient remained to support Na^+-dependent Cl^-–HCO_3^- exchange. The residual apparent Cl^- activity recorded in solution buffered with 3% CO_2–14 mM HCO_3^- was 3·3 mM (Aickin & Vermuë, 1983). However, cells showed undiminished recovery from an acidosis of up to 0·7 pH units. This represents an efflux of a minimum of 9·8 mequiv H^+ (calculated from a buffering power of 14 mequiv H^+ (pH unit)$^{-1}$ l^{-1}, assuming no contribution of CO_2–HCO_3^- to intracellular buffering; Aickin, 1994*a*), which would require an efflux of 4·9 mequiv Cl^- if the Na^+-dependent Cl^-–HCO_3^- exchanger was responsible. Since Cl^- is not significantly bound by smooth muscle tissue constituents (1·3–1·5 mmol (l cell water)$^{-1}$ measured by Ag precipitation after 1 h in Cl^--free solution: Casteels, 1971; Aickin & Brading, 1982), it must be

concluded that the recovery from acidosis was Cl^- independent.

It is possible that recovery in Cl^--free conditions could have been mediated by Na^+–H^+ exchange rather than by a HCO_3^--dependent mechanism. Certainly experiments described in the preceding paper (Aickin, 1994*a*) show that Na^+–H^+ exchange accounts for the recovery from acidosis when the HCO_3^--dependent mechanism was inhibited by the nominal absence of CO_2–HCO_3^-. If Na^+–H^+ exchange was involved, the recovery from acidosis should have been inhibited by application of amiloride. However, amiloride had no discernible effect (see Fig. 1), as indeed it had no effect on the recovery from acidosis in the presence of CO_2–HCO_3^- in Cl^--containing solution (see Figs 10 and 12 of Aickin, 1994*a*).

Effect of DIDS on the recovery from acidosis

Na^+–HCO_3^- cotransport, suggested by the above results, is inhibited by the disulphonic stilbenes in other preparations (e.g. Boron & Boulpaep, 1983; Deitmer & Schlue, 1989). Therefore, the effect of DIDS was tested on the recovery from acidosis. In the experiments shown in Fig. 2, readdition of solution buffered with 5% CO_2–21 mM HCO_3^-, after equilibration of the tissue in nominally CO_2–HCO_3^--free solution, was used to produce intracellular acid loading. In all cells tested, application of DIDS (130 μM–1 mM) had no effect on the steady-state pH_i recorded in CO_2–HCO_3^--buffered solution but caused a slowly developing hyperpolarization. In thirteen of eighteen cells, pre-equilibration with DIDS had no effect

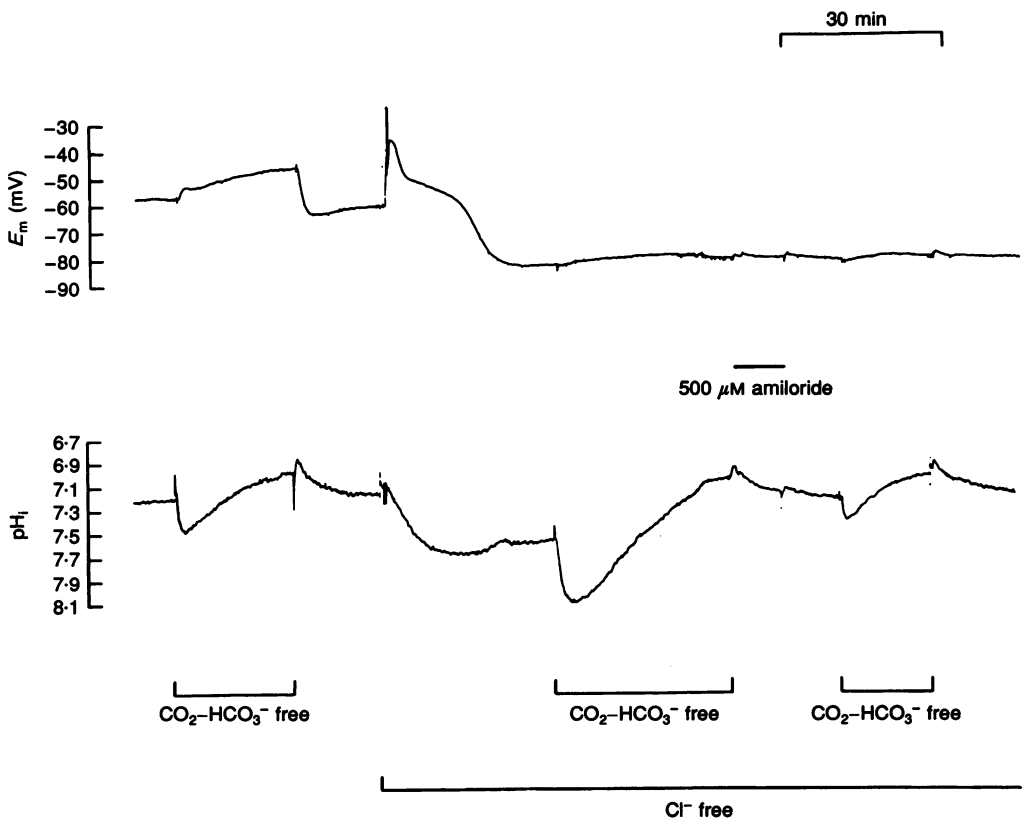

Figure 1. Pen recordings of an experiment to investigate the effect of Cl^--free conditions on the recovery of pH_i from acidosis in a smooth muscle cell of guinea-pig ureter made using a double-barrelled pH-sensitive microelectrode

Membrane potential (E_m) was recorded using a reference liquid ion exchanger in one barrel of the electrode and pH_i was recorded using a proton cocktail in the other barrel. Intracellular acidosis was induced by switching from nominally CO_2–HCO_3^--free solution (equilibrated with 100% O_2 and buffered with 21 mM NaHepes) to solution buffered with 5% CO_2–21 mM HCO_3^-. Recovery from acidosis was first recorded under normal conditions before the preparation was superfused with Cl^--free solution, as indicated. Note that after pH_i had stabilized following removal of external Cl^-, recovery from acidosis was unaffected and was insensitive to the presence of amiloride. Action potentials, observed soon after removal of external Cl^-, are truncated due to the low frequency response of both 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.

on the pH_i transients recorded on changing to nominally CO_2–HCO_3^--free solution or on returning to CO_2–HCO_3^--buffered solution (Fig. 2*A*). Recovery from the imposed acidosis was not discernibly affected. In the remaining five cells (Fig. 2*B*), pre-equilibration with DIDS caused an increase in the extent of the alkalosis observed on switching to nominally CO_2–HCO_3^--free solution and greatly inhibited the subsequent fall in pH_i in the continued presence of nominally CO_2–HCO_3^--free solution. Consequently, on return to CO_2–HCO_3^--buffered solution, little or no overshoot in pH_i occurred and so the effect on recovery from acidosis could not be determined. In all cells, pre-equilibration with DIDS converted the depolarization normally seen on switching to nominally CO_2–HCO_3^--free solution into a small hyperpolarization, which was followed by a slow decay of potential, and completely blocked the pronounced hyperpolarization normally seen on return to CO_2–HCO_3^--buffered solution.

The effect of DIDS was also tested on the HCO_3^--dependent Na^+-independent recovery from extreme acidosis observed in the preceding paper (Aickin, 1994*a*). As described in the preceding paper (Aickin, 1994*a*),

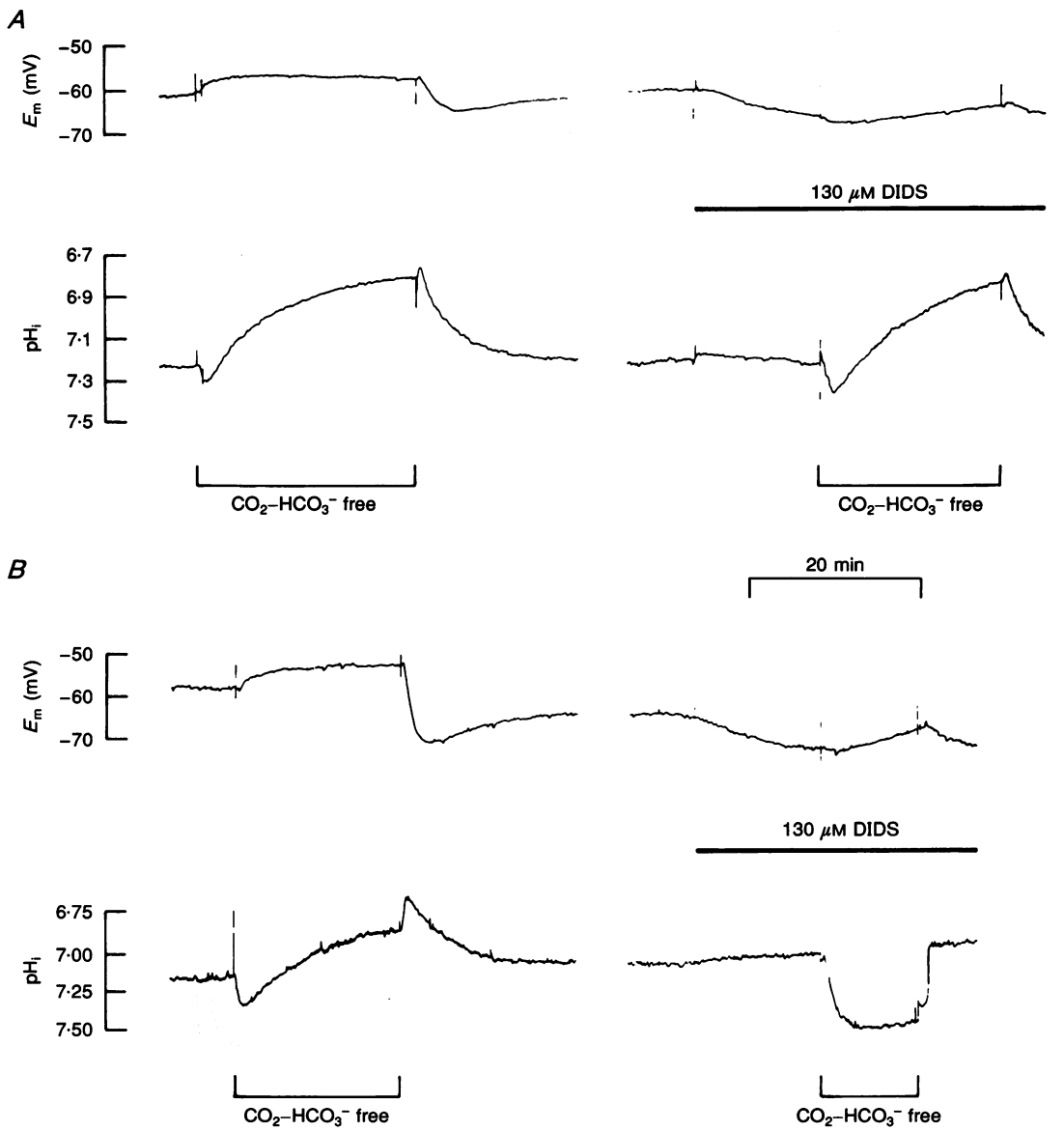

Figure 2. Pen recordings of parts of two experiments to examine the effect of DIDS on the recovery from acidosis

Both preparations were superfused with modified Krebs solution, buffered with 5% CO_2–21 mM HCO_3^- except for the intervals indicated when nominally CO_2–HCO_3^--free solution, equilibrated with 100% O_2 and buffered to the same pH with 21 mM NaHepes was applied. The gaps in the recordings represent a 45 min period in *A* and a 70 min period in *B*, during which other experimental manoeuvres were performed.

removal of Na_o^+ results in a fall in pH_i which is accelerated by the nominal absence of CO_2–HCO_3^- (see Fig. 3 of this paper). However, whereas pH_i stabilizes at approximately 6·4 in the presence of CO_2–HCO_3^-, it continues to fall in the nominal absence of CO_2–HCO_3^-. In the experiment shown in Fig. 3, pH_i was still falling at an alarming rate at approximately 6·0 in the nominal absence of CO_2–HCO_3^-. Return to CO_2–HCO_3^--buffered solution, still in the absence of Na_o^+, then caused a recovery of pH_i to about 6·3. Cl^-–HCO_3^- exchange, sensitive to DIDS and at least partially responsible for the high intracellular Cl^- in this preparation (Aickin & Vermuë, 1983), could underlie this recovery if it were forced into reversed mode by very low pH_i (very low intracellular HCO_3^-), then exchanging intracellular Cl^- for extracellular HCO_3^- (see Vanheel, de Hemptinne & Leusen, 1984). Clearly, Cl^-–HCO_3^- exchange can operate in this direction when Cl_o^- is removed (see Fig. 1). However, as illustrated in Fig. 3, 14 min pre-equilibration with DIDS had no effect on the HCO_3^--dependent recovery at extreme acidosis.

Is Na^+–HCO_3^- cotransport electrogenic?

The Na^+–HCO_3^- cotransporter, first identified in renal proximal tubule cells (Boron & Boulpaep, 1983) and subsequently found in several epithelial tissues (for references see La Cour, 1989), is strongly electrogenic with a coupling ratio of two, or probably even three HCO_3^- ions to one Na^+ ion. Because of the prevailing ion gradients and the membrane potential (E_m), this transporter normally acid loads the epithelial cell, i.e. it normally operates in the opposite direction to the Na^+- and HCO_3^--dependent mechanism found in these experiments. However, the gradients across the smooth muscle membrane of the guinea-pig ureter are such that the mechanism would be geared to affect recovery from intracellular acidosis. On the simplest model, the reversal potential for the electrogenic cotransporter (E_{rev}) is given by the following equation:

$$E_{rev} = \frac{n E_{HCO_3} - E_{Na}}{n - 1},$$

where E_{HCO_3} and E_{Na} are the equilibrium potentials for HCO_3^- and Na^+, respectively and n is the coupling ratio. So long as E_{rev} is negative in relation to E_m, the thermodynamic driving force acting on the transporter will favour Na^+–HCO_3^- influx. Under normal conditions, E_{rev} would be about −100 mV with a coupling ratio of 2 HCO_3^- : 1 Na^+ and would approximate to the value of E_m (about −58 mV) with a coupling ratio of 3 HCO_3^- : 1 Na^+. It should be noted that intracellular acidosis makes E_{HCO_3}, and therefore E_{rev}, more negative. Not only do the prevailing gradients make an electrogenic Na^+–HCO_3^- cotransport feasible, but also the marked hyper-

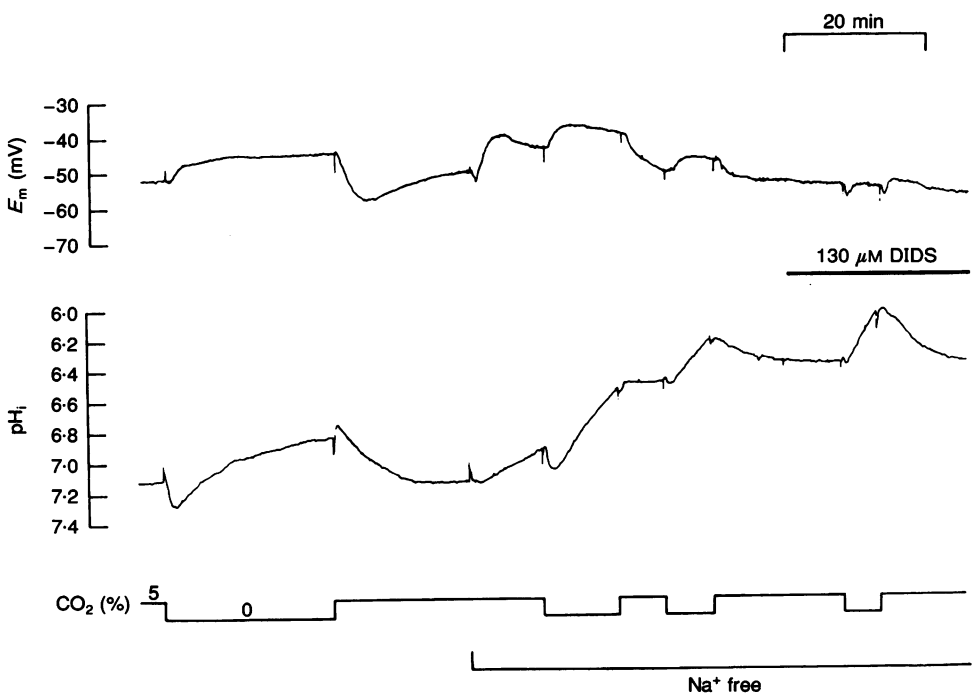

Figure 3. Pen recordings of an experiment to investigate the effect of DIDS on the Na^+-independent HCO_3^--dependent recovery from extreme acidosis

Acidosis was induced by changing from nominally CO_2–HCO_3^--free solution (buffered with 21 mM NaHepes or NMDG Hepes) to a solution buffered with 5% CO_2–21 mM HCO_3^-. Na^+ was replaced with $NMDG^+$.

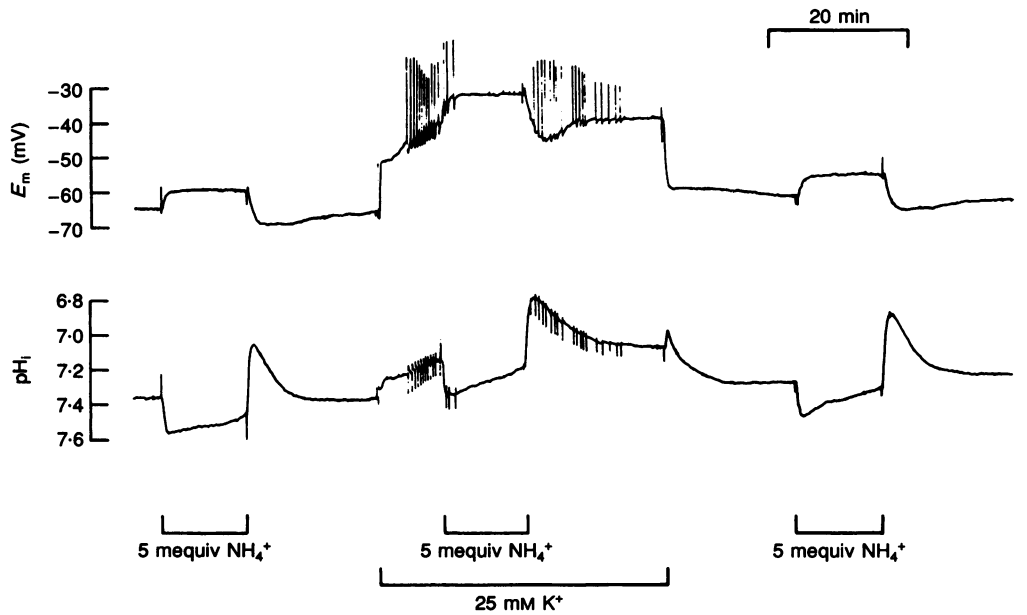

Figure 4. Pen recordings showing the effect of depolarization induced by increasing K_o^+ from 5·9 to 25 mM (isosmotic reduction in Na_o^+) on pH_i and on the rate of recovery from acidosis produced by application and removal of 2·5 mM $(NH_4)_2SO_4$
The preparation was maintained in solutions buffered with 5% CO_2–21 mM HCO_3^- throughout.

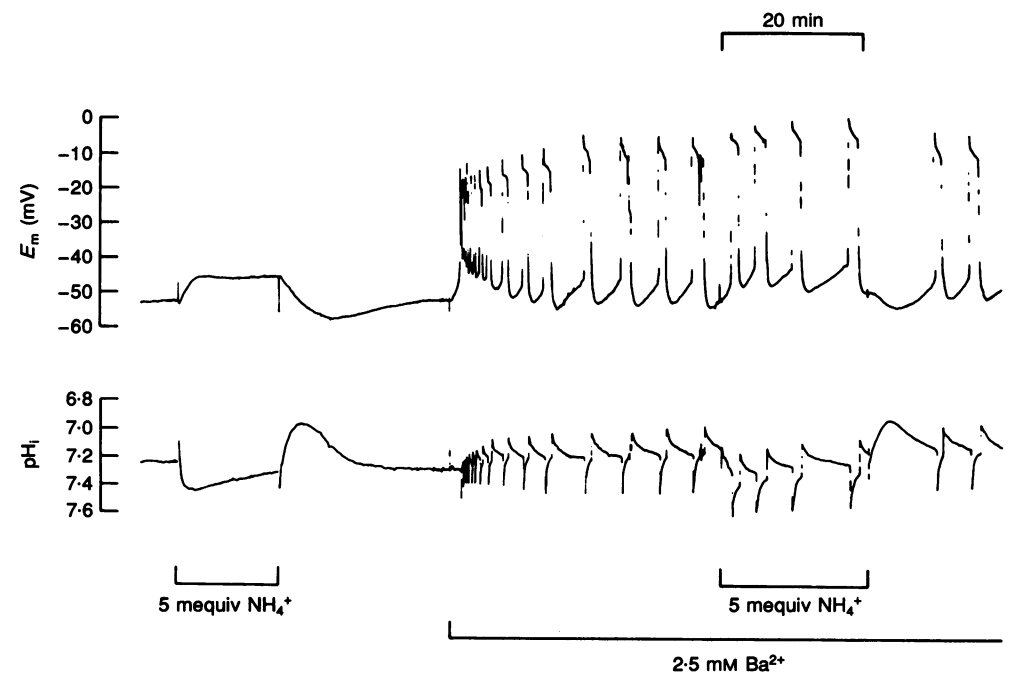

Figure 5. Pen recordings of an experiment to test the effect of 2·5 mM Ba^{2+} (added to modified Krebs solution) on the hyperpolarization associated with the recovery from an acid load induced by application and removal of 5 mM NH_4Cl
All solutions were buffered with 5% CO_2–21 mM HCO_3^-. Contraction during this experiment was considerable.

polarization which accompanies recovery from acidosis in the presence of CO_2–HCO_3^- is consistent with the operation of such a mechanism.

Effect of depolarization

If the Na^+- and HCO_3^--dependent mechanism was electrogenic, depolarization might favour inward transport and thus should increase both the steady-state pH_i and the rate of recovery from acidosis (see Deitmer & Szatkowski, 1990). However, as shown in Fig. 4, depolarization by elevation of K_o^+ caused a fall in pH_i and a decrease in the rate of recovery from acidosis. The mean pH_i in CO_2–HCO_3^--buffered solution containing 25 mM K_o^+ was 7·06 ± 0·04 ($n = 6$; E_m, −42·2 ± 2·0 mV) compared with 7·21 ± 0·08 ($n = 6$; E_m, −62·3 ± 2·3 mV; paired data) in normal (5·9 mM) K_o^+, while the half-time for recovery from acidosis increased to 3·6 ± 1·0 from 2·2 ± 0·9 min ($n = 5$; paired data). While thermodynamics cannot predict kinetics, the apparent inhibition of the ability to extrude acid equivalents is difficult to reconcile with the theoretically enhanced conditions for effective acid extrusion by the putative electrogenic mechanism. However, depolarization may cause an increase in intrinsic intracellular loading, as suggested by previous reports of an increase in both O_2 consumption (Saito, Sakai, Ikeda & Urakawa, 1968) and lactate release (Ishida & Paul, 1990) on elevation of K_o^+. Significantly, elevation of K_o^+ in the nominal absence of CO_2–HCO_3^- caused a similar acid shift and decrease in the rate of recovery from acidosis as observed in CO_2–HCO_3^--buffered solution.

Effect of channel blockers

Although the above result cannot be taken as conclusive proof that the Na^+- and HCO_3^--dependent mechanism is not electrogenic, it would suggest that it was not. What then underlies the hyperpolarization associated with the effective extrusion of acid equivalents? One possibility is a change in membrane conductance, for example an increase in the K^+ conductance. Such a change is indeed suggested by the loss of the usual overshoot in potential under conditions when the steady-state E_m was hyperpolarized, for example in Cl^--free solution (Fig. 1), after equilibration with DIDS (Fig. 2) and in the presence of 2-deoxyglucose (Fig. 5 of Aickin, 1989). Ba^{2+}, commonly used to block K^+ conductances (Sperelakis, Schneider & Harris, 1967), caused an immediate depolarization (Fig. 5) and spontaneous activity which, over a period of about 20 min, settled into a pattern of a contraction every 5–6 min associated with an action potential of almost 2 min duration (see also Lang, 1990); pH_i fell by about 0·1 unit. Nevertheless, recovery from acidosis was not measurably slowed and was accompanied by a hyperpolarization which delayed onset of the subsequent action potential for 10 min.

Recently, Lang (1990) characterized membrane currents in freshly dispersed cells of the guinea-pig ureter and indicated that all voltage-activated currents can be

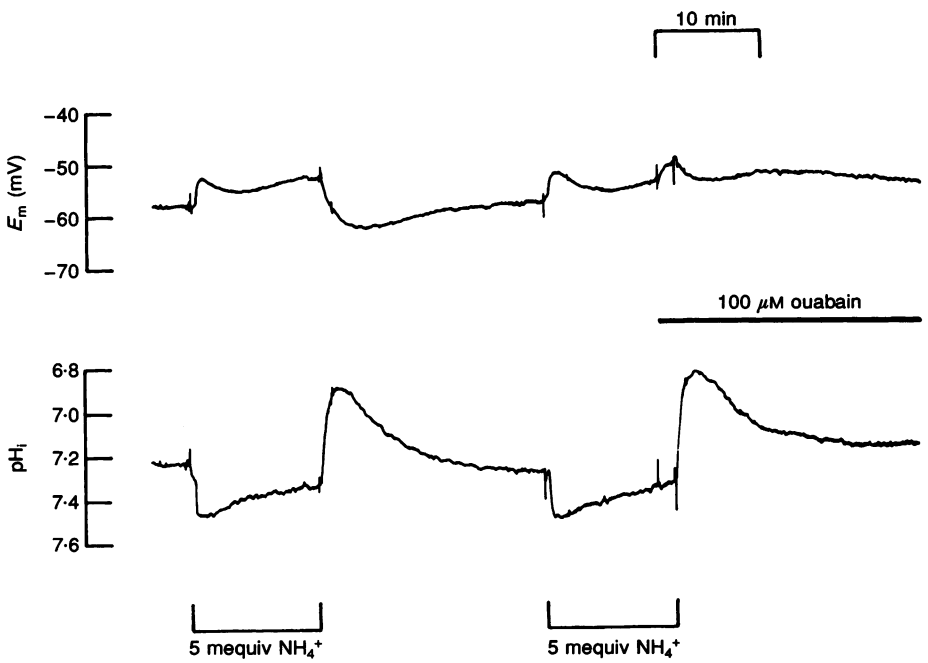

Figure 6. Pen recordings illustrating the effect of application of ouabain (100 μM) on the recovery from acidosis and on the associated hyperpolarization
Acidosis was induced by application and removal of 2·5 mM $(NH_4)_2SO_4$ and the preparation was superfused with 5% CO_2–21 mM HCO_3^--buffered solutions throughout.

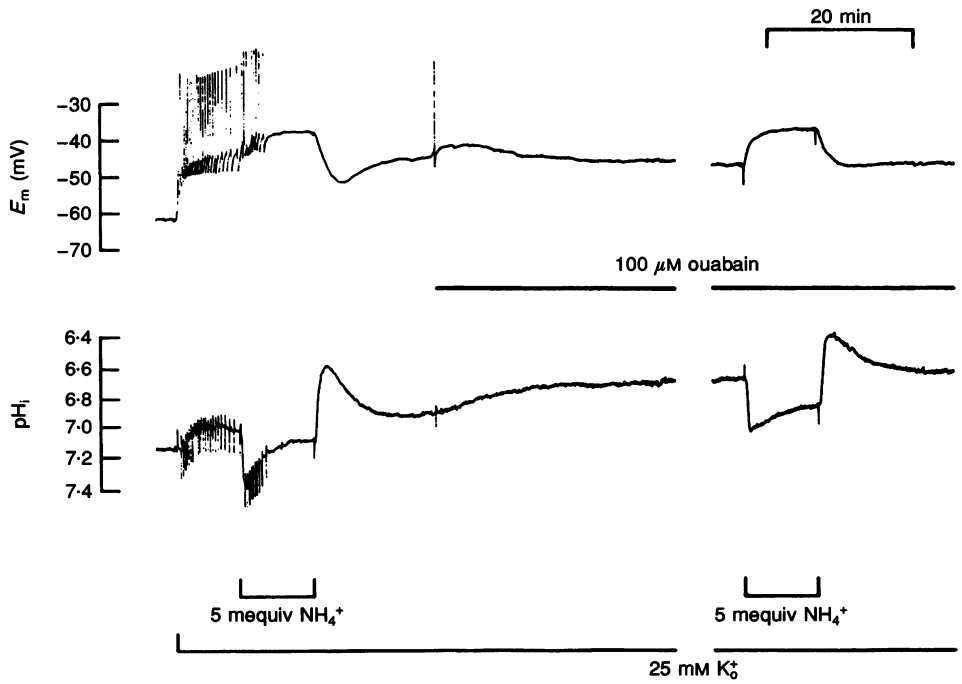

Figure 7. Pen recordings of an experiment to determine the effect of prolonged exposure to ouabain on the hyperpolarization associated with recovery from an acid load
K_o^+ was increased to 25 mM (isosmotic reduction in Na_o^+) and all solutions were buffered with 5% CO_2–21 mM HCO_3^-. The gap in the recording represents a period of 40 min during which little change in E_m or pH_i occurred.

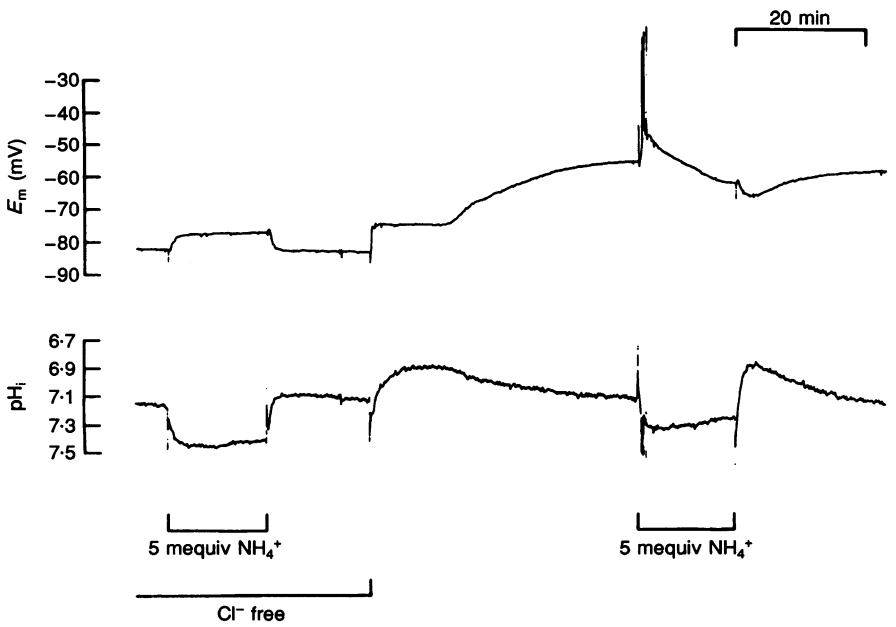

Figure 8. Pen recordings of part of an experiment showing the effect of Cl^--free conditions on the recovery from alkalosis during the continued presence of 2·5 mM $(NH_4)_2SO_4$
The preparation had been maintained in Cl^--free solution for 1·5 h before the recordings shown. All solutions were buffered with 5% CO_2–21 mM HCO_3^-.

blocked by Co^{2+} (substituting for Ca^{2+}, 2·5 mM), TEA (tetraethylammonium, 5 mM) and 4AP (4-aminopyridine, 1 mM). None of these agents abolished the overshoot in potential on removal of an ammonium salt or affected the rate of recovery of pH_i and neither did glibenclamide (10^{-6} M) or apamine (10^{-7} M).

Effect of ouabain

Since the results suggested that the hyperpolarization associated with recovery from an acid load was not caused by current flow through membrane channels, it seemed possible that it might indeed be due to an electrogenic mechanism – not the putative Na^+–HCO_3^- cotransport but the Na^+–K^+-ATPase. Stimulation of the mechanisms for effective extrusion of acid equivalents will cause an increased influx of Na^+ which may lead to stimulation of the Na^+ pump. Inhibition of the Na^+ pump by application of ouabain causes a sequence of changes in E_m in this preparation, commencing with an immediate depolarization and culminating in a maintained hyperpolarization (Aickin, 1987), which complicates investigation of the effect of ouabain on the hyperpolarization associated with recovery from acidosis. In the experiment shown in Fig. 6, ouabain (100 μM) was applied 2 min before the ammonium salt was removed. Thus, the depolarization due to application of ouabain was complete and the transmembrane Na^+ gradient hardly affected when the acid load was applied. Although the rate of pH_i recovery was essentially unaffected, the associated overshoot in potential was abolished. The 2–3 mV hyperpolarization observed on removal of the ammonium salt simply reflects removal of the depolarizing influence of NH_4^+, while the subsequent slow decline and then build up in potential is a common feature of prolonged exposure to ouabain in this preparation (see Fig. 2 of Aickin, 1987).

Figure 7 illustrates a different protocol in which the effect of Na^+ pump inhibition was investigated after the ouabain-induced changes in E_m had subsided, some 80 min after application of the drug. E_m in normal K_o^+ at this time is considerably more negative than under normal conditions and at such potentials in other conditions (e.g. in the absence of Cl^- and after equilibration with DIDS) the hyperpolarization associated with the effective extrusion of acid equivalents was inhibited. Therefore, K_o^+ was increased to 25 mM in the experiment shown so that E_m was within the normal range after prolonged exposure to ouabain. Again, the overshoot in potential normally associated with recovery from acidosis was essentially inhibited. In this case, the recovery of pH_i was slightly slowed (half-time of 3·3 compared with 2·2 min before application of ouabain), possibly due to reduction in the transmembrane Na^+ gradient. Intracellular Na^+ stabilizes at a relatively low level after inhibition of the Na^+ pump in these cells, due to operation of Na^+–Ca^{2+} exchange (Aickin, 1987; Aickin, Brading & Walmsley, 1987). It is well known that this mechanism is electrogenic and its

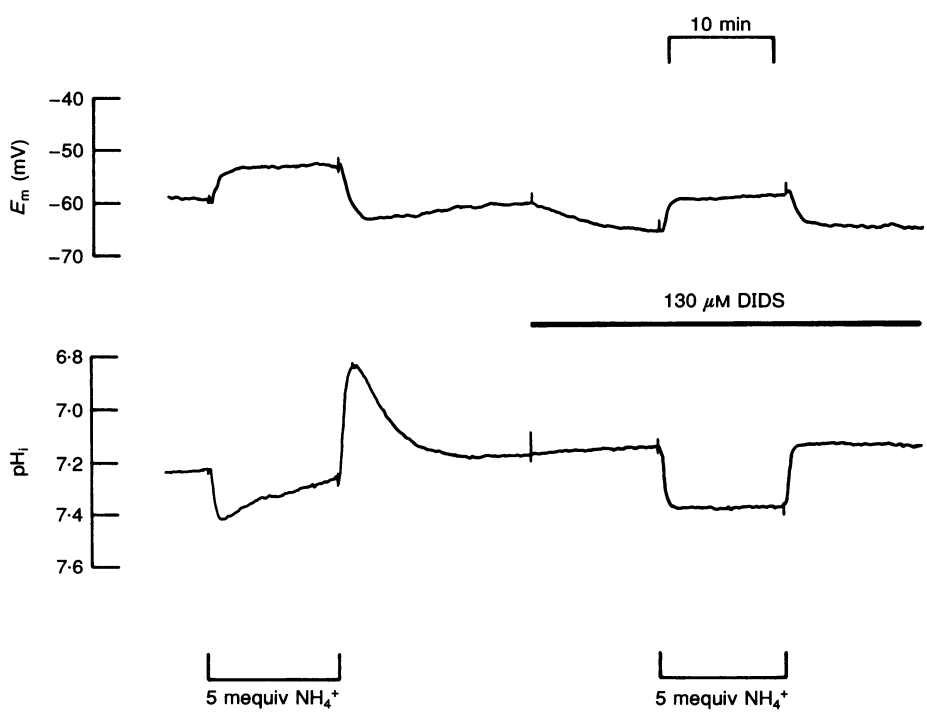

Figure 9. Pen recordings showing the effect of 130 μM DIDS on the recovery from alkalosis during the continued presence of 2·5 mM $(NH_4)_2SO_4$
All solutions were buffered with 5% CO_2–21 mM HCO_3^-.

stimulation on increased influx of Na^+ during the recovery from acidosis could account for the residual overshoot in potential observed (approximately 1 mV). It is notable that pH_i fell slowly on application of ouabain and stabilized after about 40 min, $0{\cdot}15 \pm 0{\cdot}22$ units ($n = 4$) more acidic than before drug application.

Effect of Cl^--free conditions and DIDS on the recovery from alkalosis

All the results described so far in this and the preceding paper (Aickin, 1994*a*) have been concerned with the characteristics of the recovery of pH_i from acid displacement. Recovery from alkaline displacement could be caused by passive leakage and/or metabolic production of acid equivalents and has frequently been ignored for this reason (but see Vaughan-Jones, 1982). Certainly, the relatively rapid acidification observed on inhibition of the acid extrusion mechanisms in these cells (e.g. Fig. 3) would suggest a substantial intrinsic intracellular acid loading that might well be expected to account for the return of pH_i to normal levels following alkalosis. Nevertheless, Cl^--free conditions largely blocked the acidification normally observed in the continued presence of an ammonium salt in CO_2–HCO_3^--buffered solution, as shown in Fig. 8. Note that, as a result, there was only a minimal overshoot in pH_i on removal of the ammonium salt. Similarly, pre-equilibration with DIDS prevented recovery of pH_i during continued exposure to an ammonium salt (Fig. 9) or trimethylamine (Fig. 4 of Aickin, 1994*a*) in the presence of CO_2–HCO_3^- and hence eliminated any overshoot in pH_i on return to normal solution. Taken together, these results suggest that extracellular Cl^-–intracellular HCO_3^- exchange is stimulated by intracellular alkalosis (increased intracellular HCO_3^-) and thus tends to restore pH_i towards a more acidic value.

DISCUSSION

Na^+–HCO_3^- cotransport

The finding that pH_i repeatedly recovered from acidosis at an undiminished rate after intracellular Cl^- had been maximally depleted by prolonged exposure to Cl^--free solution suggests that Na^+–HCO_3^- cotransport rather than Na^+-dependent Cl^-–HCO_3^- exchange was present in the smooth muscle of the guinea-pig ureter. This result alone cannot be taken as conclusive evidence since inhibition of the HCO_3^--dependent mechanism by the nominal absence of CO_2–HCO_3^- still left a brisk recovery from acidosis via Na^+–H^+ exchange. However, the failure of amiloride to affect the recovery of pH_i in Cl^--free conditions in the presence of CO_2–HCO_3^- rules out the possibility that Na^+–H^+ exchange was responsible in this case.

Na^+–HCO_3^- cotransport described in other preparations, whether involved in intracellular acid loading or effective acid extrusion, was initially found to be strongly electrogenic (e.g. Boron & Boulpaep, 1983; Deitmer & Schlue, 1989). Results in the smooth muscle of guinea-pig ureter, however, indicate that although the effective extrusion of acid equivalents is accompanied by a transient hyperpolarization, consistent with rheogenic Na^+–HCO_3^- cotransport, the mechanism is probably electroneutral. First, depolarization caused a fall in pH_i and a decrease in the rate of recovery from acidosis, exactly the opposite of what would be expected if the mechanism was electrogenic and voltage sensitive in the physiological range (see Deitmer & Szatkowski, 1990). This result may, however, be confused by a depolarization-induced increase in intracellular acid loading. Nevertheless, it is notable that the rate of recovery from acidosis was unaffected by the significant hyperpolarization on prolonged exposure to Cl^--free solutions, when a decrease in the rate of recovery would be expected if the mechanism was electrogenic. But second, inhibition of the electrogenic Na^+ pump eliminated the hyperpolarization associated with the effective extrusion of acid equivalents without affecting the rate of recovery of pH_i. Significantly, this result also indicates that the effective extrusion of acid equivalents is not only dependent on Na_o^+ but is accompanied by an influx of Na^+. This is supported by recording of a transient increase in the intracellular Na^+ activity both following removal of an ammonium salt from the superfusing solution and on changing from nominally CO_2-free solution to one buffered with 5% CO_2–21 mM HCO_3^- (C. C. Aickin, unpublished observations).

Cl^-–HCO_3^- exchange

The presence of anion exchange in the smooth muscle of guinea-pig ureter has previously been indicated by experiments concerned with the distribution of Cl^- ions (Aickin & Vermuë, 1983). The present recordings of intracellular alkalinization (influx of HCO_3^-) on removal of Cl_o^- (Fig. 1) and acidification (efflux of HCO_3^-) on reapplication of Cl_o^- (Fig. 7) supports the role of the exchanger in transmembrane movements of Cl^-. It is, however, interesting to note that the alkalosis caused by removal of Cl_o^- was not maintained, unlike in the vas deferens (Aickin & Brading, 1984) or the sheep heart Purkinje fibre (Vaughan-Jones, 1979), preparations in which the anion exchanger has been more fully investigated. Since most, if not all, intracellular Cl^- is lost on prolonged exposure to Cl^--free solution, extracellular Cl^-–intracellular HCO_3^- exchange must ultimately cease. Intuitively, one would expect that pH_i would then fall back towards the normal steady-state value, governed by the intrinsic intracellular acid loading (metabolic production and passive leakage) and the activity of the mechanisms for effective extrusion of acid equivalents, i.e. as observed in the ureter. Although this point has not been considered before, it is more difficult to explain why pH_i remains elevated in Cl^--free solutions in the vas deferens and cardiac Purkinje fibre.

Cl^-–HCO_3^- exchange, driven simply by the transmembrane gradients of Cl^- and HCO_3^-, would operate to acid load the cell under normal conditions, since the Cl^- equilibrium potential (E_{Cl}) is more negative (−18·6 mV; Aickin & Vermuë, 1983) than the HCO_3^- equilibrium potential (E_{HCO_3}, −14 mV), but would reverse, effectively to extrude acid equivalents when pH_i fell below about 7·1 (E_{HCO_3} then more negative than E_{Cl}). This would clearly assist in the regulation of pH_i, but at the expense of the transmembrane Cl^- gradient. However, the lack of effect of DIDS on the recovery from moderate (Fig. 2) or even extreme (Fig. 3) acidosis indicates that anion exchange was not involved. This raises the question of what underlies the HCO_3^--dependent, but Na^+-independent recovery of pH_i seen at extreme acidosis (below about 6·4). Significantly, this recovery was to a pH_i close to that predicted by a passive distribution and therefore could have been caused by passive movement of HCO_3^-, although clearly this conductance was not DIDS senstive. Inward movement of HCO_3^- ions on such a conductance would tend to hyperpolarize the membrane, as was indeed observed. It is notable that calculation of the HCO_3^- permeability from the rate of change of pH_i (assuming a buffering power of 14 mequiv H^+ (pH unit)$^{-1}$ l^{-1}) gives a value of $4{\cdot}8 \times 10^{-8}$ cm s^{-1} which, when added to the constant field equation (using values for intracellular concentrations and permeabilities to Na^+, K^+ and Cl^- previously determined under normal conditions; see Aickin, 1987), would predict a hyperpolarization of about 2 mV on changing from nominally CO_2–HCO_3^--free solution to one buffered with 5% CO_2–21 mM HCO_3^-. This is consistent with the magnitude of change recorded. It should, however, be noted that under normal conditions such a permeability to HCO_3^- would cause only a very slow fall in pH_i.

Although the evidence suggests no role for Cl^-–HCO_3^- exchange in the recovery from intracellular acidosis, there is good indication for its involvement in the restoration of pH_i from an alkaline displacement. This is apparent in the sensitivity of the recovery during exposure to trimethylamine both to the presence of CO_2–HCO_3^- and to DIDS. Recovery during exposure to an ammonium salt was also largely inhibited by the presence of DIDS but unexpectedly little affected by the presence or nominal absence of CO_2–HCO_3^-. The reason for this lack of sensitivity is unclear but it may reflect an increased permeability to NH_4^+ in the nominal absence of CO_2–HCO_3^- (note that E_m was less negative in the nominal absence than presence of CO_2).

Comparison with other preparations

Some of the findings in this and the preceding paper (Aickin, 1994*a*) closely resemble those in other preparations and particularly in other smooth muscles, notably the presence and properties of the Na^+–H^+ exchanger (at least as observed in the nominal absence of CO_2–HCO_3^-) and the contribution of Cl^-–HCO_3^- exchange to recovery from alkalosis. Of more interest, however, are the characteristics of the HCO_3^--dependent mechanism for the effective extrusion of acid equivalents which are at variance with those described in any other preparation. Recent results in rat mesenteric resistance arteries show the greatest similarity, with a considerable contribution to the effective extrusion of acid equivalents by an electroneutral Cl^--independent Na^+–HCO_3^- cotransport (Aalkjær & Cragoe, 1988; Aalkjær & Hughes, 1991). Unlike in the ureter, however, this mechanism was inhibited by DIDS. A smaller contribution to the extrusion of acid equivalents by a disulphonic stilbene-sensitive cotransport has been suggested in cultured mesangial cells (about 10%; Boyarsky, Ganz, Sterzel & Boron, 1988*b*) and a smooth muscle-like cell line (about 20%; Putnam, 1990). However, recovery in the former could have been due to passive HCO_3^- movement, as suspected to have occurred in the present experiments, since recovery was only observed to very low pH_i values. It should be emphasized that since no measurement of membrane potential was made in the mesangial cells, there is no indication of the pH_i expected from a passive distribution in any of the experimental conditions. In the latter, on the other hand, it is debatable whether intracellular Cl^- had been sufficiently depleted for the recovery not to reflect activity of the Na^+-dependent, Cl^-–HCO_3^- exchanger found to be largely responsible for the recovery from acidosis in these cells. Although nothing is known about Cl^- transport in these cultured cells, the relatively short exposure (25 min) to Cl^--free solution in the nominal absence of CO_2–HCO_3^- is inadequate to deplete intracellular Cl^- maximally in other smooth muscles (Aickin & Vermuuë, 1983; Aickin & Brading, 1984; Boyarsky *et al.* 1988*b*). Na^+-dependent Cl^-–HCO_3^- exchange was also found to play a major role in the recovery from acidosis in the mesangial cell (Boyarsky *et al.* 1988*b*). A novel Na^+- and HCO_3^--dependent mechanism, sensitive to the amiloride derivative EIPA (ethyl-isopropylamiloride) but insensitive to brief exposure to the disulphonic stilbenes, has recently been proposed in cells cultured from human internal mammary arteries (Neylon, Little, Cragoe & Bobik, 1990) but evidence for the HCO_3^- dependence was not compelling. Other cultured vascular cells apparently lack any HCO_3^--dependent mechanism for effective acid extrusion (Korbmacher, Helbig, Stahl & Wiederholt, 1988; Vigne, Breittmayer, Frelin & Lazdunski, 1988).

Electroneutral Na^+–HCO_3^- cotransport has recently been identified, contributing to the effective extrusion of acid equivalents, in isolated sheep heart Purkinje fibres (Dart & Vaughan-Jones, 1992) and guinea-pig ventricular myocytes (Lagadic-Gossmann, Buckler & Vaughan-Jones, 1992). Many similarties between the mechanisms involved in ion transport in mammalian smooth and cardiac muscle have already been noted (Aickin & Brading, 1984; Aickin *et al.* 1987) and it appears that the cotransporter revealed in

these cardiac preparations is similar to the mechanism in the guinea-pig ureter. In neither cardiac preparation, however, does the mechanism appear to be as important for the effective extrusion of acid equivalents as it is in the ureter, accounting for about 20% of the total acid equivalent efflux in the Purkinje fibre (Dart & Vaughan-Jones, 1992) and about 40% in guinea-pig ventricular myocytes (Lagadic-Gossmann *et al.* 1992). But it is worth noting that the cotransport has been reported to be the dominant mechanism in the rabbit sino-atrial node cell (Lagadic-Gossman *et al.* 1992). The striking difference between the mechanism described in these cardiac cells and that in the guinea-pig ureter is in the sensitivity to DIDS. The mechanism in the smooth muscle cells of the ureter is peculiar, with the exception of that found in cultured mouse oligodendrocytes (Kettenmann & Schlue, 1988), in its insensitivity to DIDS, even after prolonged equilibration with the drug. Na^+–HCO_3^- cotransport desribed in all other cells, whether electrogenic or electroneutral, whether mediating efflux or influx, is inhibited by the disulphonic stilbenes (see La Cour, 1989; Deitmer & Schlue, 1989; Aalkjær & Hughes, 1991; Lagadic-Gossmann *et al.* 1992).

REFERENCES

Aalkjær, C. & Cragoe, E. J. (1988). Intracellular pH regulation in resting and contracting segments of rat mesenteric resistance vessels. *Journal of Physiology* **402**, 391–410.

Aalkjær, C. & Hughes, A. (1991). Chloride and bicarbonate transport in rat resistance arteries. *Journal of Physiology* **436**, 57–73.

Aickin, C. C. (1987). Investigation of factors affecting the intracellular sodium activity in the smooth muscle of guinea-pig ureter. *Journal of Physiology* **385**, 483–505.

Aickin, C. C. (1988). Movement of acid equivalents across the mammalian smooth muscle membrane. In *Proton Passage Across Cell Membranes.* CIBA Foundation Symposium **139**, ed. Bock, G. & Marsh, J., pp. 3–22. John Wiley, Chichester, UK.

Aickin, C. C. (1989). Mechanisms involved in control of intracellular pH in smooth muscle. *Verhandlungen der Deutschen Zoologischen Gesellschaft* **82**, 121–129.

Aickin, C. C. (1994*a*). Regulation of intracellular pH in the smooth muscle of guinea-pig ureter: Na^+ dependence. *Journal of Physiology* **479**, 301–316.

Aickin, C. C. (1994*b*). Regulation of intracellular pH in smooth muscle cells of the guinea-pig femoral artery. *Journal of Physiology* **479**, 331–340.

Aickin, C. C. & Brading, A. F. (1982). Measurement of intracellular chloride in guinea-pig vas deferens by ion analysis, ^{36}chloride efflux and micro-electrodes. *Journal of Physiology* **326**, 139–154.

Aickin, C. C. & Brading, A. F. (1984). The role of chloride–bicarbonate exchange in the regulation of intracellular chloride in guinea-pig vas deferens. *Journal of Physiology* **349**, 587–606.

Aickin, C. C., Brading, A. F. & Walmsley, D. (1987). An investigation of sodium–calcium exchange in the smooth muscle of guinea-pig ureter. *Journal of Physiology* **391**, 325–346.

Aickin, C. C. & Vermuë, N. A. (1983). Microelectrode measurement of intracellular chloride activity in smooth muscle cells of guinea-pig ureter. *Pflügers Archiv* **397**, 25–28.

Ammann, D., Lanter, F., Steiner, R. A., Schulthess, P., Shijo, Y. & Simon, W. (1981). Neutral carrier based hydrogen ion selective microelectrode for extra- and intracellular studies. *Analytical Chemistry* **53**, 2267–2269.

Boron, W. F. & Boulpaep, E. L. (1983). Intracellular pH regulation in the renal proximal tubule of the salamander. Basolateral HCO_3^- transport. *Journal of General Physiology* **81**, 53–94.

Boron, W. F., McCormick, W. C. & Roos, A. (1981). pH regulation in barnacle muscle fibres: dependence on extracellular sodium and bicarbonate. *American Journal of Physiology* **240**, C80–89.

Boron, W. F. & Russell, J. M. (1983). Stoichiometry and ion dependencies of the intracellular-pH-regulating mechanism in squid giant axons. *Journal of General Physiology* **81**, 373–399.

Boyarsky, G., Ganz, M. B., Sterzel, R. B. & Boron, W. F. (1988*a*). pH regulation in single glomerular mesangial cells. I. Acid extrusion in absence and presence of HCO_3^-. *American Journal of Physiology* **255**, C844-C856.

Boyarsky, G., Ganz, M. B., Sterzel, R. B. & Boron, W. F. (1988*b*). pH regulation in single glomerular mesangial cells. II. Na^+-dependent and -independent Cl^-–HCO_3^- exchangers. *American Journal of Physiology* **255**, C857–869.

Buckler, K. J., Denyer, J. C., Vaughan-Jones, R. D. & Brown, H. F. (1990). Intracellular pH regulation in rabbit isolated sino-atrial node cells. *Journal of Physiology* **426**, 22*P*.

Casteels, R. (1971). The distribution of chloride ions in the smooth muscle of the guinea-pig's taenia coli. *Journal of Physiology* **214**, 225–243.

Casteels, R. (1981). Membrane potential in smooth muscle cells. In *Smooth Muscle: An Assessment of Current Knowledge*, ed. Bülbring, E., Brading, A. F., Jones, A. W. & Tomita, T., pp. 105–126. Edward Arnold, London.

Dart, C. & Vaughan-Jones, R. D. (1992). Na^+–HCO_3^- symport in the sheep cardiac Purkinje fibre. *Journal of Physiology* **451**, 365–385.

Deitmer, J. W. & Schlue, W.-R. (1989). An inwardly directed electrogenic sodium–bicarbonate co-transport in leech glial cells. *Journal of Physiology* **411**, 179–194.

Deitmer, J. W. & Szatkowski, M. (1990). Membrane potential dependence of intracellular pH regulation by identified glial cells in the leech central nervous system. *Journal of Physiology* **421**, 617–631.

Ishida, Y. & Paul, R. J. (1990). Effects of hypoxia on high-energy phosphagen content, energy metabolism and isometric force in guinea-pig taenia caeci. *Journal of Physiology* **424**, 41–56.

Kettenmann, H. & Schlue, W.-R. (1988). Intracellular pH regulation in cultured mouse oligodendrocytes. *Journal of Physiology* **406**, 147–162.

Korbmacher, C., Helbig, H., Stahl, F. & Wiederholt, M. (1988). Evidence for Na/H exchange and Cl/HCO_3 exchange in A10 vascular smooth muscle cells. *Pflügers Archiv* **412**, 29–37.

La Cour, M. (1989). Rheogenic sodium–bicarbonate co-transport across the retinal membrane of the frog retinal pigment epithelium. *Journal of Physiology* **419**, 539–553.

Lagadic-Gossman, D., Buckler, K. L. & Vaughan-Jones, R. D. (1992). Role of bicarbonate in pH recovery from intracellular acidosis in the guinea-pig ventricular myocyte. *Journal of Physiology* **458**, 361–384.

Lang, R. J. (1990). The whole-cell Ca^{2+} channel current in single smooth muscle cells of the guinea-pig ureter. *Journal of Physiology* **423**, 453–473.

NEYLON, C. B., LITTLE, P. J., CRAGOE, E. J. & BOBIK, A. (1990). Intracellular pH in human arterial smooth muscle. Regulation by Na^+/H^+ exchange and a novel 5-(*N*-ethyl-*N*-isopropyl) amiloride-sensitive Na^+ and HCO_3^--dependent mechanism. *Circulation Research* **67**, 814–825.

PUTNAM, R. W. (1990). pH regulatory transport systems in a smooth muscle-like cell line. *American Journal of Physiology* **285**, C470–479.

SAITO, Y., SAKAI, Y., IKEDA, M. & URAKAWA, N. (1968). Oxygen consumption during potassium induced contracture in guinea pig taenia coli. *Japanese Journal of Physiology* **18**, 321–331.

SPERELAKIS, N., SCHNEIDER, M. F. & HARRIS, E. J. (1967). Decreased K^+ conductance produced by Ba^{2+} in frog sartorius muscle. *Journal of General Physiology* **50**, 1565–1583.

THOMAS, R. C. (1977). The role of bicarbonate, chloride and sodium ions in the regulation of intracellular pH in snail neurones. *Journal of Physiology* **273**, 317–338.

THOMAS, R. C. & COHEN, C. J. (1981). A liquid ion exchanger alternative to KCl for filling intracellular reference microelectrodes. *Pflügers Archiv* **390**, 96–98.

VANHEEL, B., DE HEMPTINNE, A. & LEUSEN, I. (1984). Analysis of Cl^-–HCO_3^- exchange during recovery from intracellular acidosis in cardiac Purkinje strands. *American Journal of Physiology* **246**, C391–400.

VAUGHAN-JONES, R. D. (1979). Regulation of chloride in quiescent sheep-heart Purkinje fibres studied using intracellular chloride and pH-sensitive micro-electrodes. *Journal of Physiology* **295**, 111–137.

VAUGHAN-JONES, R. D. (1982). Chloride activity and its control in skeletal and cardiac muscle. *Philosophical Transactions of the Royal Society London* B **299**, 537–548.

VAUGHAN-JONES, R. D. (1986). An investigation of chloride–bicarbonate exchange in the sheep cardiac Purkinje fibre. *Journal of Physiology* **379**, 377–406.

VIGNE, P., BREITTMAYER, J.-P., FRELIN, C. & LAZDUNSKI, M. (1988). Dual control of the intracellular pH in aortic smooth muscle cells by a cAMP-sensitive HCO_3^-/Cl^- antiporter and a protein kinase C-sensitive Na^+/H^+ antiporter. *Journal of Biological Chemistry* **263**, 18023–18029.

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