Bicarbonate secretion in interlobular ducts from guinea-pig pancreas

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1. The transport of HCO_3^- across the luminal membrane of pancreatic duct cells was studied by monitoring the luminal pH of isolated guinea-pig interlobular ducts after microinjection of an extracellular fluoroprobe, the dextran conjugate of 2′7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF–dextran). Luminal Cl^- concentration was also measured by microfluorometry following microinjection of the dextran conjugates of 6-methoxy-*N*-(4-aminoalkyl)quinolinium bromide (ABQ–dextran) and Cl-NERF (Cl-NERF–dextran).

2. When HCO_3^-/CO_2 was admitted to the bath, a transient acidification of the duct lumen was observed, followed by a marked alkalinization. The latter was abolished when the luminal Cl^- concentration was reduced to 25–35 mM by replacement with glucuronate and may, therefore, be attributed to Cl^-–HCO_3^- exchange at the luminal membrane.

3. Secretin, forskolin and acetylcholine stimulated HCO_3^- secretion into the lumen even when the luminal Cl^- concentration was reduced to approximately 7 mM. Furthermore, agonist-evoked HCO_3^- secretion was not inhibited by luminal glibenclamide, dihydro-4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (H_2DIDS) or 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB). These observations are not easily reconciled with HCO_3^- transport across the luminal membrane being mediated by Cl^-–HCO_3^- exchange in parallel with a Cl^- conductance.

4. Agonist-stimulated HCO_3^- secretion was blocked by omitting Na^+ from the bath but not by addition of *N*-methyl-*N*-isobutylamiloride (MIA) or bafilomycin A_1. This supports our previous conclusion that HCO_3^- entry into duct cells from the extracellular fluid requires Na^+ but is not dependent on Na^+–H^+ exchange or vacuolar-type H^+-ATPase activity.

5. The three actions of secretin on guinea-pig pancreatic duct cells described in this and the accompanying paper – stimulation of a relatively Cl^--insensitive luminal HCO_3^- efflux pathway, stimulation of basolateral Na^+–HCO_3^- cotransport, and lack of effect on intracellular pH – require the current model of pancreatic HCO_3^- secretion to be modified.

Studies on the perfused cat pancreas (Case, Scratcherd & Wynne, 1970; Case, Hotz, Hutson, Scratcherd & Wynne, 1979) and isolated rabbit pancreas (Kuijpers, Van Nooy, De Pont & Bonting, 1984*a*) demonstrated that pancreatic fluid secretion is absolutely dependent upon the presence of HCO_3^- in the perfusion/incubation fluid. They also demonstrated a dependence on Cl^-. Replacement of Cl^- in the external fluid by isethionate inhibited fluid secretion by 70% in the cat and 50% in the rabbit. These data suggested that pancreatic fluid and HCO_3^- secretion were in some way linked to Cl^- transport, and the involvement of a Cl^-–HCO_3^- exchanger was tentatively invoked (Case *et al.* 1979).

More recently, studies on microperfused interlobular ducts isolated from the rat pancreas have shown that Cl^-–HCO_3^- exchangers are present at the luminal membrane of the duct cells (Zhao, Star & Muallem, 1994) and have also indicated the presence there of a Cl^- conductance (Novak & Greger, 1988). Patch-clamp studies on the same cells have confirmed the presence of this luminal conductance and shown it to be mediated by a small-conductance Cl^- channel, analogous to the human cystic fibrosis gene product (CFTR), which is activated by cyclic AMP following stimulation with secretin (Gray, Greenwell & Argent, 1988; Gray, Harris, Coleman, Greenwell & Argent, 1993).

These and other data have led to a model for HCO_3^- secretion across the luminal membrane of the pancreatic duct in which Cl^- first enters the lumen via Cl^- channels and is then exchanged for HCO_3^- by Cl^-–HCO_3^- exchange: in effect the cycling of Cl^- drives HCO_3^- exit from the cell.

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While such a model may explain HCO_3^- secretion in a species such as the rat, where the maximum observed HCO_3^- concentration in pancreatic juice is 70 mM (Sewell & Young, 1975), it is unclear whether it can adequately account for HCO_3^- secretion in species like the guinea-pig (and human) where the luminal HCO_3^- concentration may reach 150 mM (Padfield, Garner & Case, 1980). Under these conditions it seems unlikely that the inward Cl^- gradient across the luminal membrane is sufficient to drive HCO_3^- efflux on the Cl^-–HCO_3^- exchanger.

If the model is correct, HCO_3^- secretion into the lumen should be dependent upon the presence of Cl^- in the lumen. In this paper we describe experiments to test this hypothesis. By adapting a method devised for studies on intrahepatic bile ductules (Roberts, Kuntz, Gores & LaRusso, 1993) we have measured luminal pH in interlobular ducts isolated from the guinea-pig pancreas. Luminal alkalinization, following the addition of HCO_3^-/CO_2 to the bath, has been used as an index of HCO_3^- secretion. This has enabled us to demonstrate that the ducts secrete HCO_3^- in response to stimulation with both secretin and acetylcholine, and to examine the dependence of HCO_3^- secretion on luminal Cl^-. It has also allowed us to investigate further the contributions of Na^+–H^+ exchange and vacuolar-type H^+-ATPase activity to HCO_3^- uptake across the basolateral membrane, which we have discussed in the preceding paper (Ishiguro, Steward, Lindsay & Case, 1996). Taken together, the data in the two papers suggest that, at least for the guinea-pig, the currently accepted model is an inadequate description of pancreatic HCO_3^- secretion.

Some of the data presented here have been reported to The Physiological Society (Ishiguro, Wilson, Steward & Case, 1995*a*, *b*).

METHODS

Isolation and culture of interlobular ducts

Guinea-pigs (300–400 g) of either sex from the Manchester University breeding colony were killed by cervical dislocation. The body and tail of the pancreas were removed and interlobular ducts isolated and cultured using methods described in the accompanying paper (Ishiguro *et al.* 1996).

Solutions

Bath solution. The standard Hepes-buffered solution 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 standard HCO_3^--buffered solution contained (mM): 115 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose and 25 $NaHCO_3$, equilibrated with 95% O_2–5% CO_2. Cl^--free solutions were made by replacing Cl^- with glucuronate. The Na^+-free, Cl^--free, HCO_3^--buffered solution contained (mM): 115 *N*-methyl-D-glucamine ($NMDG^+$), 115 glucuronate, 25 choline bicarbonate, 10 D-glucose, 2·5 K_2HPO_4, 1 $MgSO_4$ and 1 $CaSO_4$, and included 10 μM atropine to prevent the possible activation of muscarinic receptors by choline. All solutions were adjusted to pH 7·4 at 37 °C.

Luminal injection solution. The standard solution contained (mM): 139 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose and 1 Hepes. Cl^--free solutions were made by replacing Cl^- with glucuronate. The injection solutions were adjusted to pH 7·2 at 37 °C. Dextran conjugates of the fluorescent indicator dyes were dissolved in the injection solution and stored at −20 °C for up to 1 month.

Micropuncture of the lumen

The lumen of the cultured interlobular ducts was micropunctured using a modification of the method developed for measuring fluid secretion in interlobular ducts isolated from the pancreas of copper-deficient rats (Argent, Arkle, Cullen & Green, 1986). Double-barrelled micropipettes (tip diameter 10–12 μm) were constructed from θ-type glass capillaries. One barrel was filled with oil (light paraffin oil saturated with water and coloured with Oil Red-O; Sigma). The other barrel was filled with the injection solution containing an extracellular fluoroprobe. The first 100 μm of the tip was then filled with oil to prevent contamination of the injection solution. The duct was transferred to a 750 μl perfusion chamber on a Nikon Diaphot inverted microscope and was immobilized by applying a suction pipette. The duct was superfused at 3 ml min^{-1} with a Hepes-buffered HCO_3^--free solution at 37 °C. The lumen was then punctured with the double-barrelled micropipette and a small volume of oil was injected via one barrel to confirm the position of the tip and, later, to help seal the puncture site. The pipette tip was pushed forward through the oil droplet and the luminal fluid was withdrawn via the same barrel so that the lumen collapsed. The dye solution was then injected via the other barrel until the duct had regained its original volume. The process of emptying and refilling the duct was then repeated to ensure that the original luminal fluid was almost completely replaced by the experimental injection solution. Leakage around the pipette, which would have led to a time-dependent loss of the fluorescence signal, was prevented by the oil droplet.

Measurement of luminal pH

The pH of the duct lumen (pH_L) was estimated by microfluorometry (Tsien, 1989) after the lumen had been filled with a weakly buffered HCO_3^--free solution (1 mM Hepes, pH 7·2) containing a dextran conjugate of the pH-sensitive fluoroprobe 2′7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF–dextran; 70 kDa, 20 μM; Roberts *et al.* 1993). A small region of the lumen was illuminated alternately at 440 and 490 nm and fluorescence was measured at 520 nm (F_{440} and F_{490}). Values of pH_L were calculated at 5 s intervals from the F_{490}/F_{440} ratios, after correction for endogenous tissue fluorescence. Calibration was performed *in situ* by permeabilizing the duct with digitonin (2·5 mM) for 2 min and varying the pH of the superfusate (Fig. 1*A*). Figure 1*B* shows the relationship between the measured fluorescence ratio in the lumen and the pH of the bath.

Measurement of luminal Cl^- concentration

Luminal Cl^- concentration ($[Cl^-]_L$) was estimated by microfluorometry in single-excitation, dual-emission mode. As in the pH_L measurements, we used a ratiometric fluorescence method (Xia, Persson & Spring, 1995) to measure $[Cl^-]_L$ so as to avoid artifacts resulting from changes in luminal volume and/or dye leakage. The lumen of the duct was filled with a solution containing dextran conjugates of a Cl^--sensitive fluoroprobe, 6-methoxy-*N*-(4-aminoalkyl)quinolinium bromide (ABQ–dextran; 10 kDa, 200 μM) and a Cl^--insensitive dye, Cl-NERF (Cl-NERF–dextran; 10 kDa, 100 μM). Since ABQ is not a ratiometric dye, Cl-NERF, conjugated to a dextran of the same molecular weight, was used as the

ratiometric reference. A small region of the lumen was illuminated at 380 nm at 5 s intervals and fluorescence was measured at 440 and 530 nm (F_{440} and F_{530}). The F_{440} signal was due to the fluorescence of ABQ–dextran and the F_{530} signal to the fluorescence of Cl-NERF–dextran. Values of $[Cl^-]_L$ were calculated from the F_{440}/F_{530} ratios after correction for endogenous tissue fluorescence. Calibration was performed *in situ* by superfusing the duct with solutions containing 5 μM nigericin and 10 μM tributyltin chloride (a chloride–hydroxyl exchange ionophore) and by varying the perfusate Cl^- concentration by substitution with glucuronate (Verkman, 1990). Calibrations in the lower range of Cl^- concentrations (0, 20 and 50 mM; Fig. 2*A*) and in the higher range (142, 120 and 90 mM; Fig. 2*B*) were performed separately because the glucuronate which replaced the luminal Cl^- does not permeate the duct epithelium and therefore calibration over wide concentration ranges was slow and resulted in large changes of luminal volume. Figure 2*C* shows the resulting calibration curve. Xia *et al.* (1995) reported that, although Cl-NERF–dextran does not respond to Cl^- concentration changes, crossover emission from ABQ–dextran may distort the Cl-NERF–dextran emission spectrum at low-Cl^- concentrations in aqueous solutions. However, our calibration data were linear on a Stern–Volmer plot (Fig. 2*D*) suggesting that this effect was, at most, small.

Measurement of intracellular pH

Intracellular pH (pH_i) was estimated by BCECF microfluorometry (Tsien, 1989). The cells were loaded with BCECF by superfusion for 10 min with the acetoxymethyl ester BCECF AM (2 μM), and then the lumen was filled with the weakly buffered HCO_3^--free injection solution (1 mM Hepes, pH 7·2). Small regions of the duct epithelium (10–20 cells) were illuminated alternately at 440 and 490 nm and fluorescence was measured at 530 nm (F_{440} and F_{490}). Values of pH_i were calculated at 5 s intervals from the F_{490}/F_{440} ratios, after correction for endogenous tissue fluorescence. Calibration data were obtained using the high-K^+–nigericin technique (Thomas, Buchsbaum, Zimniak & Racker, 1979).

Materials

Culture media were obtained from Flow Laboratories (Irvine, UK) and Sigma; collagenase (type CLSPA) from Worthington Biochemical Corporation; BCECF AM, BCECF–dextran (70 kDa), ABQ–dextran (10 kDa), Cl-NERF–dextran (10 kDa) and dihydro-4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (H_2DIDS) from Molecular Probes; glibenclamide, *N*-methyl-*N*-isobutylamiloride (MIA) and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) from Research Biochemicals International; secretin (porcine) from Bachem California (Walden, Essex, UK); forskolin from Calbiochem; and bafilomycin A_1, hyaluronidase and soybean trypsin inhibitor from Sigma.

Statistics

Averaged data are presented as the 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.

RESULTS

Luminal pH changes in unstimulated ducts

During overnight culture, the ends of interlobular duct segments isolated from the guinea-pig pancreas seal spontaneously thus isolating the luminal space from the bathing medium. In order to detect HCO_3^- secretion into the lumen, the luminal fluid was removed by micropuncture and replaced by a weakly buffered solution containing the pH-sensitive fluoroprobe BCECF–dextran. Switching the bath solution from a HCO_3^--free solution to one containing 25 mM HCO_3^- (equilibrated with 5% CO_2) had the effect of acidifying the lumen from 7·34 ± 0·08 to 6·54 ± 0·15 pH units ($n = 5$) as CO_2 diffused quickly from the bath into the luminal fluid (Fig. 3*A*). The resulting pH difference between

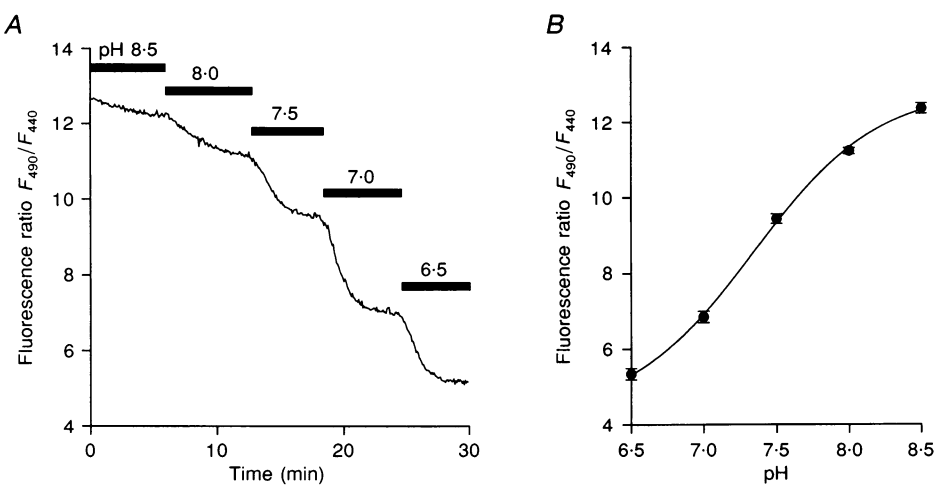

Figure 1. Calibration of BCECF–dextran fluorescence for measurement of luminal pH in superfused interlobular ducts

A, calibration performed *in situ* by treating the duct with digitonin (2·5 mM) for 2 min and stepping the bath pH between the values shown. *B*, relationship between the measured fluorescence ratio of BCECF–dextran in the lumen (F_{490}/F_{440}) and the pH of the bath solution. Data are means ± S.D. ($n = 4$) and are fitted with a sigmoid curve.

the bath and the lumen established a gradient for HCO_3^- diffusion into the lumen so, not surprisingly, pH_L then began to increase. The increase continued, however, to a value significantly greater than the bath pH. After 10 min, pH_L had reached 8·06 ± 0·11 pH units and was still increasing. If we assume that P_{CO_2} in the lumen was the same as that in the bath, this would correspond to a luminal HCO_3^- concentration, at equilibrium, of approximately 110 mM.

To determine whether the flux of HCO_3^- into the lumen was dependent on the presence of Cl^-, ducts were filled with a solution in which all of the Cl^- was replaced by glucuronate. As before, pH_L decreased following exposure to HCO_3^-/CO_2 but this time pH_L remained at a low value: 6·86 ± 0·13 pH units ($n = 4$) after 10 min (Fig. 3*B*). The precise time course of the changes in pH_L following the switch to HCO_3^-/CO_2 varied between ducts: in some cases the acidification was followed by a slight recovery while in others there was further acidification (see Figs 3*B* and 4*A–C*). These differences may reflect variations in how completely the luminal solution was replaced with the injection solution. Nonetheless, the results suggest that the HCO_3^- flux into the lumen of the unstimulated duct requires luminal Cl^- and may therefore be mediated by a Cl^-–HCO_3^- exchanger in the luminal membrane.

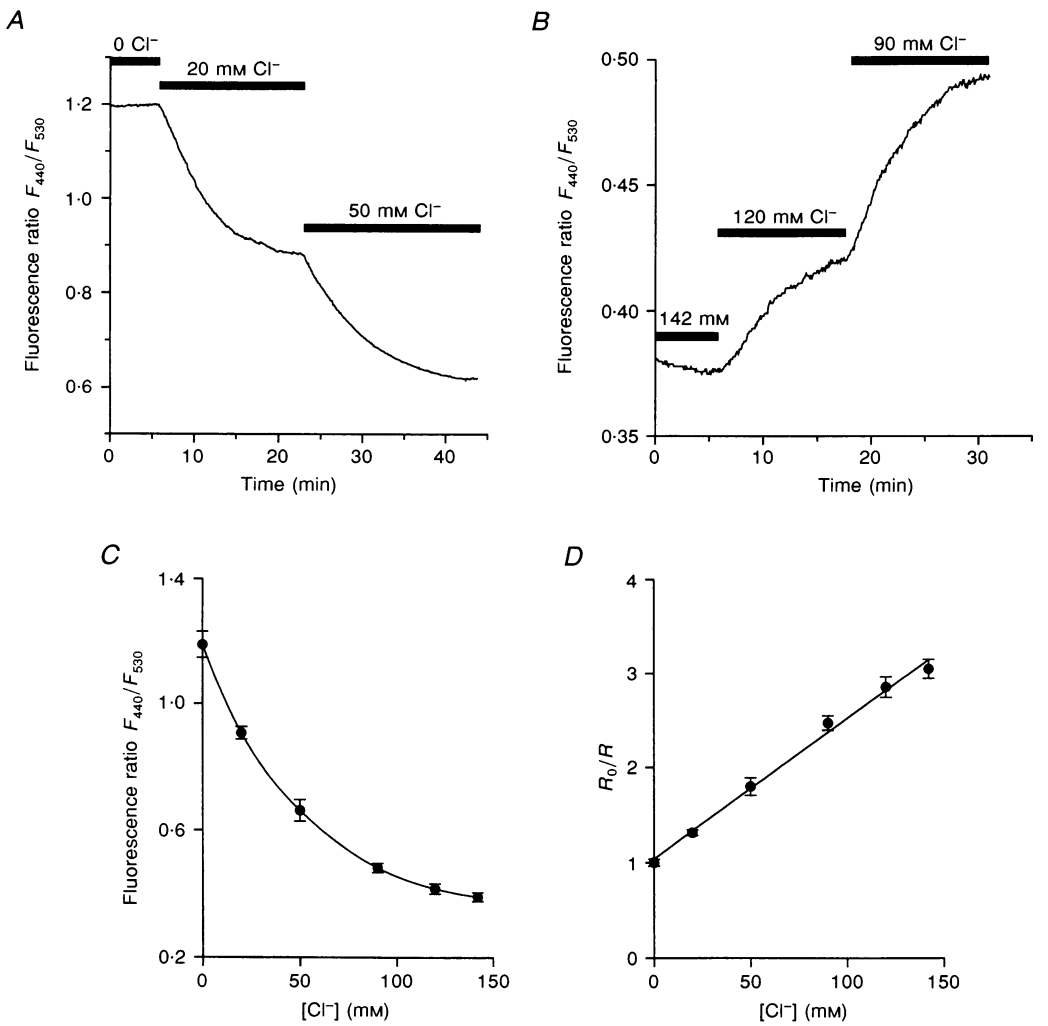

Figure 2. Calibration of ABQ– and Cl-NERF–dextran fluorescence for measurement of luminal Cl^- concentrations in superfused interlobular ducts

A and *B*, calibration experiments performed *in situ* by superfusing the duct with solutions containing 10 μM tributyltin chloride and 5 μM nigericin, and by varying the perfusate Cl^- concentration (replaced by glucuronate). Calibration of the lower range (*A*: 0, 20 and 50 mM Cl^-) and the higher range (*B*: 142, 120 and 90 mM Cl^-) were performed in separate experiments. *C*, relationship between the measured fluorescence ratio in the lumen (F_{440}/F_{530}) and the Cl^- concentration of the bath solution. Data are means ± S.D. ($n \geqslant 4$) and are fitted with a single exponential. *D*, Stern–Volmer plot of the calibration data (means ± S.D., $n \geqslant 4$). R and R_0 are the values of the fluorescence ratio (F_{440}/F_{530}) in the presence and absence of Cl^-, respectively. The Stern–Volmer constant for Cl^- in the lumen was 15 M^{-1}.

Luminal pH and Cl^- concentration in ducts during stimulation

The abolition of the HCO_3^- flux into the lumen when the ducts were filled with a Cl^--free solution provided an opportunity to examine whether HCO_3^- secretion could be elicited by secretomotor agonists under the same conditions. Figure 4 shows the results of stimulation with secretin (10 nM), which elicits fluid secretion by elevating intracellular cAMP, forskolin (1 μM), which directly activates adenylate cyclase, and acetylcholine (1 μM), which is believed to act through the elevation of intracellular Ca^{2+}. Ducts filled with the Cl^--free solution were bathed first in the Hepes solution and then switched to the HCO_3^-/CO_2 bath solution. After a further 5 min, the agonists were added to the bath. In each case, pH_L quickly increased and, after 10 min, reached 8·15 ± 0·18 pH units with secretin ($n = 4$), 7·96 ± 0·14 pH units with forskolin ($n = 4$) and 8·06 ± 0·13 pH units with acetylcholine ($n = 4$).

Although the rise in pH_L elicited by secretomotor agonists appeared to be due to a Cl^--independent secretory mechanism, it seemed possible that Cl^- was entering the lumen either from the epithelial cells or from the bath, where the Cl^- concentration was 142 mM. The luminal Cl^- concentration ($[Cl^-]_L$) was therefore measured using the Cl^--sensitive fluoroprobe ABQ–dextran in an experiment that, in other respects, was identical to the pH experiment shown in Fig. 4*B*. The results (Fig. 4*D*) show that, from the outset, the Cl^- concentration in the lumen was greater than zero and was steadily increasing, presumably as a result of Cl^- diffusion into the lumen from the epithelial cells and/or the bath. At the time when the bath was switched from the Hepes solution to the HCO_3^-/CO_2 solution, $[Cl^-]_L$ was in the range 25–35 mM. Stimulation with forskolin did not cause any further increase in $[Cl^-]_L$. Although these values were clearly greater than zero, we may nonetheless conclude that, whereas the unstimulated HCO_3^- flux was inhibited by low luminal Cl^- concentrations, agonist-evoked HCO_3^- secretion was not.

pH_L, $[Cl^-]_L$ and intracellular pH in ducts superfused with Cl^--free solutions

To test more rigorously the Cl^- dependence of forskolin-evoked HCO_3^- secretion, ducts were subsequently superfused with a Cl^--free, Hepes-buffered solution for at least 15 min prior to the microinjection of the Cl^--free solution into the lumen. The changes in pH_L following the switching of the bath solution to HCO_3^-/CO_2 and the subsequent addition of 1 μM forskolin to the bath are shown in Fig. 5*A*. These results, obtained in the nominal absence of both luminal and bath Cl^-, are essentially the same as those shown in Fig. 4*B*. Five minutes after switching to HCO_3^-/CO_2, the low value of pH_L (6·80 ± 0·08 pH units, $n = 4$) indicated that the unstimulated HCO_3^- flux was blocked as before. Furthermore, the response to forskolin was also undiminished: pH_L increased to 8·06 ± 0·10 pH units after 5 min of stimulation.

The changes in $[Cl^-]_L$ measured with ABQ–dextran under these conditions are shown in Fig. 5*B*. Although still detectable, $[Cl^-]_L$ remained below 10 mM throughout these experiments. Prior to stimulation with forskolin, $[Cl^-]_L$ was 7·2 ± 0·7 mM ($n = 3$), and after 5 min of stimulation it had decreased to 6·1 ± 0·3 mM, probably through the diluting effect of fluid secretion into the lumen. In two experiments, the calibration of the ABQ–dextran fluorescence was checked

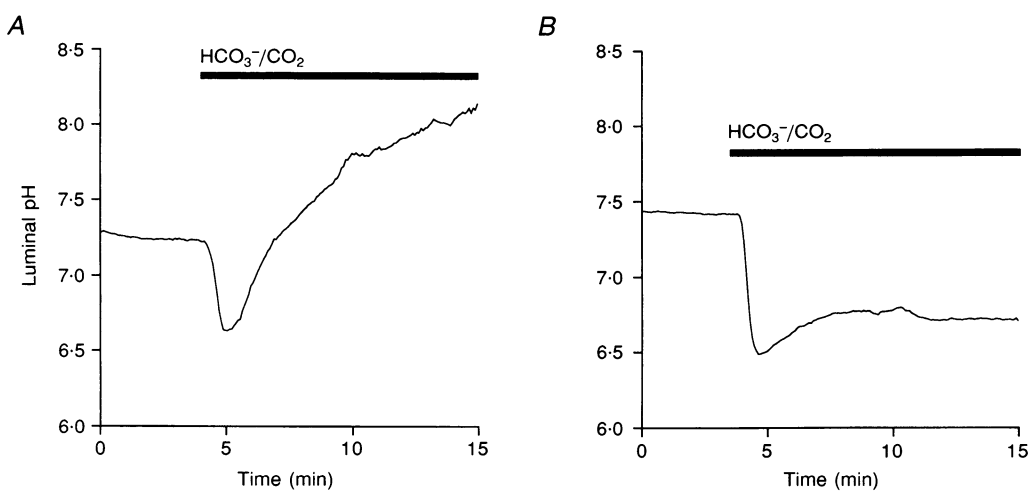

Figure 3. Changes in luminal pH in unstimulated ducts following exposure to HCO_3^-/CO_2

Ducts filled with a weakly buffered HCO_3^--free injection solution (1 mM Hepes, pH 7·2) containing 20 μM BCECF–dextran were superfused initially with a Hepes-buffered, HCO_3^--free solution. After a short equilibration period, the bath solution was switched to a solution containing 25 mM HCO_3^- equilibrated with 5% CO_2. *A*, changes in luminal pH in a duct filled with an injection solution containing a normal Cl^- concentration (148 mM). One of five experiments. *B*, changes in luminal pH in a duct filled with a Cl^--free solution (replaced with glucuronate). One of four experiments.

shortly after switching the bath solution to HCO_3^-/CO_2. Figure 5*C* shows the changes in the fluorescence ratio F_{440}/F_{530}, which is inversely related to $[Cl^-]_L$. Upon application of the ionophores tributyltin and nigericin, there was a slight increase in F_{440}/F_{530} (i.e. reduction in $[Cl^-]_L$), presumably due to the efflux of the residual luminal Cl^- to the bath. The larger subsequent decrease in the fluorescence ratio when 20 mM Cl^- was added to the bath confirms that the value of $[Cl^-]_L$ in these nominally Cl^--free experiments was closer to zero than to 20 mM. This therefore supports our conclusion that the increase in pH_L evoked by forskolin occurred with very low concentrations of Cl^- in the lumen.

Because the conditions in these experiments were somewhat different from those in the preceding paper (Ishiguro *et al.* 1996), we also examined the changes in intracellular pH (pH_i) in response to forskolin stimulation. Three experiments were carried out using the same Cl^--free solutions as those used to obtain the pH_L data shown in Fig. 5*A*, but this time BCECF was loaded into the epithelial cells and BCECF–dextran was omitted from the luminal injection solution. Intracellular pH (Fig. 5*D*) decreased from 7·44 ± 0·04 to 7·20 ± 0·01 pH units ($n = 3$) upon switching the bath solution to HCO_3^-/CO_2, presumably as a result of CO_2 diffusion into the cells, and then recovered slightly. As we observed previously, however, 1 μM forskolin had no effect on pH_i: the values immediately before stimulation (7·38 ± 0·04 pH units, $n = 3$) and after 5 min of stimulation (7·33 ± 0·03 pH units) were not significantly different. This result suggests that, under conditions in which forskolin-evoked HCO_3^- secretion raised pH_L to 8·0, equivalent to a luminal HCO_3^- concentration of approximately 100 mM, the intracellular concentration of HCO_3^- remained at approximately 20 mM.

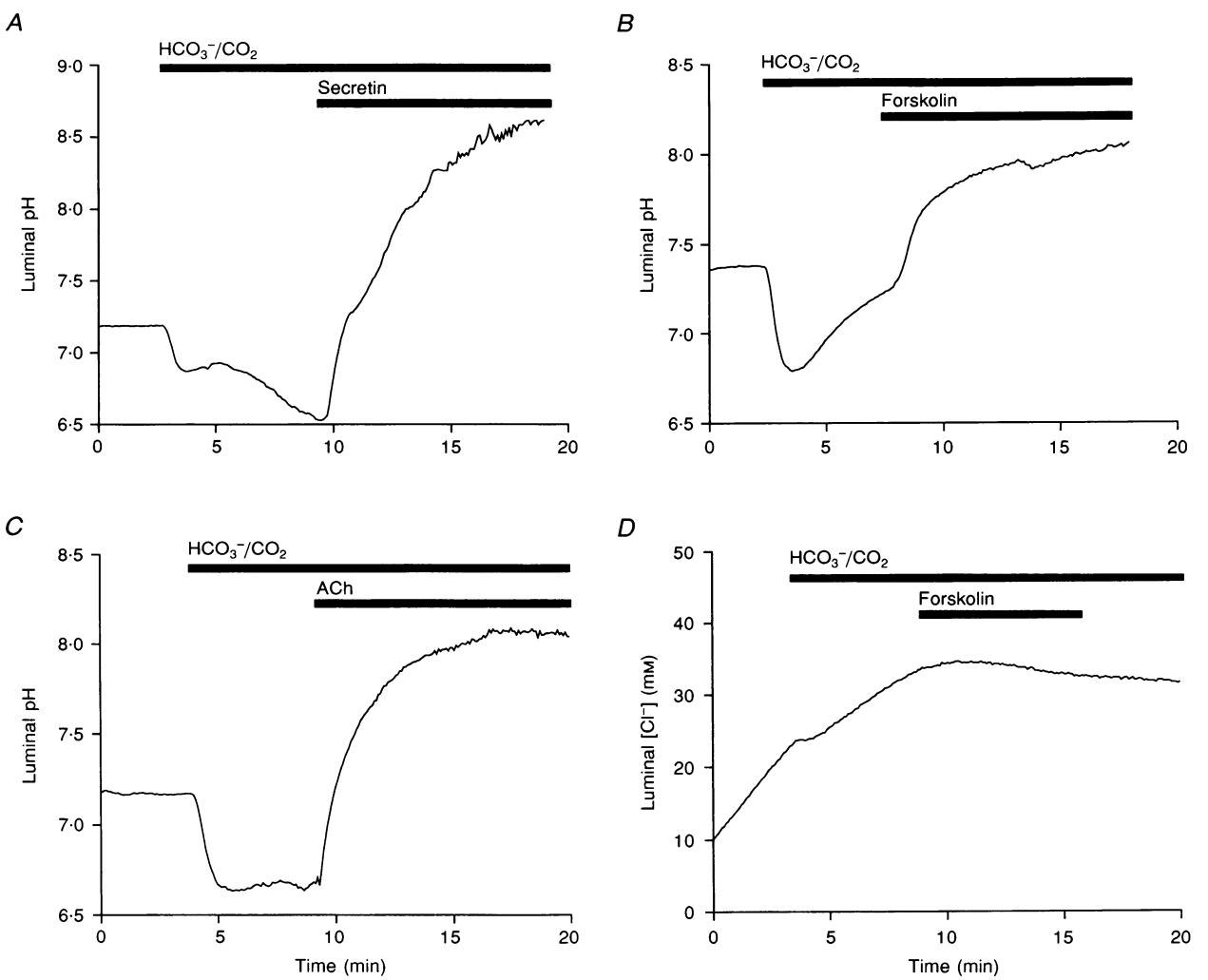

Figure 4. Luminal pH and luminal Cl^- concentrations in ducts during agonist-evoked secretion

Changes in luminal pH following stimulation with 10 nM secretin (*A*), 1 μM forskolin (*B*), or 1 μM acetylcholine (ACh; *C*). The lumen was initially filled with a Cl^--free solution, and the agonist applied after switching the bath from the Hepes solution to the HCO_3^-/CO_2 solution. Each trace is representative of four experiments. *D*, changes in luminal Cl^- concentration estimated from ABQ– and Cl-NERF–dextran fluorescence in an experiment of the same protocol as *B*. One of five experiments.

Effects of luminal inhibitors on the secretory response to forskolin

In a preliminary attempt to identify the luminal transport mechanisms that might be responsible for the secretion of HCO_3^- in the nominal absence of Cl^-, a number of anion channel blockers were tested for their ability to inhibit the increase in pH_L following stimulation with forskolin. The compounds used were glibenclamide, which has been reported to be an effective blocker of CFTR channels (Sheppard & Welsh, 1992; Valverde, O'Brien, Sepúlveda, Ratcliff, Evans & Colledge, 1993), H_2DIDS, an established blocker of various anion transporters, and NPPB, a non-specific Cl^- channel blocker which has been reported to block the cAMP-stimulated whole-cell Cl^- current in rat pancreatic duct cells (Gray *et al.* 1993). In experiments using the same protocol as in Fig. 5*A*, but with the inhibitors included in the luminal injection solution, the pH_L increase due to forskolin stimulation was not blocked by 100 μM glibenclamide (Fig. 6*A*), by 500 μM H_2DIDS (Fig. 6*B*), or by 10 μM NPPB (Fig. 6*C*).

To explore the possibility that the increase in pH_L was due to H^+ transport out of the lumen via an H^+-ATPase, 1 μM bafilomycin A_1, an inhibitor of the vacuolar-type H^+-ATPase (V-ATPase) activity previously demonstrated in pig pancreatic ducts (Villanger, Veel & Ræder, 1995), was included in the luminal injection solution and the bath. Under these conditions, the increase in pH_L in response to forskolin in the nominal absence of Cl^- was again undiminished (Fig. 6*D*).

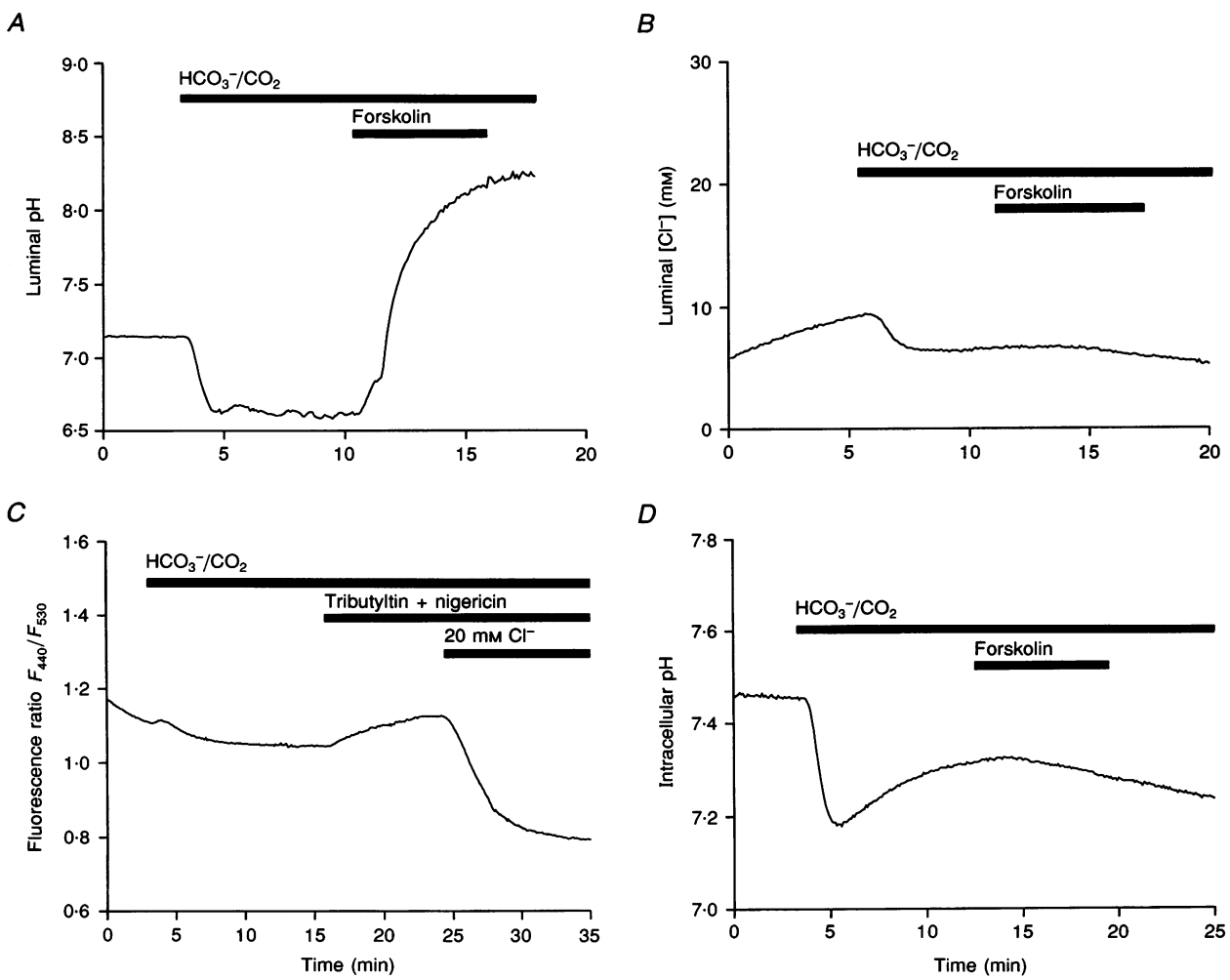

Figure 5. Effects of forskolin stimulation on luminal pH, luminal Cl^- concentration and intracellular pH in ducts superfused with a Cl^--free solution

Ducts were superfused with a Cl^--free, Hepes-buffered solution for at least 15 min and then the luminal fluid replaced by a Cl^--free injection solution. In *A*, *B* and *D*, 1 μM forskolin was applied after switching the bath from the Hepes solution to a Cl^--free HCO_3^-/CO_2 solution. *A*, luminal pH (one of four experiments); *B*, luminal Cl^- concentration (one of three experiments); *C*, ABQ–dextran : Cl-NERF–dextran fluorescence ratio (F_{440}/F_{530}) showing the results of *in situ* calibration (one of two experiments); *D*, intracellular pH changes under the same experimental conditions (one of three experiments).

Effects of Na^+ substitution and an amiloride derivative on the secretory response to forskolin

During agonist-evoked HCO_3^- secretion, HCO_3^- must be accumulated in the cell either by HCO_3^- influx or by H^+ extrusion across the basolateral membrane. To date, the identification of the basolateral transporters responsible for the accumulation of intracellular HCO_3^- has largely been based on measurements of the rate of recovery of pH_i following acid loading (Zhao *et al.* 1994; Villanger *et al.* 1995; Ishiguro *et al.* 1996). Evidence has variously been obtained for the existence in the basolateral membrane of a Na^+–H^+ exchanger, a V-ATPase and a Na^+–HCO_3^- co-transporter. Having established the methodology to detect transepithelial HCO_3^- secretion, we next sought to clarify the relative importance of these transporters by examining the effects of Na^+ substitution and of inhibiting Na^+–H^+ exchange with an amiloride derivative.

The experiments used the same protocol as in Fig. 5*A* except that, shortly after switching to HCO_3^-/CO_2, the bath Na^+ was replaced by $NMDG^+$ to block any Na^+-dependent basolateral transporters (Fig. 7*A*). Under these conditions, forskolin (1 μM) had no effect on pH_L. However, when basolateral Na^+ was restored in the presence of forskolin, pH_L increased rapidly indicating that the forskolin-evoked secretion of HCO_3^- into the lumen is dependent on the presence of basolateral Na^+.

In the experiment shown in Fig. 7*B*, 10 μM *N*-methyl-*N*-isobutylamiloride (MIA), a specific and potent inhibitor of Na^+–H^+ exchange (Dixon, Cohen, Cragoe & Grinstein, 1987), was included in the bath solution. In the presence of MIA, forskolin stimulation caused pH_L to increase rapidly by 1·05 ± 0·08 pH units ($n = 3$), which was not significantly different from the increase in the corresponding control experiments (1·27 ± 0·17 pH units, $n = 4$; Fig. 5*A*). This

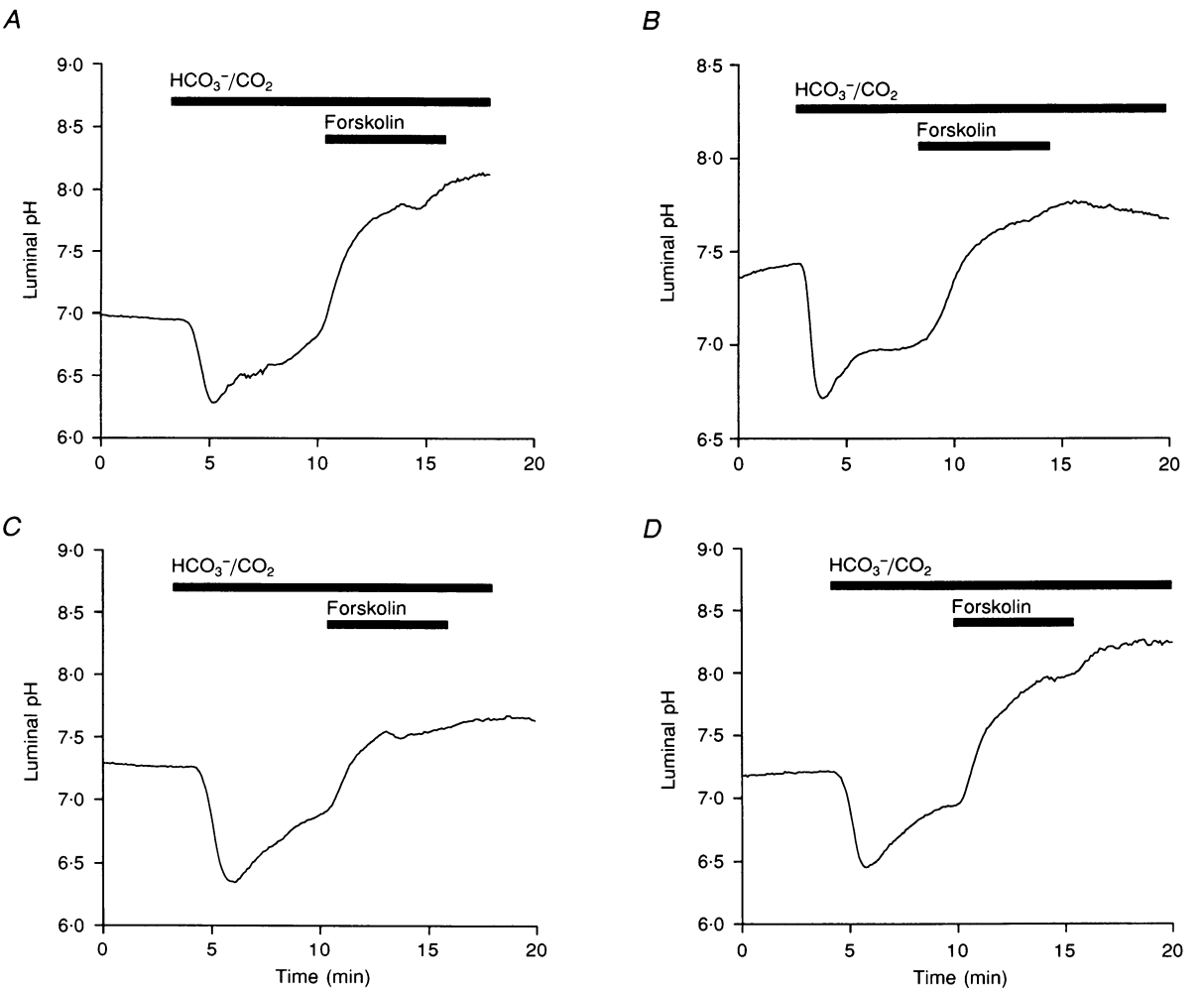

Figure 6. Effects of luminal inhibitors on forskolin-evoked HCO_3^- secretion

Ducts were superfused with a Cl^--free, Hepes-buffered solution and the luminal fluid replaced by a Cl^--free injection solution containing 100 μM glibenclamide (*A*; one of five experiments), 500 μM H_2DIDS (*B*; one of four experiments), 10 μM NPPB (*C*; one of four experiments) or 1 μM bafilomycin A_1 (*D*; one of four experiments). Forskolin (1 μM) was applied after switching the bath from the Hepes solution to a Cl^--free HCO_3^-/CO_2 solution. In *D*, 1 μM bafilomycin A_1 was also included in the bath.

result shows that agonist-evoked HCO_3^- secretion is not dependent on basolateral Na^+–H^+ exchange. Since the pH_L increase evoked by forskolin was also not inhibited by bafilomycin A_1 in the bath solution (Fig. 6*D*), these data support the conclusion of the accompanying paper (Ishiguro *et al.* 1996) that, during agonist-stimulated HCO_3^- secretion, Na^+–HCO_3^- cotransport contributes more to HCO_3^- accumulation in the cell than do Na^+–H^+ exchange and V-ATPase activity.

DISCUSSION

Measurement of luminal pH and Cl^- concentration by microfluorometry

In this paper we have described an experimental strategy which allows the detection of HCO_3^- secretion into the pancreatic duct lumen under controlled conditions. Although it is possible to collect and analyse the fluid secreted by isolated ducts using micropuncture techniques (Argent *et al.* 1986), the long collection periods required and the difficulty of repetitive sampling preclude the investigation of dynamic changes in HCO_3^- secretion. For the present study, therefore, we have adopted the alternative strategy of measuring changes in luminal pH fluorometrically following microinjection of BCECF–dextran into the duct lumen. To do this, we have modified a method introduced by Roberts *et al.* (1993) for studies of HCO_3^- secretion in isolated intrahepatic bile ducts.

Using a double-barrelled micropipette, it is possible to first empty and then refill the interlobular pancreatic ducts with a fluoroprobe-containing solution of chosen composition. This allows unilateral ion substitution experiments to be performed as well as pharmacological studies of the luminal membrane transporters. Inevitably the composition of the luminal fluid will change with time after injection so, for measurements of intracellular parameters, a microperfusion method is preferable (Novak & Greger, 1988; Zhao *et al.* 1994). However, for measurements of transepithelial fluxes, the microinjection of luminal fluoroprobes has many advantages. Full quantification of the fluxes would require measurements of the changes in luminal volume as well as luminal ion concentrations, but volume flow measurements may be complicated by the limited elasticity of the duct wall and the build-up of intraluminal pressure. In the present study, therefore, we confined our measurements to changes in luminal ion concentrations alone over relatively short time periods.

By using ratiometric fluorescence methods both for pH_L and for $[Cl^-]_L$, artifacts resulting from changes in luminal volume and/or dye leakage were avoided. Nonetheless it remains a possibility that the fluorescence of ABQ–dextran, like that of SPQ (6-methoxy-*N*-(3-sulphopropyl)quinolinium), was quenched slightly by HCO_3^- and by glucuronate (Verkman, 1990). In particular, this could have affected the estimation of the residual Cl^- concentration in the duct lumen following replacement of both luminal and bath Cl^- by glucuronate. However, *in situ* calibration measurements (Fig. 5*C*) confirmed that the residual Cl^- concentration in the duct lumen under these conditions was 6–8 mM. This presumably was due either to the efflux of Cl^- from the epithelial cells or the incomplete replacement of the original luminal fluid.

Unstimulated and agonist-evoked HCO_3^- secretion

In ducts filled initially with a solution containing 148 mM Cl^-, the luminal pH increased rapidly when HCO_3^-/CO_2

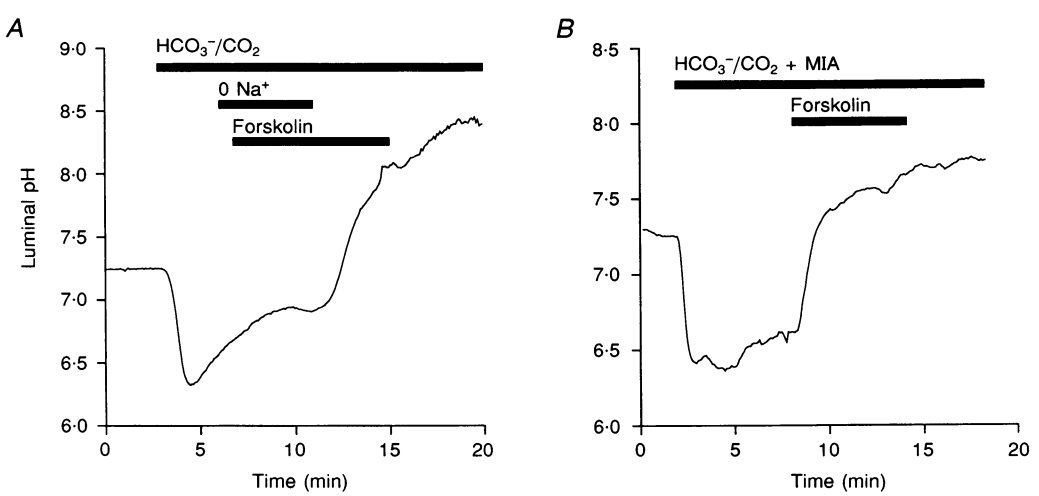

Figure 7. Effects of Na^+ substitution and MIA on forskolin-evoked HCO_3^- secretion

Ducts were superfused with a Cl^--free, Hepes-buffered solution and the luminal fluid replaced by a Cl^--free injection solution. Forskolin (1 μM) was applied after switching the bath from the Hepes solution to a Cl^--free HCO_3^-/CO_2 solution. *A*, changes in luminal pH when the bath Na^+ was replaced by $NMDG^+$ prior to forskolin stimulation and then restored (one of four experiments). *B*, effects of forskolin stimulation in the presence of 10 μM *N*-methyl-*N*-isobutylamiloride (MIA) in the bath (one of three experiments).

was admitted to the system in the absence of exogenous stimulation (Fig. 3A). Given the steep gradients for Cl^- and HCO_3^- that existed initially across the luminal membrane, much of the increase in pH_L is readily explained by a simple 1:1 exchange of luminal Cl^- and intracellular HCO_3^- without any net solute or fluid secretion. An anion exchanger is undoubtedly present in the luminal membrane; for example, the replacement of luminal Cl^- by glucuronate causes an intracellular alkalinization that is abolished by H_2DIDS (H. Ishiguro, unpublished observations). However, it is unclear whether Cl^-–HCO_3^- exchange alone can account for the high steady-state pH_L values that are attained in the unstimulated ducts. If we assume that P_{CO_2} in the lumen parallels that in the bath, a pH_L value of 8·06 corresponds to a luminal HCO_3^- concentration of 110 mM. To attain such a high value by passive exchange of Cl^- and HCO_3^- across the luminal membrane, the intracellular Cl^- concentration would have to be around 10 mM (assuming that the luminal concentration had decreased to 40 mM). Although low, this value could be sustained if Cl^- was able to recycle across the luminal membrane via a Cl^- conductance, as in the secretory model proposed for the rat pancreatic duct (Gray *et al.* 1988; Novak & Greger, 1988). It would also give rise to a net secretion of HCO_3^- and fluid. This would be consistent with earlier *in vivo* observations (Padfield *et al.* 1989) and would account for the gradual swelling of the ducts that is observed during culture.

The alkalinization of the unstimulated duct lumen was abolished when the luminal Cl^- concentration was reduced to 25–35 mM by replacement with glucuronate. This dependence on luminal Cl^- is consistent with the hypothesis that, in the absence of stimulation, HCO_3^- enters the lumen mainly by Cl^-–HCO_3^- exchange. More importantly, it provided an opportunity to examine the effects of secretomotor agonists on HCO_3^- secretion without a background of HCO_3^- efflux into the lumen via the exchanger. Under these low $[Cl^-]_L$ conditions, secretin and forskolin were able to evoke HCO_3^- secretion and raise pH_L to a value equivalent to a luminal HCO_3^- concentration of 100–150 mM. This concentration is much higher than that measured in the fluid collected by micropuncture from isolated rat pancreatic ducts during secretin stimulation (less than 25 mM; Argent *et al.* 1986). The difference suggests that the pancreatic duct cells of the guinea-pig possess additional HCO_3^- transport mechanisms that are absent in the rat.

Acetylcholine also stimulated HCO_3^- secretion and raised the luminal pH_L to comparable values. The response of the pancreas *in vivo* to cholinergic stimulation or carbachol injection varies greatly between species (Case & Argent, 1993). In some, a chloride-rich secretion is evoked. In others, a HCO_3^--rich secretion is observed. In general, the source of this fluid (whatever its composition) is unknown. However, recently it has been shown that acetylcholine increases intracellular Ca^{2+} concentration and stimulates fluid secretion in isolated rat pancreatic ducts (Ashton, Evans, Elliott, Green & Argent, 1993), but whether this secretion is Cl^--rich or HCO_3^--rich is unknown. Pahl & Novak (1993) also reported that rat pancreatic duct cells respond not only to secretin but also to carbachol by depolarization of the basolateral membrane following an increase in luminal conductance. This suggests that cholinergic stimulation may regulate HCO_3^- secretion via a Ca^{2+}-activated anion conductance (Gray, Winpenny, Porteous, Dorin & Argent, 1994). Our present data unequivocally demonstrate that acetylcholine evokes HCO_3^--transport in guinea-pig pancreatic duct cells.

Cl^- dependence of ductal HCO_3^- secretion

The observation that agonist-evoked HCO_3^- secretion is unaffected when the luminal Cl^- concentration is reduced to very low values raises important questions when comparison is made with earlier studies. In the isolated perfused cat pancreas, secretin-stimulated fluid secretion is reduced by 70% during replacement of perfusate Cl^- by the impermeant anion isethionate (Case *et al.* 1979). In the isolated rabbit pancreas, spontaneous fluid secretion is inhibited by 50% (but HCO_3^- output by only 20%) during replacement of Cl^- by isethionate (Kuijpers *et al.* 1984*a*), while fluid secretion from isolated, secretin-stimulated rat pancreatic ducts is reduced by 70% during replacement of Cl^- by gluconate (Ashton, Argent & Green, 1991).

It is difficult to compare our studies on the isolated guinea-pig duct, in which HCO_3^- secretion was monitored over a few minutes, with those in the rat duct, where the secretion, a Cl^--rich fluid, was collected by micropuncture over a period of 1 h (Argent *et al.* 1986). Comparison with the rabbit data is not really possible because, for reasons that are still not clear, the isolated rabbit pancreas exhibits a high spontaneous rate of fluid secretion (sixfold greater than that observed *in vivo*) whose origin (ductal or acinar) is uncertain and which is only slightly increased by secretin (Rothman & Brooks, 1965). Comparison with our earlier publication on the perfused cat pancreas (Case *et al.* 1979) is more valid because the available evidence (indirect though much of it is) suggests that secretin acts exclusively on the ducts in this species to evoke a HCO_3^--rich secretion. While it is certainly true that replacement of Cl^- by isethionate inhibited fluid secretion by 70%, the concentration of HCO_3^- in the secreted fluid increased from 121 to 151 mM – so the output of HCO_3^- was only reduced by 63%. Clearly, therefore, the perfused cat pancreas is able to secrete significant amounts of HCO_3^- in the complete absence of extracellular Cl^-. This is consistent with our findings in the guinea-pig ducts. However, the fact that two-thirds of the HCO_3^- output in the cat pancreas was abolished by Cl^- replacement suggests either that there is a substantial component of HCO_3^- secretion that is directly dependent upon Cl^-, or that Cl^- has other roles in duct cell homeostasis. It is worth noting that Cl^- has been ascribed a regulatory role, influencing the activity of membrane transport proteins, in a number of epithelia (e.g. Robertson & Foskett, 1994; Nakahari & Marunaka, 1995*a*, *b*; Dinudom, Komwatana, Young & Cook, 1995).

What is the role of Cl^- in the efflux of HCO_3^- across the luminal membrane of the guinea-pig pancreatic duct? There is no doubting the existence of cyclic AMP-regulated Cl^- channels (CFTR) in the luminal membrane of rat ductal cells (Gray *et al.* 1988, 1994) and there is no reason to suppose that the same is not true in the guinea-pig. Furthermore, our own data support the existence of a luminal Cl^-–HCO_3^- exchanger in as much as the spontaneous alkalinization of the duct lumen was inhibited when the luminal concentration of Cl^- was reduced to 25–35 mM. However, our most significant finding is that the secretin-stimulated component of HCO_3^- transport in the guinea-pig duct is maintained at very low luminal Cl^- concentrations (less than 8 mM; Fig. 5) and is not abolished by luminal H_2DIDS (Fig. 6*B*). This suggests that the luminal transporter responsible for agonist-stimulated HCO_3^- transport is different from that responsible for the spontaneous alkalinization of the duct lumen.

HCO_3^- transport across the luminal membrane

What Cl^--independent HCO_3^- transport mechanisms might account for HCO_3^- secretion in the absence of Cl^-–HCO_3^- exchanger activity? One possibility is that, in the absence of Cl^-, HCO_3^- is transported through the CFTR channel even though the HCO_3^-/Cl^- permeability ratio of the CFTR channel is known to be relatively low (0·2–0·25; Gray, Pollard, Harris, Coleman, Greenwell & Argent, 1990; Poulsen, Fischer, Illek & Machen, 1994). The failure of glibenclamide and NPPB to inhibit HCO_3^- secretion at low Cl^- concentrations does not totally exclude this possibility since their efficacy as blockers of CFTR in native tissue is not well documented. Another possibility is that the pancreatic duct cells of the guinea-pig possess a non-specific anion channel similar to that expressed in rabbit mandibular secretory cells (Brown, Elliott & Lau, 1989), bovine parotid cells (Lee & Turner, 1992) and sheep parotid secretory endpieces (Poronnik, Schumann & Cook, 1995). Such channels are relatively insensitive to most Cl^- channel blockers at normal membrane potentials (T. Ishikawa, personal communication). Interestingly, a high-conductance anion channel, equally permeable to HCO_3^- and Cl^-, has recently been described in a cell line (Capan 1) derived from the human pancreatic duct (Mahieu, Becq, Wolfensberger, Gola, Carter & Hollande, 1994). Pancreatic duct cells of the guinea-pig may express a similar HCO_3^- channel since the guinea-pig pancreas resembles the human pancreas in its capacity to generate a high secretory HCO_3^- concentration.

There is another, theoretical reason for questioning whether Cl^-–HCO_3^- exchange operating in parallel with a Cl^- conductance can achieve a luminal HCO_3^- concentration of 150 mM. For HCO_3^- efflux to the lumen to occur by Cl^-–HCO_3^- exchange, the following inequality has to be satisfied:

$$\frac{[HCO_3^-]_i}{[HCO_3^-]_L} > \frac{[Cl^-]_i}{[Cl^-]_L}, \quad (1)$$

where subscripts i and L represent the intracellular and luminal compartments, respectively. At the same time, an outwardly directed electrochemical gradient for Cl^- across the luminal membrane would require that:

$$\frac{RT}{F} \ln\left(\frac{[Cl^-]_i}{[Cl^-]_L}\right) > V_a, \quad (2)$$

where V_a is the luminal (apical) membrane potential. Combining these two expressions yields the following inequality:

$$\frac{[HCO_3^-]_i}{[HCO_3^-]_L} > \exp\left(\frac{V_a F}{RT}\right), \quad (3)$$

which predicts that the maximum luminal concentration of HCO_3^- that could be achieved by such a mechanism is limited by the requirement that:

$$[HCO_3^-]_L < [HCO_3^-]_i \exp\left(-\frac{V_a F}{RT}\right). \quad (4)$$

This means that in the resting condition, at a membrane potential of −60 mV and with an intracellular HCO_3^- concentration of 20 mM (equivalent to an intracellular pH of approximately 7·3), the maximum attainable HCO_3^- concentration in the lumen would be 190 mM. However, upon stimulation with secretin, which in the rat leads to a depolarization of approximately 30 mV (Novak & Pahl, 1993), the maximum luminal HCO_3^- concentration would drop to 60 mM – a figure that is comparable with *in vivo* measurements in the rat (Sewell & Young, 1975) but difficult to reconcile with the much higher concentrations achieved in the guinea-pig.

It should be noted that expressions (3) and (4) would also apply to a model in which HCO_3^- efflux across the luminal membrane was by diffusion through an anion channel rather than by exchange with Cl^-. For example, with 150 mM HCO_3^- in the lumen and 20 mM in the cell, depolarization of the luminal membrane potential to −30 mV during stimulation would result in the electrochemical gradient for HCO_3^- being directed into rather than out of the cell. Therefore, for either of the luminal transport mechanisms discussed above to achieve the high secretory HCO_3^- concentrations that are observed in the guinea-pig, it would be essential that the membrane potential remained large enough during secretin stimulation to satisfy expression (3). Until electrophysiological data are available for the guinea-pig ducts, it is impossible to say whether or not this is the case.

HCO_3^- accumulation across the basolateral membrane

The accompanying paper (Ishiguro *et al.* 1996) shows that, during secretin stimulation, HCO_3^- accumulation across the basolateral membrane of guinea-pig pancreatic duct cells is driven largely by Na^+–HCO_3^- cotransport. This concept is further strengthened by data in this paper which show that luminal HCO_3^- secretion, although absolutely dependent on extracellular Na^+, is not affected by blockers of Na^+–H^+ exchange or inhibitors of V-ATPase activity. Taken together, the data in the two papers strongly suggest that,

during secretin-stimulated HCO_3^- secretion, Na^+–HCO_3^- cotransport contributes more to HCO_3^- accumulation in the cell than does either Na^+–H^+ exchange or V-ATPase activity. This conclusion supports earlier conclusions drawn from indirect studies on the isolated pancreas of rabbit (Kuijpers, Van Nooy, De Pont & Bonting, 1984*b*) and cat (Ammar, Hutson & Scratcherd, 1987).

Given the likely importance of Na^+–HCO_3^- cotransport in mediating HCO_3^- entry into the cell, why is carbonic anhydrase (CA) present in the duct cells (Mahieu *et al.* 1994) and why is secretion inhibited by acetazolamide (Case *et al.* 1970; Ammar *et al.* 1987)? One possibility is that acetazolamide inhibits Na^+–HCO_3^- cotransport. Alternatively, since immunocytochemical studies indicate that the CA IV isoenzyme is localized on the luminal membrane of Capan I cells (Mahieu *et al.* 1994), it is possible that CA dimers form channel-like transporters for HCO_3^- as has been postulated for red blood cells (Widdas & Baker, 1995). In this case, the effects of acetazolamide would be easily explained.

In summary, we have developed a new experimental protocol for investigating the mechanisms and control of HCO_3^- secretion in interlobular ducts isolated from the guinea-pig pancreas. The results presented in this paper confirm that both secretin and cholinergic stimulation evoke HCO_3^- secretion in cultured ducts, and they provide further support for the involvement of Na^+–HCO_3^- cotransport in HCO_3^- accumulation across the basolateral membrane. They also highlight the difficulty of accounting for HCO_3^- efflux across the luminal membrane in the presence of very low luminal Cl^- concentrations. It is clear that Cl^-–HCO_3^- exchange in parallel with a Cl^- conductance will only generate high luminal HCO_3^- concentrations if both (a) the effective K_m of the exchanger for Cl^- is extremely low, and (b) secretin stimulation does not depolarize the luminal membrane potential beyond about −50 mV.

Finally, it should be recalled that the pancreatic physiology of the rat and guinea-pig are markedly different (Case & Argent, 1993) so it should not be surprising if the guinea-pig proves to have mechanisms for HCO_3^- transport additional to or different from those described for the rat. The challenge now is to characterize these mechanisms and quantify their relative importance.

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