Accumulation of intracellular HCO_3^- by Na^+–HCO_3^- cotransport in interlobular ducts from guinea-pig pancreas

H. Ishiguro, M. C. Steward*, A. R. G. Lindsay and R. M. Case

School of Biological Sciences, University of Manchester, G.38 Stopford Building, Manchester M13 9PT, UK

1. Short segments of interlobular duct were microdissected from guinea-pig pancreas following enzymatic digestion. After overnight culture, intracellular pH (pH_i) and Na^+ concentration ($[Na^+]_i$) were measured by microfluorometry in duct cells loaded with either the pH-sensitive fluoroprobe 2′7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) or the sodium-binding benzofuran isophthalate (SBFI).

2. The transporters responsible for maintaining pH_i above equilibrium were investigated by using the NH_4Cl pulse technique to acid load the cells. In the absence of HCO_3^-/CO_2, the recovery of pH_i was Na^+ dependent, abolished by 0·2 mM amiloride and by 10 μM *N*-methyl-*N*-isobutylamiloride and was therefore attributed to Na^+–H^+ exchange.

3. In the presence of HCO_3^-/CO_2, amiloride only partially inhibited the recovery from acid loading. The amiloride-insensitive component was abolished by 0·5 mM H_2DIDS and unaffected by depletion of intracellular Cl^- and was therefore attributed to Na^+–HCO_3^- cotransport.

4. Stimulation with 10 nM secretin did not cause a significant change in pH_i despite a significant increase in HCO_3^- efflux. However, in the presence of secretin, addition of 0·5 mM H_2DIDS caused a decline in pH_i that was three times more rapid than that obtained with 0·2 mM amiloride.

5. In secretin-stimulated ducts, Na^+ uptake increased when HCO_3^-/CO_2 was added to the bath and this increase was strongly inhibited by 0·5 mM H_2DIDS.

6. We conclude that Na^+–HCO_3^- cotransport contributes approximately 75% of the HCO_3^- taken up by guinea-pig pancreatic duct cells during stimulation with secretin. It is proposed that electrical coupling between HCO_3^- efflux at the luminal membrane and electrogenic Na^+–HCO_3^- cotransport at the basolateral membrane explains why secretin causes little change in pH_i.

Electrophysiological data have led to a model for HCO_3^- secretion by the rat pancreatic duct in which the supply of intracellular HCO_3^- ions is derived from carbonic acid as a result of the extrusion of protons across the basolateral membrane by Na^+–H^+ exchange (Case & Argent, 1993). Although this may be true in the rat, the lack of effect of amiloride (an inhibitor of Na^+–H^+ exchange) on ductal secretion in other species suggests that it may not be the only mechanism for HCO_3^- accumulation across the basolateral membrane (Kuijpers, Van Nooy, De Pont & Bonting, 1984; Grotmol, Buanes, Brørs & Ræder, 1986). Some evidence suggests that basolateral proton extrusion might, in part, involve a vacuolar-type H^+-ATPase (Villanger, Veel & Ræder, 1995). Other mechanisms that might be involved in HCO_3^- accumulation include Na^+-dependent HCO_3^- uptake from the blood either by Na^+–HCO_3^- cotransport or by Na^+-dependent Cl^-–HCO_3^- exchange (Boron, 1986).

In order to identify the transporters involved in HCO_3^- accumulation in pancreatic duct cells, we first examined the recovery of intracellular pH (pH_i) after acid loading by the NH_4Cl pulse technique. These studies were performed on interlobular ducts isolated from the guinea-pig pancreas which, for studies of HCO_3^- secretion, may be a more representative experimental model than the rat pancreas. The fluid secreted by the guinea-pig pancreas in response to secretin, like that of many other species, contains approximately 150 mM HCO_3^- (Padfield, Garner & Case, 1989), which is double the concentration secreted by the rat pancreas (Sewell & Young, 1975).

* To whom correspondence should be addressed.

Our data reveal the existence of a Na^+–HCO_3^- cotransporter in the guinea-pig pancreatic duct. The effects on pH_i and intracellular Na^+ concentration of inhibitors applied during stimulation with secretin indicate that Na^+–HCO_3^- cotransport contributes more to the accumulation of intracellular HCO_3^- for secretion than does Na^+–H^+ exchange. In the paper that follows (Ishiguro, Steward, Wilson & Case, 1996), we examine the mechanism of HCO_3^- efflux across the luminal membrane.

Parts of this work have been reported previously to The Physiological Society (Ishiguro, Lindsay, Steward & Case, 1994; Ishiguro, Steward & Case, 1995).

METHODS

Isolation and culture of interlobular ducts

Interlobular ducts were prepared by a modification of the methods developed originally for isolating ducts from copper-deficient rats (Arkle, Lee, Cullen & Argent, 1986; Argent, Arkle, Cullen & Green, 1986). Guinea-pigs (300–600 g) of either sex, raised on a normal pellet diet, were obtained from the Manchester University breeding colony and killed by cervical dislocation. The body and tail of the pancreas were removed and injected with a digestion buffer consisting of Dulbecco's modified Eagle's medium (DMEM) containing 80 U ml^{-1} collagenase, 400 U ml^{-1} hyaluronidase, 0·2 mg ml^{-1} soybean trypsin inhibitor and 2 mg ml^{-1} bovine serum albumin. The tissue was chopped coarsely with scissors into approximately 1 mm^3 pieces, gassed with 5% CO_2–95% O_2 and incubated at 37 °C for 35 min and then in fresh digestion buffer for a further 30 min. The digested tissue was washed with DMEM and resuspended in DMEM containing 0·2 mg ml^{-1} soybean trypsin inhibitor and 3% (w/v) bovine serum albumin. Interlobular ducts (diameter 100–130 μm) were microdissected from samples of tissue suspension under a dissection microscope using sharpened needles. The ducts were placed on polycarbonate membrane filters (Cyclopore) floating on McCoy's 5A tissue culture medium supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 0·1 mg ml^{-1} soybean trypsin inhibitor, 0·1 i.u. ml^{-1} insulin and 4 μg ml^{-1} dexamethasone. They were cultured at 37 °C in 5% CO_2 in air for up to 24 h.

Solutions

The standard Hepes-buffered solution used in these experiments contained (mM): 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose and 10 Hepes, and was equilibrated with 100% O_2. The Na^+-free Hepes-buffered solution contained *N*-methyl-D-glucamine ($NMDG^+$) in place of Na^+. The standard HCO_3^--buffered solution contained (mM): 115 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose and 25 $NaHCO_3$, and was equilibrated with 95% O_2–5% CO_2. The Na^+-free HCO_3^--buffered solution contained NMDG-Cl in place of NaCl, and choline bicarbonate in place of $NaHCO_3$. It also included 10 μM atropine to prevent the possible activation of muscarinic receptors by choline. Cl^--free solutions were made by replacing Cl^- with glucuronate. In solutions containing NH_4^+, the concentration of Na^+ was reduced to maintain osmolarity. All solutions were adjusted to pH 7·4 at 37 °C.

Measurement of intracellular pH

Intracellular pH was estimated by microfluorometry in ducts loaded with the pH-sensitive fluoroprobe 2′7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF). After overnight culture, the ducts were attached to glass coverslips pretreated with Cell-Tak. The coverslips were then mounted on the base of a 500 μl Perspex chamber which was perfused at 1·5 ml min^{-1} with the standard Hepes-buffered solution and maintained at 37 °C on the stage of a Nikon Diaphot inverted microscope. The cells were loaded with BCECF by superfusion for 10 min with buffer containing the acetoxymethyl ester BCECF AM (2 μM). Small regions of the ductal epithelium (10–20 cells) were illuminated alternately at 440 and 490 nm; fluorescence was measured at 530 nm (F_{440} and F_{490}). Values of pH_i were calculated at 2 s intervals from the F_{490}/F_{440} fluorescence ratio after correction for the endogenous tissue fluorescence measured prior to loading with BCECF. Calibration data were obtained by the high-K^+–nigericin technique (Thomas, Buchsbaum, Zimniak & Racker, 1979) using a separate perfusion

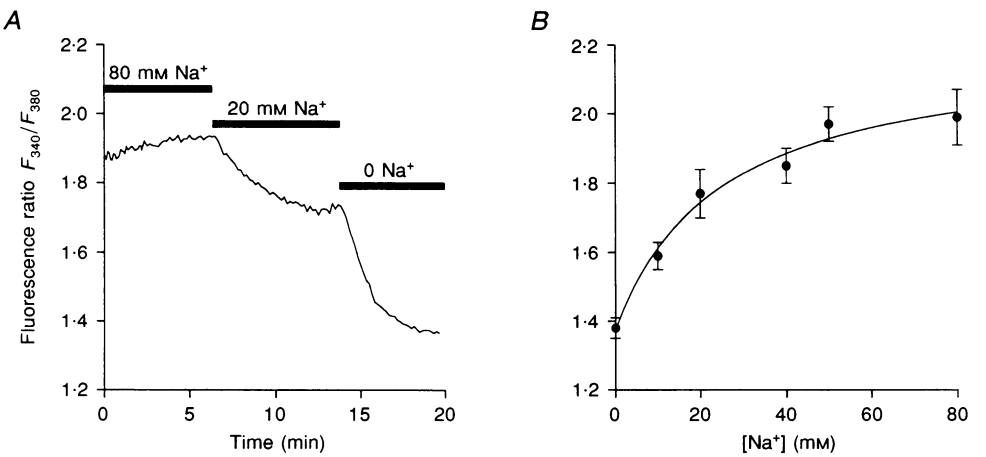

Figure 1. *In situ* calibration of SBFI fluorescence in interlobular ducts isolated from guinea-pig pancreas

A, calibration was performed *in situ* by first treating the SBFI-loaded duct with a combination of gramicidin D (5 μM), nigericin (10 μM), monensin (10 μM) and ouabain (1 mM). The concentration of Na^+ in the superfusate was stepped between the values shown. *B*, the relationship between the measured fluorescence ratio F_{340}/F_{380} and the Na^+ concentration of the bath. Data are plotted as the means ± S.D. of at least four values.

line to avoid nigericin contamination (Richmond & Vaughan-Jones, 1993).

Measurement of intracellular Na^+ concentration

Intracellular Na^+ concentration ($[Na^+]_i$) was estimated by microfluorometry in ducts loaded with the sodium-binding benzofuran isophthalate (SBFI; Harootunian, Kao, Eckert & Tsien, 1989). The cultured pancreatic ducts were incubated for 90 min at room temperature (19–22 °C) with the acetoxymethyl ester SBFI AM (10 μM) in a Hepes-buffered solution containing 1% BSA (w/v) and equilibrated with 100% O_2. The SBFI AM was dissolved first at a concentration of 10 mM in dimethyl sulphoxide containing pluronic acid (25% w/v). The ducts were washed and kept for 60 min at room temperature in fresh Hepes-buffered solution to allow completion of hydrolysis. The ducts were stored at 4 °C until required for use.

Microfluorometry was performed on small regions of the duct epithelium which were illuminated alternately at 340 and 380 nm. The fluorescence intensities were measured at 510 nm (F_{340} and F_{380}). Calibration of the F_{340}/F_{380} fluorescence ratio was performed *in situ* by application of a combination of gramicidin D (5 μM), nigericin (10 μM), monensin (10 μM) and ouabain (1 mM). The calibration solutions were prepared by mixing a high-Na^+ solution (containing (mM): 115 sodium glucuronate, 10 Na-Hepes, 15 NaCl, 1 $MgSO_4$ and 1 $CaCl_2$) and a high-K^+ solution (containing (mM): 115 potassium glucuronate, 10 Hepes, 10 KOH, 15 KCl, 1 $MgSO_4$ and 1 $CaCl_2$). The pH of both was adjusted to 7·4 with concentrated HCl. A low-chloride concentration was chosen in order to prevent cell swelling.

Figure 1*A* shows representative calibration data and Fig. 1*B* is a plot of the pooled data fitted with the function (Grynkiewicz, Poenie & Tsien, 1985):

$$F_{340}/F_{380} = (R_{max}[Na^+]_i + R_0K'_d)/(K'_d + [Na^+]_i),$$

where R_0 is the fluorescence ratio at zero Na^+ concentration, R_{max} is the ratio when SBFI is saturated with Na^+, and K'_d is the apparent dissociation coefficient, which was found to be 25·4 mM.

Statistics

Averaged data are presented as means ± S.E.M. unless otherwise indicated. Tests for statistically significant differences were made with Student's *t* test for paired or unpaired data as appropriate.

Materials

Culture media were obtained from Flow Laboratories (Irvine, UK) and Sigma. Collagenase (type CLSPA) was obtained from Worthington Biochemical Corporation (Freehold, NJ, USA), BCECF AM, SBFI AM, pluronic acid (Pluronic™ F-127) and dihydro-4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (H_2DIDS) from Molecular Probes, *N*-methyl-*N*-isobutylamiloride (MIA) from Research Biochemicals International, Cell-Tak from Becton Dickinson Labware (Bedford, MA, USA), and hyaluronidase, soybean trypsin inhibitor, amiloride hydrochloride, nigericin, gramicidin D, monensin and ouabain from Sigma.

RESULTS

The morphology of the interlobular duct segments isolated from the guinea-pig pancreas was similar to that of interlobular ducts obtained from rats maintained on a copper-deficient diet (Arkle *et al.* 1986; Argent *et al.* 1986). As with the ducts from the rat, overnight culture led to a dilatation of the lumen. This occurred as a result of fluid secretion into the closed luminal space following the spontaneous sealing of the cut ends. Stimulation with secretin (10 nM) led to a further increase in duct diameter (data not shown) indicating that the secretory response of the tissue to secretin was preserved.

Intracellular pH in unstimulated ducts superfused with a HCO_3^--free, Hepes-buffered solution was 7·54 ± 0·06 pH units (mean ± S.E.M., $n = 5$). When the ducts were superfused with a HCO_3^--buffered solution, pH_i was significantly lower, with a mean value of 7·28 ± 0·01 pH units ($n = 12$; $P < 0·01$, Student's *t* test).

Recovery of intracellular pH from an acid load in the absence of HCO_3^-/CO_2

In order to identify the transport mechanisms responsible for maintaining pH_i above electrochemical equilibrium, we examined the recovery of pH_i from an acid load. This was achieved by exposing ducts to a 2 min pulse of 20 mM NH_4Cl which, following a brief increase in pH_i, led to a marked acidification of the cells. To assess the contribution of the Na^+–H^+ exchanger, we first examined the recovery of pH_i in ducts superfused with the Hepes-buffered solution, i.e. in the nominal absence of HCO_3^- and CO_2 (Fig. 2). Under such conditions, there was no recovery of pH_i when the NH_4Cl pulse was followed by a Na^+-free solution. Upon restoration of extracellular Na^+, pH_i returned rapidly to the resting value. In the presence of 0·2 mM amiloride – a reversible inhibitor of Na^+–H^+ exchange – the initial rate of recovery was reduced by 80 ± 7% ($n = 5$), but when amiloride was then withdrawn, pH_i increased rapidly towards the resting value. When extracellular Na^+ was restored in the presence of 10 μM *N*-methyl-*N*-isobutyl-amiloride (MIA) – a more specific but less rapidly reversible inhibitor of Na^+–H^+ exchange (Dixon, Cohen, Cragoe & Grinstein, 1987) – the initial recovery rate was reduced by 86 ± 5% ($n = 5$) but, as expected, withdrawal of MIA led to a rather slower recovery compared with amiloride. This experiment indicates that, in the absence of HCO_3^-/CO_2, pH_i is maintained above equilibrium by Na^+–H^+ exchange.

Recovery of intracellular pH from an acid load in the presence of HCO_3^-/CO_2

The experiment was then repeated in the presence of HCO_3^- and CO_2. As shown in the first part of Fig. 3, there was again no recovery from an acid load in the absence of extracellular Na^+, but there was a rapid recovery of pH_i when extracellular Na^+ was restored in the presence of 0·2 mM amiloride. The initial rate of recovery was reduced by only 37 ± 10% ($n = 5$) compared with the recovery from the first pulse. When Na^+ was restored in the presence of MIA the recovery was still only partially inhibited. The initial rate was reduced by 44 ± 8% ($n = 5$) compared with the recovery from the first pulse.

These results show that in the presence of HCO_3^- and CO_2 an additional mechanism contributes to the maintenance of pH_i above electrochemical equilibrium and thus also to the supply of HCO_3^- for secretion. This component is Na^+

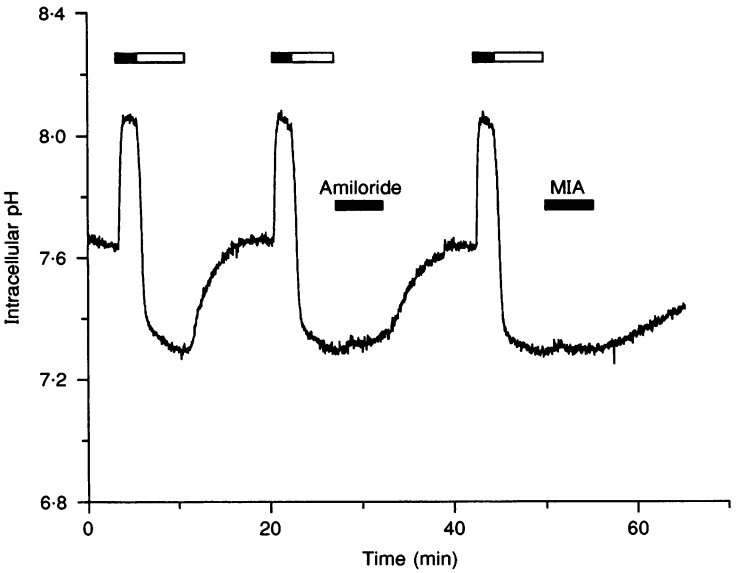

Figure 2. Effects of amiloride (0·2 mM) and MIA (10 μM) on the recovery from an acid load in the nominal absence of HCO_3^-/CO_2

Duct cells were acid loaded by exposure to 2 min pulses of 20 mM NH_4Cl (filled bar) followed by 5 min of Na^+-free solution (open bar). One of five experiments.

dependent, HCO_3^- dependent and insensitive to amiloride and MIA. If it is due to a Na^+-dependent anion transporter, it should be inhibited by the reversible, non-fluorescent disulphonic stilbene H_2DIDS (Lepke, Fasold, Pring & Passow, 1976). To test this, we examined the H_2DIDS sensitivity of the recovery of pH_i following an NH_4Cl pulse in ducts superfused with the HCO_3^--buffered solution (Fig. 4). When extracellular Na^+ was restored in the presence of both 0·2 mM amiloride and 0·5 mM H_2DIDS, the recovery was almost completely blocked. When H_2DIDS was withdrawn there was an immediate recovery at a rate comparable to that previously observed in the presence of amiloride alone. Thus the HCO_3^--dependent component of the recovery appears to involve anion transport, and is most probably due to HCO_3^- uptake.

Dependence of HCO_3^- accumulation on Cl^-

If the recovery mechanism involves HCO_3^- uptake via a Na^+-dependent Cl^-–HCO_3^- exchanger (Boron, 1986), it should show a dependence on intracellular Cl^-. To test this, ducts were depleted of Cl^- by replacement of all the superfusate Cl^- with glucuronate (Fig. 5). To allow free access of the superfusate to the lumen, the ends of the ducts were cut off with sharpened needles. The initial effect of Cl^- replacement was a marked increase in pH_i due to the accumulation of intracellular HCO_3^-. This increase was blocked by H_2DIDS (data not shown) and was probably due to Cl^- efflux via a Na^+-independent Cl^-–HCO_3^- exchanger.

To ensure that the cells were thoroughly depleted of Cl^-, they were exposed to three 4 min pulses of 10 mM $(NH_4)_2SO_4$, each followed by a 5 min exposure to the Na^+-free solution. The rationale for this protocol is as follows. After each $(NH_4)_2SO_4$ pulse, the resulting acidification would be expected to reduce $[HCO_3^-]_i$. This in turn would reduce the outward HCO_3^- gradient opposing Cl^-–HCO_3^- exchange and therefore favour further Cl^- efflux in exchange for HCO_3^- uptake. This would account for the partial

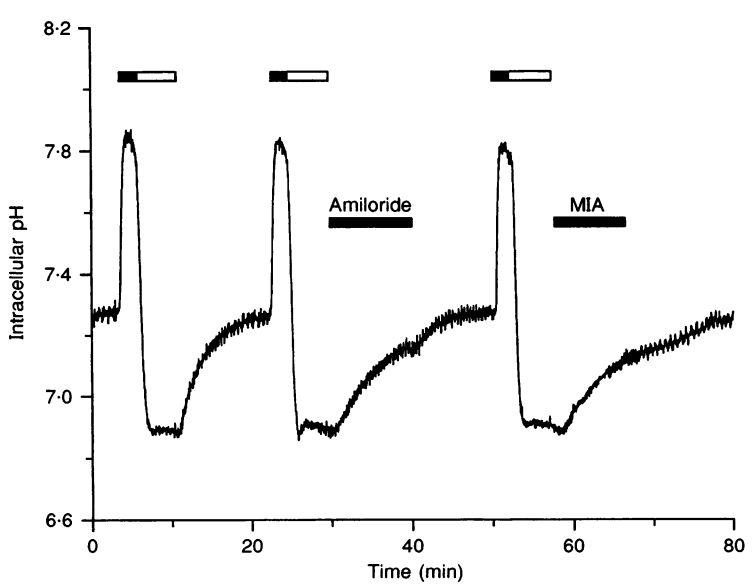

Figure 3. Effects of amiloride (0·2 mM) and MIA (10 μM) on recovery from acid load in the presence of 25 mM HCO_3^-

Duct cells were acid loaded by exposure to 2 min pulses of 20 mM NH_4Cl (filled bar) followed by 5 min of Na^+-free solution (open bar). One of five experiments.

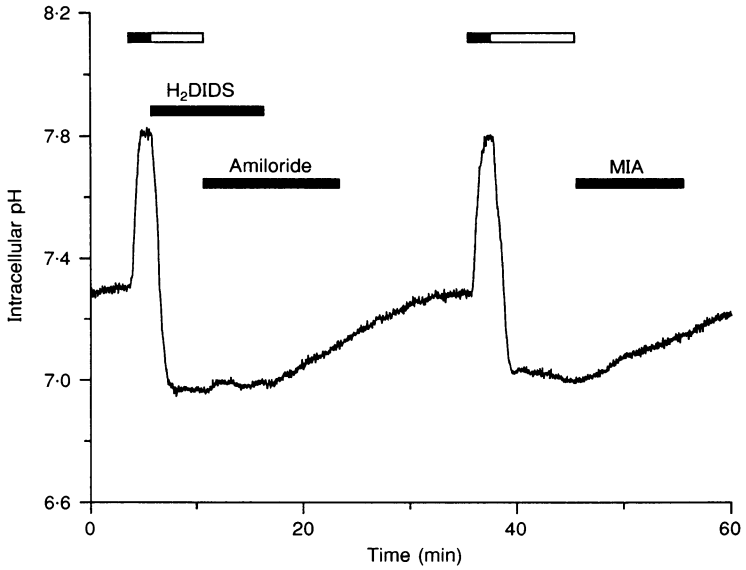

Figure 4. Effects of amiloride (0·2 mM) and H_2DIDS (0·5 mM) on recovery from acid load in the presence of 25 mM HCO_3^-

Duct cells were acid loaded by exposure to 2 min pulses of 20 mM NH_4Cl (filled bar) followed by 5 min of Na^+-free solution (open bar). One of seven experiments.

recovery of pH_i in the absence of extracellular Na^+ immediately after the first $(NH_4)_2SO_4$ pulse (Fig. 5). After the second pulse, this effect was reduced to a small transient, and after the third it was absent altogether. The equilibrium value of pH_i in the absence of Na^+ also gradually returned to normal (6·8–7·0) suggesting that the gradient for Cl^- efflux, and therefore HCO_3^- uptake, via the exchanger was largely abolished. It is therefore reasonable to suppose that, by this stage, the cells were thoroughly depleted of Cl^-. Consequently, after the third $(NH_4)_2SO_4$ pulse, the sensitivity of the recovery of pH_i to amiloride was examined. Once again amiloride only partially inhibited the recovery. The initial recovery rate, from a comparable pH_i value, was not significantly different from that observed in the presence of Cl^-. Thus the HCO_3^- uptake mechanism appears to be Cl^- independent but H_2DIDS sensitive, fulfilling another of the criteria for assignment to a Na^+–HCO_3^- cotransporter.

Effects of amiloride and H_2DIDS on steady-state pH_i

The next series of experiments was designed to evaluate the relative contributions of Na^+–H^+ exchange and Na^+–HCO_3^- cotransport to the maintenance of pH_i, and therefore the supply of HCO_3^- for secretion, both in unstimulated ducts and during stimulation with secretin. If Na^+–H^+ exchange is the dominant mechanism for HCO_3^- accumulation, addition of amiloride should lead to a steeper decline in pH_i than addition of H_2DIDS. Conversely, if Na^+–HCO_3^- cotransport is dominant, the opposite should be true.

A representative experiment on unstimulated duct cells is shown in Fig. 6*A*. After a small transient increase, pH_i declined hardly at all in the presence of 0·2 mM amiloride. The rate of acidification (Table 1) was very similar when the experiment was repeated with 0·5 mM H_2DIDS. When both inhibitors were applied simultaneously, the rate of acidification was approximately twice that observed with

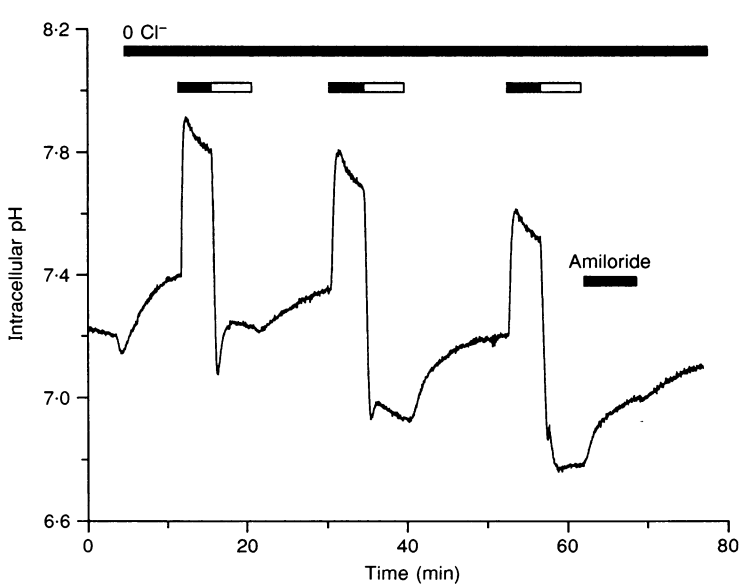

Figure 5. Recovery from acid load in duct cells depleted of Cl^-

Duct cells were acid loaded by exposure to 4 min pulses of 10 mM $(NH_4)_2SO_4$ (filled bar) followed by 5 min of Na^+-free solution (open bar). Cl^- was replaced by equimolar glucuronate. Solutions contained 25 mM HCO_3^-. One of five experiments.

Table 1. Initial rate of acidification following inhibition of acid/base transporters in unstimulated and secretin-stimulated ducts

Inhibitor	Unstimulated		Secretin (10 nM)	
	Initial pH_i	dpH_i/dt ((pH unit) min^{-1})	Initial pH_i	dpH_i/dt ((pH unit) min^{-1})
0·2 mM amiloride	7·34 ± 0·02	0·008 ± 0·001	7·36 ± 0·05	0·009 ± 0·001
0·5 mM H_2DIDS	7·37 ± 0·04	0·009 ± 0·002	7·34 ± 0·04	0·027 ± 0·002
0·2 mM amiloride + 0·5 mM H_2DIDS	7·35 ± 0·03	0·016 ± 0·002	7·37 ± 0·04	0·036 ± 0·002

The rate of acidification was estimated as the initial rate of decrease in pH_i following the addition of amiloride and/or H_2DIDS. Values represent the mean ± S.E.M. of five experiments. The ducts were superfused with the standard HCO_3^-/CO_2 solution. Initial values of pH_i prior to the addition of the inhibitors are also shown and were not significantly different in any of the groups ($P > 0·05$).

either inhibitor alone (Fig. 6*A*). These results indicate that, in unstimulated cells, the rate of acid loading as a result of HCO_3^- efflux, H^+ influx and metabolic H^+ production is relatively slow. Furthermore, the Na^+–H^+ exchanger and the Na^+–HCO_3^- cotransporter appear to contribute approximately equally in counteracting the tendency of the cells to acidify.

Stimulation with secretin

Perhaps surprisingly, stimulation with secretin at a concentration (10 nM) known to evoke maximal fluid secretion from rat ducts (Argent *et al.* 1986) led to little change in pH_i. In five experiments, pH_i was 7·34 ± 0·05 pH units prior to stimulation and 7·32 ± 0·05 pH units after 5 min of secretin stimulation ($P = 0·48$). However, in the presence of secretin, simultaneous application of amiloride and H_2DIDS (Fig. 6*B*) revealed an underlying increase in acid loading, presumably due to increased HCO_3^- efflux. Application of amiloride alone had no more effect on pH_i than it had in the unstimulated cells while application of H_2DIDS alone resulted in a rapid decline in pH_i (Table 1). These differences in the rate of acidification cannot be attributed to differences in the initial value of pH_i since this did not vary significantly in any of the experimental groups (Table 1). From these results, therefore, we conclude that the Na^+–HCO_3^- cotransporter contributes more to the accumulation of intracellular HCO_3^- during secretin stimulation than does Na^+–H^+ exchange.

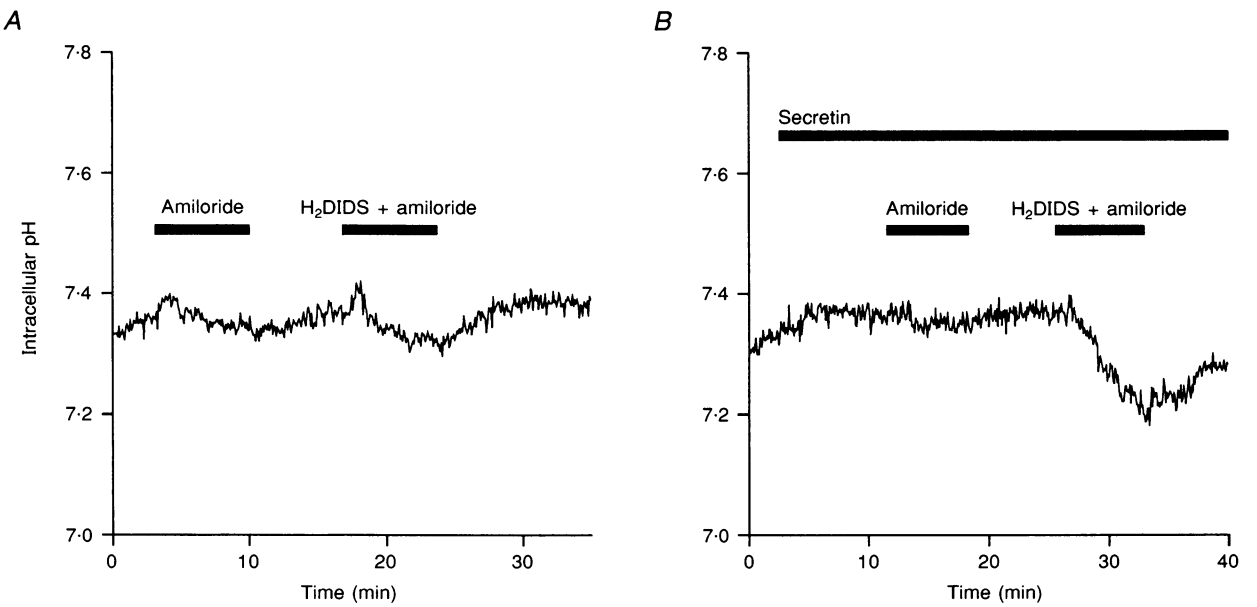

Figure 6. Estimation of the initial rate of intracellular acidification following inhibition of HCO_3^- accumulation pathways

Changes in pH_i in ducts exposed first to 0·2 mM amiloride and then simultaneously to 0·2 mM amiloride and 0·5 mM H_2DIDS. *A*, unstimulated duct. One of five experiments. *B*, duct stimulated with 10 nM secretin. One of five experiments.

H_2DIDS sensitivity of HCO_3^--dependent Na^+ uptake

As a further test of the contribution of Na^+–HCO_3^- transport to HCO_3^- accumulation during stimulation with secretin, we also monitored changes in intracellular Na^+ concentration ($[Na^+]_i$). The strategy adopted here was firstly to investigate the effect on steady-state $[Na^+]_i$ of switching from a HCO_3^--free to a HCO_3^--containing bath solution. If the net driving force for Na^+–HCO_3^- cotransport favoured HCO_3^- uptake, we would predict a concomitant increase in steady-state $[Na^+]_i$. Secondly, we measured the rate of increase in $[Na^+]_i$ upon application of 1 mM ouabain to assess the effects of secretin and H_2DIDS on steady-state Na^+ influx (Poronnik, Schumann & Cook, 1995).

$[Na^+]_i$ in unstimulated ducts superfused with the HCO_3^--free, Hepes-buffered solution was 13·3 ± 0·7 mM ($n = 4$). When the bath solution was switched to the HCO_3^--buffered solution (Fig. 7*A*), the mean value of $[Na^+]_i$ decreased slightly to 11·7 ± 1·2 mM ($n = 4$) but this change was not statistically significant. This suggests either that the Na^+–HCO_3^- cotransporter is inactive in unstimulated conditions or that the net driving force does not favour HCO_3^- uptake (see Discussion). When 1 mM ouabain was then applied to block Na^+ efflux, $[Na^+]_i$ increased steadily at a rate of 0·94 ± 0·20 mM min^{-1} ($n = 4$).

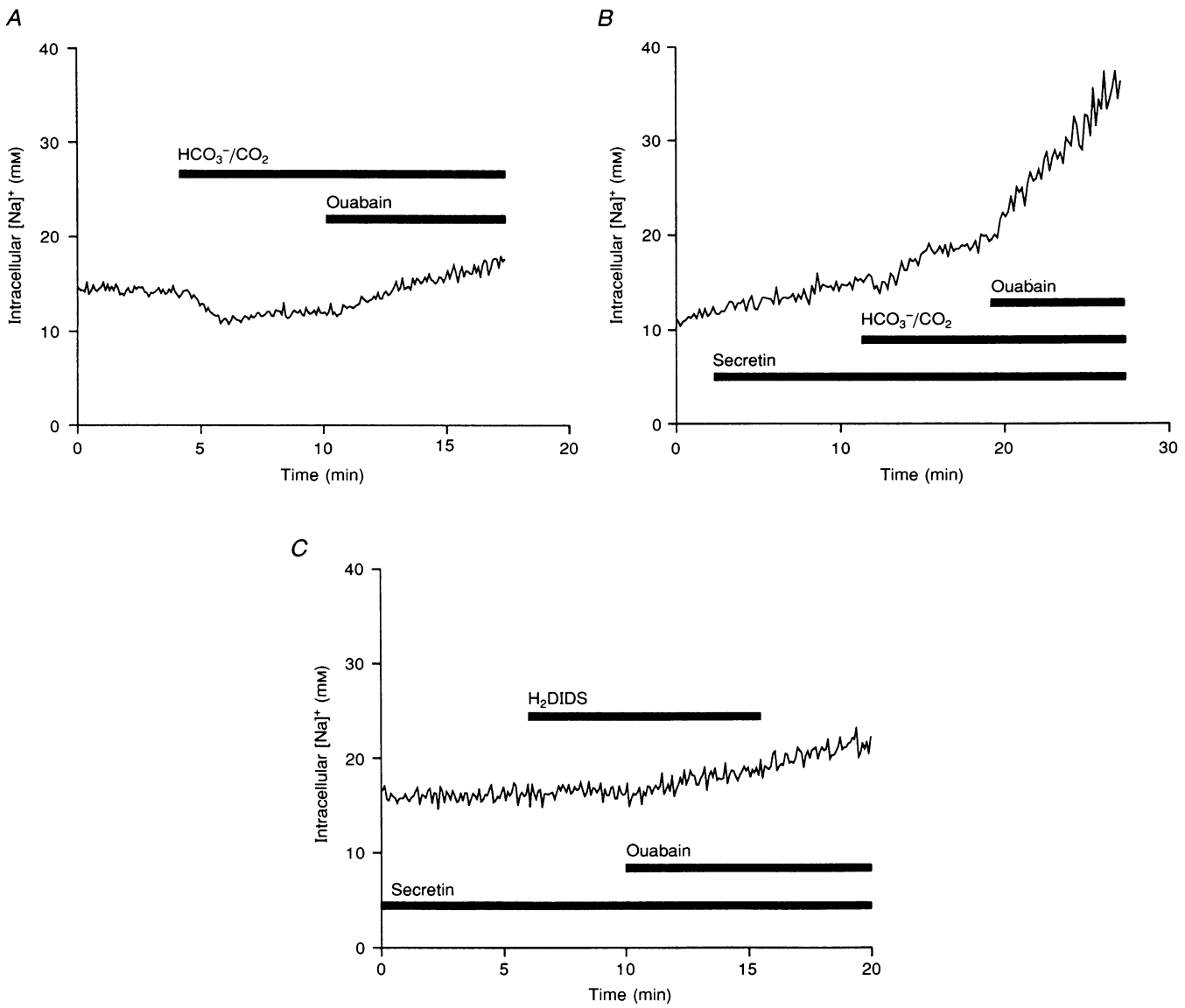

Figure 7. Effects of HCO_3^- and ouabain on intracellular Na^+ concentration

A, changes in $[Na^+]_i$ in an unstimulated duct loaded with SBFI and switched from the Hepes superfusate to the HCO_3^--buffered solution. Ouabain (1 mM) was then applied to estimate Na^+ influx. One of four experiments. *B*, a duct stimulated with secretin (10 nM) and then exposed to HCO_3^- and subsequently ouabain. One of four experiments. *C*, a secretin-stimulated duct in which ouabain was applied in the presence of H_2DIDS (0·5 mM). HCO_3^- was present throughout the experiment. One of four experiments.

When the ducts were exposed to HCO_3^-/CO_2 in the presence of 10 nM secretin, $[Na^+]_i$ increased significantly from 13·5 ± 0·8 to 17·2 ± 1·0 mM ($n = 4$, $P < 0·05$) after 5 min (Fig. 7*B*). This shows that in secretin-stimulated ducts there is significant HCO_3^--dependent uptake of Na^+. In support of this interpretation, subsequent inhibition of Na^+ efflux by ouabain caused $[Na^+]_i$ to increase at a rate of 2·01 ± 0·35 mM min^{-1} ($n = 4$), twice the rate obtained in unstimulated ducts ($P < 0·05$). Furthermore, in the presence of 0·5 mM H_2DIDS, which would be expected to inhibit Na^+ uptake via a Na^+–HCO_3^- cotransporter, the rate of increase in $[Na^+]_i$ following addition of ouabain was reduced by 75% to 0·48 ± 0·25 mM min^{-1} ($n = 4$; Fig. 7*C*). Unfortunately it was not possible to examine the effects of amiloride and its derivatives because of their strong interference with the SBFI signal. Nonetheless, these data clearly support the conclusion that, during stimulation with secretin, there is a marked increase in the activity of a Na^+- and HCO_3^--dependent transporter that is inhibited by H_2DIDS.

DISCUSSION

Our aim was to determine whether transporters other than the Na^+–H^+ exchanger contribute to the accumulation of intracellular HCO_3^- in the guinea-pig pancreatic duct. To do this, we first examined the recovery of pH_i following acid loading with an NH_4Cl pulse. In the nominal absence of HCO_3^-/CO_2, the recovery from an acid load was entirely dependent on extracellular Na^+ and was largely abolished by amiloride and MIA. We conclude that under these conditions pH_i is maintained above electrochemical equilibrium by Na^+–H^+ exchange alone.

The resting value of pH_i was significantly lower in the presence of HCO_3^-/CO_2 (*ca* 7·3) than in its absence (*ca* 7·5). This would be expected if there is a constitutive efflux pathway for HCO_3^-. If the efflux pathway is to the lumen, it could explain the spontaneous secretion that is observed in the absence of secretin (Padfield *et al.* 1989).

In the presence of HCO_3^-/CO_2, the recovery from an acid load was only partially blocked by amiloride or MIA. It was, however, totally blocked by amiloride in combination with H_2DIDS. The H_2DIDS-sensitive component of the recovery was unaffected by depletion of intracellular Cl^-, so it was unlikely to be due to a Na^+-dependent Cl^-–HCO_3^- exchanger such as that postulated to explain HCO_3^- secretion in the rabbit pancreas (Kuijpers *et al.* 1984). We therefore propose that, in the guinea-pig pancreatic duct, pH_i recovery in the presence of amiloride is the result of HCO_3^- uptake via a Na^+–HCO_3^- cotransporter. This may indicate a significant difference from the rat pancreatic duct where the existence of such a transporter has been excluded on electrophysiological grounds (Novak & Greger, 1988), although measurements of pH_i suggest that a Na^+–HCO_3^- cotransporter may nonetheless be present (Zhao, Star & Muallem, 1994).

To evaluate the relative contributions of Na^+–H^+ exchange and Na^+–HCO_3^- cotransport to the accumulation of intracellular HCO_3^-, we next measured the rates of intracellular acidification when the transporters were inhibited with amiloride and H_2DIDS, respectively. The main assumption was that the initial rate of acidification would indicate the rate of HCO_3^- uptake or H^+ efflux that had been generated by the individual transporters prior to their inhibition. The results obtained with both unstimulated and secretin-stimulated ducts suggested that the amiloride- and H_2DIDS-sensitive fluxes were additive. In the unstimulated ducts, the two transporters appeared to contribute roughly equally to a relatively small basal flux. Upon stimulation with secretin, the H_2DIDS-sensitive flux increased threefold while the amiloride-sensitive flux remained unchanged. This suggests that Na^+–HCO_3^- cotransport could contribute as much as 75% of the HCO_3^- accumulation flux during secretin stimulation.

Previous studies of pH_i regulation in both rat and pig pancreatic ducts have yielded evidence for the existence of a basolateral, vacuolar-type H^+-ATPase that is stimulated by secretin (Zhao *et al.* 1994; Villanger *et al.* 1995). Whether it makes a significant contribution to HCO_3^- secretion in those species is not clear. In our studies, the failure of pH_i to recover from acid loading in the absence of extracellular Na^+ suggests that there is little H^+-ATPase activity in the unstimulated guinea-pig ducts – either in the presence or absence of HCO_3^-/CO_2. This contrasts with observations in unstimulated ducts from the rat pancreas (Zhao *et al.* 1994) which showed that the H^+-ATPase may be activated by HCO_3^-/CO_2 through a Ca^{2+}-mediated pathway. One explanation for the difference may be that the lowest values of pH_i attained following acid loading in our studies (6·9–7·0 in the presence of HCO_3^-/CO_2) were not as low as those which elicited H^+-ATPase activity in the rat ducts (*ca* 6·25). It is possible that a larger acidification would activate a normally quiescent H^+-ATPase in the guinea-pig ducts. It may also be significant that, in the rat ducts, pH_i recovered to around 6·7 through the activity of the H^+-ATPase but apparently no higher. This suggests that, at physiological values of pH_i such as those observed during secretin stimulation, the H^+-ATPase in the rat ducts would be relatively inactive.

Although we cannot exclude the presence of a secretin-stimulated H^+-ATPase in the guinea-pig ducts, the rapid decrease in pH_i that we observed following the application of H_2DIDS (Fig. 6*B*) leaves little doubt that the Na^+–HCO_3^- cotransporter makes a major contribution to the supply of intracellular HCO_3^- during secretin stimulation. Curiously, Villanger *et al.* (1995) also presented good evidence (see their Fig. 10) for the involvement of Na^+–HCO_3^- cotransport in this process in the pig ducts. However, they dismissed its relevance for two reasons. First, they argued that a proton extrusion mechanism, rather than HCO_3^- uptake, is necessary to explain the low P_{CO_2} of pancreatic juice obtained *in vivo* (Ræder, Mo, Aune & Mathisen, 1980). As discussed below, this latter observation is in direct conflict with measurements of P_{CO_2} in the juice secreted in the

perfused cat pancreas. Second, they suggested that the cotransporter would only contribute to the small fraction of the HCO_3^- output that is secreted independently of carbonic anhydrase activity. This assumes that carbonic anhydrase inhibitors do not interfere with other components of the secretory mechanism, a point that we discuss further in the accompanying paper (Ishiguro *et al.* 1996).

Further evidence of the role of the Na^+–HCO_3^--cotransporter in the guinea-pig ducts was obtained in measurements of $[Na^+]_i$. First, the rise in $[Na^+]_i$ upon introduction of HCO_3^- during secretin stimulation was consistent with HCO_3^--dependent Na^+ uptake, but was absent in the unstimulated ducts where the gradient for the cotransporter was less favourable or possibly even reversed (see below). Second, the influx of Na^+, estimated from the initial rate of increase in $[Na^+]_i$ following application of ouabain, was markedly stimulated by secretin but largely abolished by H_2DIDS.

Our evidence for the role of Na^+–HCO_3^- cotransport in secretion by the isolated guinea-pig pancreatic duct accords with earlier observations in the isolated perfused cat pancreas. In this preparation, the rate of secretin-stimulated fluid secretion increases in proportion to the perfusate HCO_3^- concentration over the range of 0–30 mM HCO_3^- (Case, Scratcherd & Wynne, 1970). Under these conditions, the P_{CO_2} of the secreted fluid increases as secretory rate increases and is *always* elevated above the P_{CO_2} of the arterial perfusate and venous effluent (Ammar, Hutson & Scratcherd, 1987). Therefore, unless there are marked differences in the permeability of basolateral and apical membranes towards CO_2 (which is unlikely, but has been observed in gastric glands; Waisbren, Geibel, Modlin & Boron, 1994), there is no gradient for the passive movement of CO_2 from perfusate to lumen nor, therefore, from perfusate to cytosol. These data therefore support our conclusion that inward transport of HCO_3^- across the basolateral membrane on a Na^+–HCO_3^- cotransporter is more important than outward transport of H^+.

The Na^+–HCO_3^- cotransporters characterized in other tissues carry two or three HCO_3^- ions per Na^+ ion and are therefore electrogenic (Boron, 1986). They are reversible and while they mediate HCO_3^- efflux from the renal proximal tubule (Seki, Coppola & Frömter, 1993) they may be responsible for HCO_3^- uptake in other tissues (Fitz, Lidofsky & Scharschmidt, 1993). The direction in which net transport occurs will depend on both the Na^+ and HCO_3^- concentration gradients and on the membrane potential. The data in Fig. 3 suggest that, in unstimulated cells, the cotransporter may approach equilibrium at a value of pH_i below the resting value. In that case, it might even contribute to HCO_3^- *efflux* at the resting pH_i. The small decrease in $[Na^+]_i$ following exposure of unstimulated cells to HCO_3^- (Fig. 7*A*) provides some support for this idea. However, if stimulation with secretin leads to depolarization, as it does in the rat pancreatic duct (Novak & Pahl, 1993), the driving force for the cotransporter will then strongly favour HCO_3^- uptake. The most probable location for the cotransporter would therefore be in the basolateral rather than the luminal membrane. Two other observations point to a basolateral location. First, as we show in the following paper (Ishiguro *et al.* 1996), H_2DIDS applied to the luminal membrane had no effect on secretin-stimulated HCO_3^- secretion. Second, fluorometric studies of microperfused pancreatic ducts from the rat have demonstrated the presence of a Na^+–HCO_3^- cotransporter that is located in the basolateral membrane (Zhao *et al.* 1994).

One initially surprising finding of the present study was the absence of any significant change in pH_i following stimulation with secretin. This is in marked contrast to the transient acidification and subsequent alkalinization that occurs in salivary acinar cells following muscarinic stimulation (e.g. Lau, Elliott & Brown, 1989; Seo, Larcombe-McDouall, Case & Steward, 1995). An attractive feature of our modified model for HCO_3^- secretion in the pancreatic duct – which could account for this observation – is that increased HCO_3^- efflux across the luminal membrane will stimulate HCO_3^- uptake across the basolateral membrane without necessarily causing any change in pH_i. This is because the electrogenicity of the cotransporter will ensure an electrical coupling between electrogenic HCO_3^- efflux across the luminal membrane, which tends to depolarize the cell, and HCO_3^- uptake across the basolateral membrane, which would be accelerated by depolarization. If, on the other hand, HCO_3^- secretion were driven chiefly by Na^+–H^+ exchange, increased HCO_3^- accumulation (H^+ efflux) would require either a decrease in pH_i to accelerate the Na^+–H^+ exchanger or a shift in the activation curve of the exchanger to higher pH values. Both changes occur in salivary acinar cells, but we have found no evidence for either in pancreatic duct cells. This observation therefore lends further support to our conclusion that secretin-stimulated HCO_3^- secretion is driven chiefly by Na^+–HCO_3^- cotransport rather than by Na^+–H^+ exchange.

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Author's present address

H. Ishiguro: Internal Medicine II, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466, Japan.

Author's email address

M. C. Steward: m.c.steward@man.ac.uk

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