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Concurrent and Independent HCO_3^- and Cl^- Secretion in a Human Pancreatic Duct Cell Line (CAPAN-1)

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Abstract. The present study investigated both HCO_3^- and Cl^- secretions in a human pancreatic duct cell line, CAPAN-1, using the short-circuit current (I_{sc}) technique. In Cl^-/HCO_3^--containing solution, secretin (1 μM) or forskolin (10 μM) stimulated a biphasic rise in the I_{sc} which initially reached a peak level at about 3 min and then decayed to a plateau level after 7 min. Removal of external Cl^- abolished the initial transient phase in the forskolin-induced I_{sc} while the plateau remained. In HCO_3^-/CO_2-free solution, on the contrary, only the initial transient increase in I_{sc} was prominent. Summation of the current magnitudes observed in Cl^--free and HCO_3^--free solutions over a time course of 10 min gave rise to a curve which was similar, both in magnitude and kinetics, to the current observed in Cl^-/HCO_3^--containing solution. Removal of external Na^+ greatly reduced the initial transient rise in the forskolin-induced I_{sc} response, and the plateau level observed under this condition was similar to that obtained in Cl^--free solution, suggesting that Cl^--dependent I_{sc} was also Na^+-dependent. Bumetanide (50 μM), an inhibitor of the Na^+-K^+-$2Cl^-$ cotransporter, and Ba^{2+} (1 mM), a K^+ channel blocker, could reduce the forskolin-induced I_{sc} obtained in Cl^-/HCO_3^--containing or HCO_3^--free solution. However, they were found to be ineffective when external Cl^- was removed, indicating the involvement of these mechanisms in Cl^- secretion. On the contrary, the HCO_3^--dependent (in the absence of external Cl^-) forskolin-induced I_{sc} could be significantly reduced by carbonic anhydrase inhibitor, acetazolamide (45 μM). Basolateral application of amiloride (100 μM) inhibited the I_{sc}; however, a specific Na^+-H^+ exchanger blocker, 5-N-methyl-N-isobutylamiloride (MIA, 5–10 μm) was found to be ineffective, excluding the involvement of the Na^+-H^+ exchanger. However, an inhibitor of H^+-ATPase, N-ethylmaleimide did suppress the I_{sc} (IC_{50} = 22 μM). Immunohistochemical studies also confirmed the presence of a vacuolar type of H^+-ATPase in these cells. H_2DIDS (100 μM), an inhibitor of Na^+-HCO_3^- cotransporter, was without effect. Apical addition of Cl^- channel blocker, diphenylamine-2,2′-dicarboxylic acid (DPC, 1 mM), but not disulfonic acids, DIDS (100 μm) or SITS (100 μM), exerted an inhibitory effect on both Cl^- and HCO_3^--dependent forskolin-induced I_{sc} responses. Histochemical studies showed discrete stainings of carbonic anhydrase in the monolayer of CAPAN-1 cells, suggesting that HCO_3^- secretion may be specialized to a certain population of cells. The present results suggest that both HCO_3^- and Cl^- secretion by the human pancreatic duct cells may occur concurrently and independently.

Introduction

The current pancreatic secretory model based on study results obtained from the rat suggests that HCO_3^- is accumulated through the conversion of basolaterally entered CO_2 into carbonic acid by carbonic anhydrase and proton extrusion through the basolateral membrane via the Na^+-H^+ exchanger. HCO_3^- is secreted into the lumen through the Cl^--HCO_3^- exchanger working in parallel with the apical Cl^- channel (reviewed by Novak, 1990; Argent & Case, 1994). The major difficulty with the current model is that there is no mechanism for active Cl^- accumulation although a recent study using a mathematical model has predicted that the resting intracellular Cl^- concentration of the duct cells is quite high and that the current pancreatic duct model cannot support secretion of HCO_3^- at the higher concentrations found in the pancreatic juice of cats, guinea pigs, dogs and men

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(Sohma et al., 1996). In addition, recent studies have shown that other mechanisms, such as a vacuolar-type H^+-ATPase (Raeder, 1992) and a Na^+-HCO_3^- cotransporter (Zhao, Star, & Muallem, 1994; Ishiguro et al., 1996*a*), may also be involved in pancreatic HCO_3^- secretion. All these indicate that the current pancreatic secretory model requires modification. Furthermore, it remains to be determined whether the current model could be applied to the pancreatic ducts of humans since no work on human ductal cells has ever been reported.

We undertook the present study to investigate the cellular mechanisms involved in the cAMP-dependent secretory processes in a human pancreatic duct cell line, CAPAN-1, using the short-circuit current technique. CAPAN-1 was used since it had been shown to conserve most of the properties of ductal epithelial cells (Kyriazis et al., 1982; Levrat et al., 1988) and possess apical cAMP-dependent Cl^- channels which are crucial to HCO_3^- secretion (Becq et al., 1992, 1993). Therefore, CAPAN-1 appears to be a useful model for the study of pancreatic ductal secretory mechanisms of human origin considering the limited supply of intact human pancreatic ducts. The results of the present study indicate an active Cl^- accumulation mechanism which may be involved in Cl^- as well as HCO_3^- secretion by the pancreatic duct cells. Evidence is presented to suggest that the current model may not accurately describe the secretory processes in the pancreatic duct of human origin.

Materials and Methods

Materials

Hank's balanced salt solution (HBSS) was purchased from Sigma Chemical (St. Louis, MO). RPMI 1640 medium and fetal bovine serum, trypsin-EDTA were supplied by Gibco Laboratories (New York).

The following drugs were supplied by Sigma Chemical (St. Louis, MO): 4,4′-diisothiocyanatostibene-2,2′-disulfonic acid (DIDS), forskolin, secretin, Glucose, sodium bicarbonate, N-methy-D-glucamine (NMDG), calcium gluconate, N-2-hydroxethypiperazine-N′-2-ethanessulfonic acid (HEPES) and chemicals used in enzyme histochemistry: glutaraldehyde, sodium cacodylate, hydrochloric acid, Cobalt sulfate, sulfuric acid and potassium hydrogen phosphate. Amiloride hydrochloride was obtained from Merck Sharp & Dohme Research (Rahway, NJ). Calcium chloride, magnesium sulfate, potassium chloride, sodium chloride, sodium dihydrogen phosphate were obtained from Merck (Darmstadt, Germany). Potassium gluconate and sodium gluconate were from BDH Chemicals (Poole, England).

Cell Culture

Human pancreatic duct cell line, CAPAN-1, was purchased from American Type Culture Collection (Maryland) at passage of 23. Experiments were performed on cells of passages 27–38. Cells were grown in RPMI 1640 medium with 15% fetal bovine serum. When cells were disassembled from the culture flask, 0.25% trypsin-EDTA was added with extra care to avoid striking on cell layer directly. Quickly afterwards, less than 1 min, most of the trypsin was removed leaving about 0.5 ml in the flask which was then incubated for 2–3 min. Cells were then resuspended in serum-containing medium with gentle pipetting of the cells to break up the cell aggregations. The suspension was then transferred into a centrifuge tube for spinning at 800 × *g* for 5 min to remove any trypsin left. Supernatant was discarded and the cells were resuspended with a desirable volume of medium to make up to a final cell concentration of 1.5×10^6/ml. A volume of 0.25 ml of the cell suspension was then plated onto each permeable support, which was made of a Millipore filter and a silicon ring with a confined area of 0.45 cm^2, floating on culture medium and incubated at 37°C with 5% CO_2/95% O_2 in air atmosphere for 4–5 days till the monolayers reached confluence and were ready for I_{sc} measurement and histochemical staining.

Short-circuit Current Measurement

The basic principles of the short-circuit current experiments performed in the present study were the same as previously described (Ussing & Zerahn, 1951). Monolayers grown on permeable supports were clamped vertically between two halves of the Ussing chamber and bathed in Krebs-Henseleit (K-H) solutions with following composition (mM): NaCl, 117; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 24.8; $CaCl_2$, 2.56; Glucose, 11.1; with an osmalarity of 285 mOsm gassed with 95% O_2 and 5% CO_2. In some experiments, gluconate and NMDG were used to replace anions, Cl^- or HCO_3^-, and cation, Na^+, respectively. When gluconate was used, free Ca^{2+} concentration in the bath solution was measured by a Ca^{2+} electrode and titrated to a final concentration of 2.5 mM. For HCO_3^--free solution, HEPES and Tris were used and the solution was gassed in 100% O_2.

All the electrodes were connected to the voltage-current clamp amplifier (DVC-1000, World Precision Instrument, Sarasota, FL). The signal output from the amplifier was the I_{sc} measured and was recorded online by the use of a chart recorder (Kipp & Zonen, Delft, Netherlands). A 0.1-mV voltage pulse was applied intermittently across the epithelium and the transepithelial conductance was calculated from the corresponding current changes.

The Histochemical Staining Technique

The method used for the demonstration of carbonic anhydrase activity in cryostat sections was described by Rosen (1970). CAPAN-1 cell culture was fixed for approximately 1 hr in 3% glutaraldehyde and then rinsed in 0.17 M cacodylate buffer (pH 7.5) solution. Cryostat sections of 8 μm were picked up on Millipore filters and then incubated in a mixture of solutions containing 3.5 mM KH_2PO_4 with 1.75 mM $CoSO_4$, 54 mM H_2SO_4 and 157 mM $NaHCO_3$ for 20 min. Control sections were done using the similar incubation mixture containing 10 μM acetazolamide. After the staining procedure, the sections were rinsed in saline, dehydrated, and mounted with permount. Micrographs were taken with a Leica DMRBE microscope.

Immunohistochemistry

CAPAN-1 cells grown on filters were frozen in embedding medium using isopentane. Cryostat sections (8 μm) were cut and fixed in 4% paraformaldehyde for 15 min. Samples were then processed for indirect immunofluorescence technique. Samples were first incubated overnight at 4°C with monoclonal antibody (20 μg/mL) against the 60 kDa subunit of a vacuolar type of H^+-ATPase (Molecular Probes, Eugene, OR). After washing several times with PBS, samples were in-

cubated with anti-mouse IgG-fluorescein conjugated secondary antibody. Samples were then examined by confocal laser scanning microscopy.

Statistical Analysis

Results were expressed as mean ± SEM. Comparisons between groups of data were carried out using Student's unpaired *t*-test. A *P*-value less than 0.05 was considered to be statistically significant.

Results

Electrophysiological Properties of the Cultured CAPAN-1 Monolayer

Cultured monolayers were grown on permeable supports at a density of 1.5×10^6/ml for four to five days. When they were clamped in Ussing chambers bathing with normal K-H solution (Cl^-/HCO_3^--containing), a transepithelial potential difference of 0.2 ± 0.02 mV ($n = 20$) was usually observed, the apical side negative with respect to that of the basolateral side. A small basal current of 1.32 ± 0.37 μA/cm^2 ($n = 20$) was measured. The transepithelial resistance of the monolayers was 119 ± 9.7 Ω cm^2 ($n = 20$), which was somewhat higher than the values observed in isolated perfused duct of the rat (Novak & Greger, 1988*a*).

I_{sc} Induced by cAMP-evoking Agents

When CAPAN-1 monolayers were challenged with basolateral addition of secretin (1 μM), one of the physiological regulators of pancreatic secretion via stimulation of cAMP, a biphasic rise in I_{sc} was observed, with an initial transient peak followed by a plateau ($n = 8$, Fig. 1*A*). Forskolin (10 μm), an adenylate cyclase activator, also induced a rise in I_{sc} (Fig. 1*B*) with a characteristic similar to that obtained under stimulation with secretin. An initial peak level of about 2.1 ± 0.3 μA/cm^2 was reached at 3.5 min and followed by a plateau level of 1.0 ± 0.1 μA/cm^2 after 7 min ($n = 13$). No obvious change in transepithelial conductance after stimulation was observed. Since both secretin and forskolin yielded similar results, the following experiments were performed using forskolin as the agonist.

Concurrent and Independent Cl^- and HCO_3^- Secretion

Ion substitution experiments were carried out to study the ionic basis underlying the biphasic nature of the forskolin-induced I_{sc}. When external Cl^- was removed leaving HCO_3^- as the major permanent anion in the bathing solution, the initial peak of the forskolin-induced I_{sc} was not observed. Instead, a slow rise in I_{sc} reached to a level of about 1.3 ± 0.03 μA/cm^2 ($n = 10$) and remained at that level for at least 10 min. (Fig. 2*A*), indicating that HCO_3^- contributed largely to the plateau phase. When external HCO_3^- and CO_2 were removed, only a transient rise in I_{sc} was observed in response to forskolin, with a rise to a peak level of 0.9 ± 0.2 μA/cm^2 ($n = 5$) at 4 min and decayed to nearly zero level at 10 min, $n = 5$ (Fig. 2*B*), indicating the involvement of Cl^- in the initial peak phase. When both HCO_3^- and Cl^- were removed from the bathing solution, the forskolin-induced I_{sc} response was nearly abolished (96 ± 7%, $n = 6$).

The averaged forskolin-induced I_{sc} obtained in different solutions is shown in Fig. 2*C*. It is interesting to note that the summation of Cl^--dependent and HCO_3^--dependent currents gave rise to a curve which was simi-

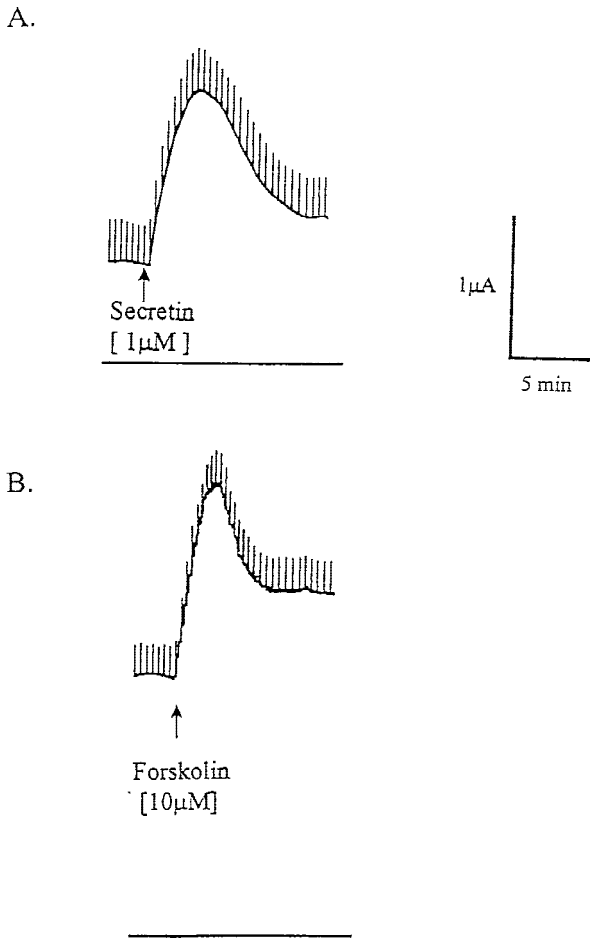


Fig. 1. I_{sc} in response to cAMP-evoking agents. (*A*) Representative recording of the I_{sc} activated by physiological regulator, secretin (1 μM, basolateral, $n = 8$), with arrow marking the time at which secretin was added and the horizontal line represents zero I_{sc}. (*B*) Mimicking the secretin-activated I_{sc} by forskolin (10 μM), an adenylate cyclase activator, ($n = 13$). Experiments were performed in K-H solution (Cl^-/HCO_3^--containing). Note the presence of biphasic characteristic in both responses.

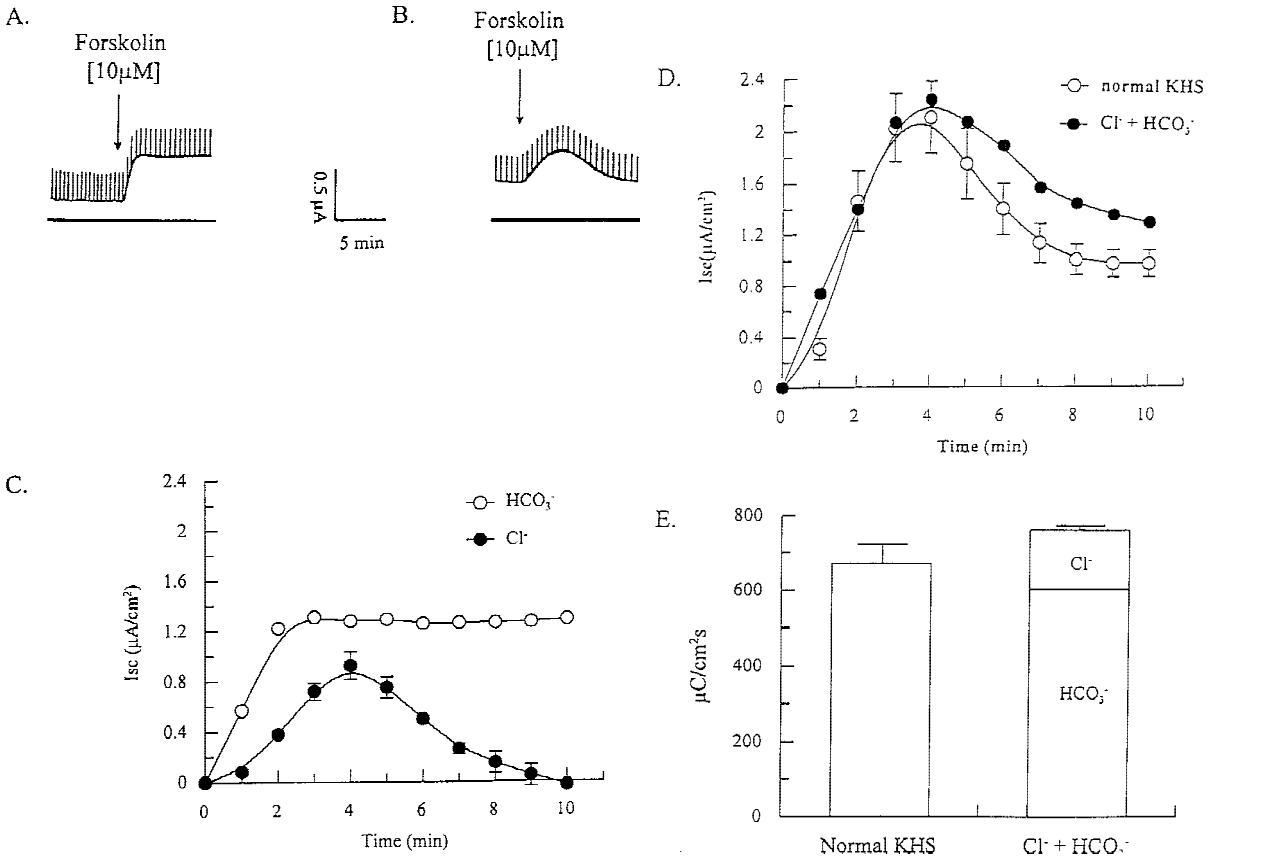


Fig. 2. Effect of Cl^-or HCO_3^- removal on the forskolin-activated I_{sc}. I_{sc} responses to forskolin (10 μM) obtained in Cl^--free (HCO_3^--containing, $n = 10$) solution (*A*), and in HCO_3^-/CO_2-free (Cl^--containing, $n = 5$) solution (*B*). (*C*) Averaged forskolin-induced I_{sc} obtained in the two solutions exhibiting different kinetic characteristics. (*D*) Comparison of the forskolin-induced I_{sc} obtained in K-H (Cl^-/HCO_3^- containing) solution and the summated I_{sc} obtained in the presence of Cl^- alone and HCO_3^- alone. (*E*) Comparison of the total charges, calculated from the area under curves, across the epithelium in various solutions. No significant difference was found.

lar to the averaged forskolin-induced I_{sc} obtained in Cl^-/HCO_3^--containing solution, both in kinetics and magnitude (Fig. 2*D* and *E*). Thus, Cl^- secretion and HCO_3^- secretion may occur in CAPAN-1 cells concurrently and independently.

Na^+ dependence of the Forskolin-induced I_{sc}

Since anion secretion may be through a Na^+-dependent secondary active transport mechanism, the Na^+-dependence of the forskolin-induced I_{sc} was also examined. In Na^+-free solution, the initial phase of the forskolin-induced I_{sc} normally observed in Cl^-/HCO_3^- solution was greatly reduced while the plateau remained as shown in Fig. 3*A*. The plateau level obtained in Na^+-free solution was similar to that obtained in Cl^--free solution (Fig. 3*A*). There was no significant difference in the total charge transfer across the monolayers, calculated from the area under curve, between the two cases (Fig. 3*B*). This suggested that the Cl^--dependent portion of the forskolin-induced I_{sc} might also be Na^+-dependent.

Cellular Mechanisms for Cl^- Secretion

As a portion of the forskolin-induced I_{sc} was dependent on both Na^+ and Cl^-, it seemed possible that Na^+-K^+-$2Cl^-$ cotransporter was involved in the secretory process in CAPAN-1 cells. Bumetanide (50 μM), an inhibitor of Na^+-K^+-$2Cl^-$ cotransporter, was applied basolaterally to the monolayers after forskolin stimulation. This resulted in a significant reduction of the forskolin-induced I_{sc} in Cl^-/HCO_3^--containing solutions from the control level of 1.0 ± 0.1 μA/cm² to 0.4 ± 0.1 μA/cm² with a total reduction of 60% ($n = 5$, Fig. 4*A* and *B*). Pretreatment of the cells with bumetanide ($n = 9$) greatly reduced the initial transient phase, but not the plateau, of the forskolin-induced I_{sc} (Fig. 4*C* and *D*). The inhibitory effect of bumetanide was also observed in HCO_3^--free solution (63.8%, $n = 5$); however, bumetanide was found to exert an insignificant effect when external Cl^- was removed (Fig. 4*E* and *F*), indicating that the effect of bumetanide was on a Cl^--dependent process. Bumetanide was also ineffective in Na^+-free solution ($n = 3$, *not shown*).

These results indicated the involvement of a basolaterally located Na^+-K^+-$2Cl^-$ cotransporter in mediating the Cl^--dependent forskolin-induced I_{sc}.

The involvement of the Na^+-K^+-$2Cl^-$ cotransporter suggested a secondary active transport mechanism present in CAPAN-1 cells. To further test this possibility, the effect of ouabain, an inhibitor of the Na^+-K^+-ATPase, on the forskolin-induced I_{sc} was examined. In monolayers 30 min after treatment with basolaterally applied ouabain (1 mM), forskolin was not able to elicit any I_{sc} ($n = 3$, *not shown*), indicating the presence of the Na^+-K^+-ATPase. The involvement of basolaterally located K^+ channels was also investigated since they play an important role in secondary active Cl^- transport. Ba^{2+} (1 mM), a K^+ channel blocker, was applied basolaterally to the monolayer after forskolin-stimulation. This induced a drastic reduction (93.3%) in the I_{sc} from the control level of 1.7 ± 0.3 μA/cm^2 to 0.1 ± 0.01 μA/cm^2, ($n = 10$, Fig. 5*A*). Similar to bumetanide, the inhibitory effect of Ba^{2+} was also observed in HCO_3^--free solution (98%, $n = 5$) but not in Cl^--free ($n = 6$, Fig. 5*B*) or Na^+-free solution ($n = 6$, *not shown*). Surprisingly, in these solutions Ba^{2+} induced a rise in the I_{sc}, indicating that blocking the K^+ channels would also affect the HCO_3^--dependent I_{sc} for a reason that was not immediately apparent (*see* Discussion). Taken together, the presence of basolaterally located Na^+-K^+-ATPase, Na^+-K^+-$2Cl^-$ cotransporter and K^+ channels suggested the involvement of a secondary active transport mechanism for Cl^- secretion in CAPAN-1 cells.

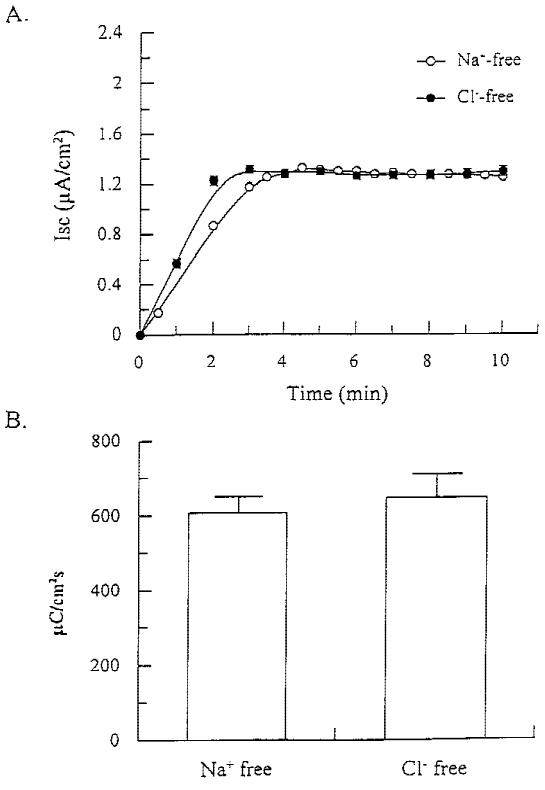


Fig. 3. Na^+ and Cl^- dependence of the forskolin-induced I_{sc}. (*A*) Comparison of averaged I_{sc} obtained in Cl^--free ($n = 8$) and Na^+-free but Cl^-/HCO_3^--containing ($n = 9$) solutions. (*B*) Comparison of total charges across the epithelium in the two solutions. No significant difference was found.

Cellular Mechanisms for HCO_3^- Secretion

The current pancreatic HCO_3^--secretion model requires the conversion of CO_2 into carbonic acid through the action of carbonic anhydrase (CA). The involvement of CA in CAPAN-1 cells was investigated using a CA inhibitor, acetazolamide. After stimulation with forskolin, acetazolamide (45–100 μm) was applied to the monolayers bathed in Cl^-/HCO_3^--containing solution and found to inhibit the I_{sc} from a level of 1.5 ± 0.03 μA/cm^2 to 0.9 ± 0.04 μA/cm^2 with a total reduction of 38.7%, $n = 6$ (Fig. 6*A* and *B*). In contrast to bumetanide or Ba^{2+}, the effect of acetazolamide was also found in Cl^--free solution (Fig. 6*C*), further indicating a role of CA in HCO_3^--secretion.

To sustain HCO_3^- secretion, H^+ must be expelled from the cell through the basolateral membrane, e.g., via the Na^+/H^+ exchanger as suggested by the current model. In Cl^-/HCO_3^--containing solution, amiloride at a concentration of 100 μm, which is known to have an inhibitory effect on Na^+/H^+ exchanger, was applied basolaterally resulting in a significant reduction (45.7%) in the I_{sc} from 1.3 ± 0.1 μA/cm^2 to 0.7 ± 0.1 μA/cm^2 ($n = 4$, Fig. 7*A*). Significant inhibition in the forskolin-induced I_{sc} was also observed in the Cl^--free solution ($n = 5$). However, MIA (10 μM), a more specific Na^+/H^+ exchanger inhibitor, was found to be ineffective in blocking the forskolin-induced I_{sc} ($n = 4$, Fig. 7*B*).

The possible involvement of H^+-ATPase was also tested using one of its inhibitors, NEM. Since NEM may also have an effect on other ATPases, we examined the concentration dependence of the inhibitory effect of NEM and a concentration-response curve was constructed (Fig. 8) with an apparent IC_{50} of 21.6 μm. This was very similar to the concentration known to inhibit H^+-ATPase in the rat kidney (Ait-Mohamed et al., 1986).

As recently suggested, a CA-independent mechanism, Na^+-HCO_3^- cotransporter, may also be involved in pancreatic ductal HCO_3^- secretion (Zhao et al., 1994; Ishiguro et al., 1996*a*). We examined the effect of H_2DIDS (150 μm), an inhibitor of Na^+-HCO_3^- cotransporter, on the forskolin-induced I_{sc}. However, no inhibitory effect was found ($n = 5$, *not shown*).

Apical Transport Mechanisms

The current model suggests that pancreatic HCO_3^- secretion requires the operation of an apically located Cl^--

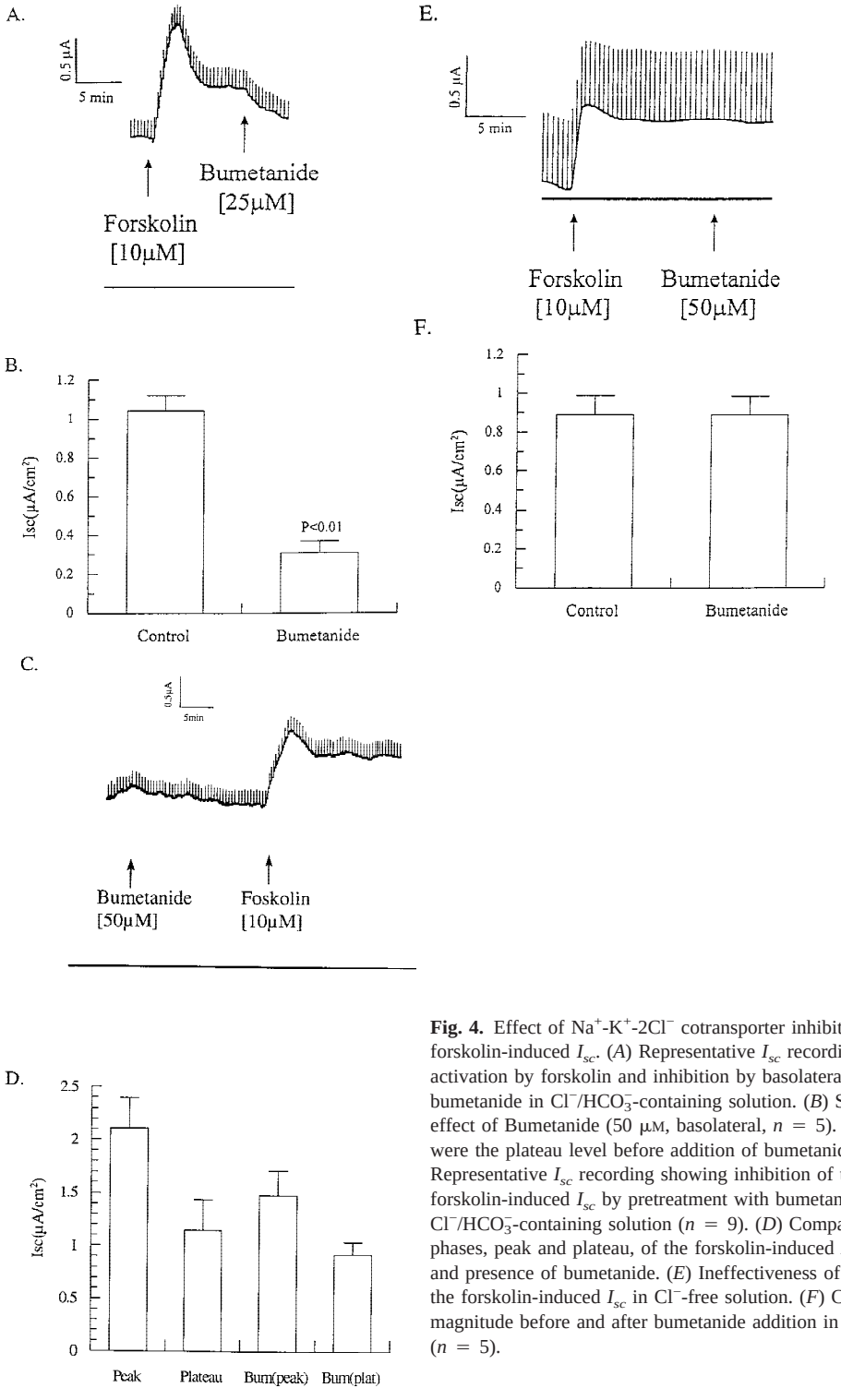


Fig. 4. Effect of Na^+-K^+-$2Cl^-$ cotransporter inhibitor on the forskolin-induced I_{sc}. (*A*) Representative I_{sc} recording showing activation by forskolin and inhibition by basolateral addition of bumetanide in Cl^-/HCO_3^--containing solution. (*B*) Summary of the effect of Bumetanide (50 μM, basolateral, $n = 5$). Control values were the plateau level before addition of bumetanide. (*C*) Representative I_{sc} recording showing inhibition of the forskolin-induced I_{sc} by pretreatment with bumetanide in Cl^-/HCO_3^--containing solution ($n = 9$). (*D*) Comparison of the two phases, peak and plateau, of the forskolin-induced I_{sc} in the absence and presence of bumetanide. (*E*) Ineffectiveness of bumetanide on the forskolin-induced I_{sc} in Cl^--free solution. (*F*) Comparison of I_{sc} magnitude before and after bumetanide addition in Cl^--free solution ($n = 5$).

HCO_3^- exchanger in parallel with an apical Cl^- channel. In the present study, an inhibitor of the Cl^--HCO_3^- exchanger, SITS (250 μM), was found to exert an insignificant effect on the forskolin-induced I_{sc} in Cl^-/HCO_3^--containing solution ($n = 3$, *not shown*). In the same solution, DIDS (200 μm), which is known to block the exchanger as well as Ca^{2+}-dependent Cl^- channel, was also ineffective in inhibiting the I_{sc} (Fig. 9*A*). However, DPC (1–2 mM), a nonselective Cl^- channel blocker with known effect on the cAMP-dependent Cl^- channel, pro-

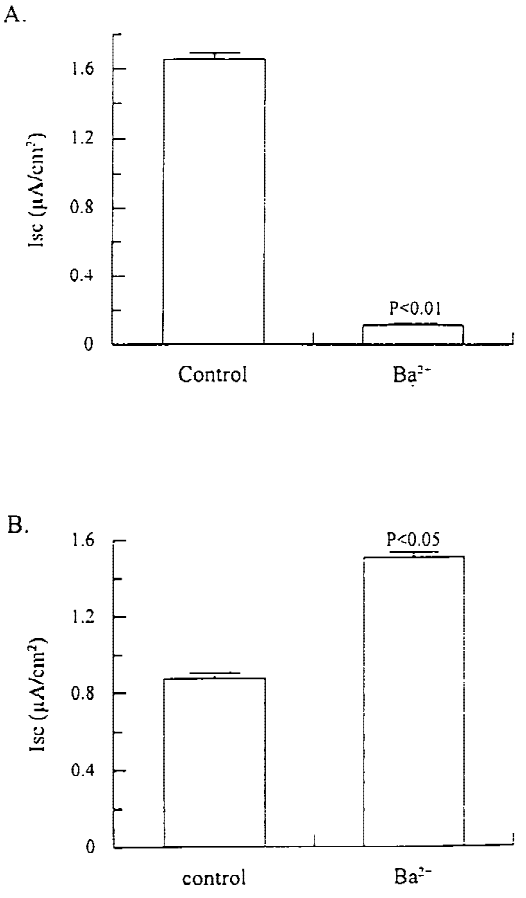


Fig. 5. Effect of K^+ channel blocker on the forskolin-induced I_{sc}. (*A*) Averaged inhibitory effect of Ba^{2+} on forskolin-induced I_{sc} in Cl^-/HCO_3^--containing solution ($n = 10$). (*B*) Effect of Ba^{2+} (1 mM, basolateral) in (Cl^--free) μM-containing solution ($n = 6$). Note that an increase rather than a decrease in I_{sc} was observed in this condition. Ba^{2+} was added 5 min after stimulation with forskolin. The effects were assessed over a 5-min period after addition of Ba^{2+}.

duced a potent inhibitory effect on the forskolin-induced I_{sc} (Fig. 9*A*), reducing the I_{sc} from 2.2 ± 0.5 ± 0.04 μA/cm^2 ($n = 10$). The effects of DIDS and DPC are summarized in Fig. 9*B*.

We further examined how DPC affected different processes, e.g., the peak (mainly Cl^--dependent) or the plateau (mainly HCO_3^--dependent) phase of the forskolin-induced I_{sc}. Experiments were conducted by pretreating the monolayers with DPC in different bathing solutions. In Cl^-/HCO_3^--containing solution, pretreatment of the monolayers ($n = 6$) with apical addition of DPC (1 mM) for 10 min resulted in large suppression of the forskolin-stimulated I_{sc}, the initial peak as well as the plateau phase (Fig. 10*A*).

When monolayers were bathed in Cl^--free solution, the HCO_3^--dependent I_{sc} was also suppressed by DPC treatment ($n = 4$) (Fig. 10*B*), indicating that DPC-sensitive Cl^- channels may be important for both Cl^- and HCO_3^- secretion in CAPAN-1 cells.

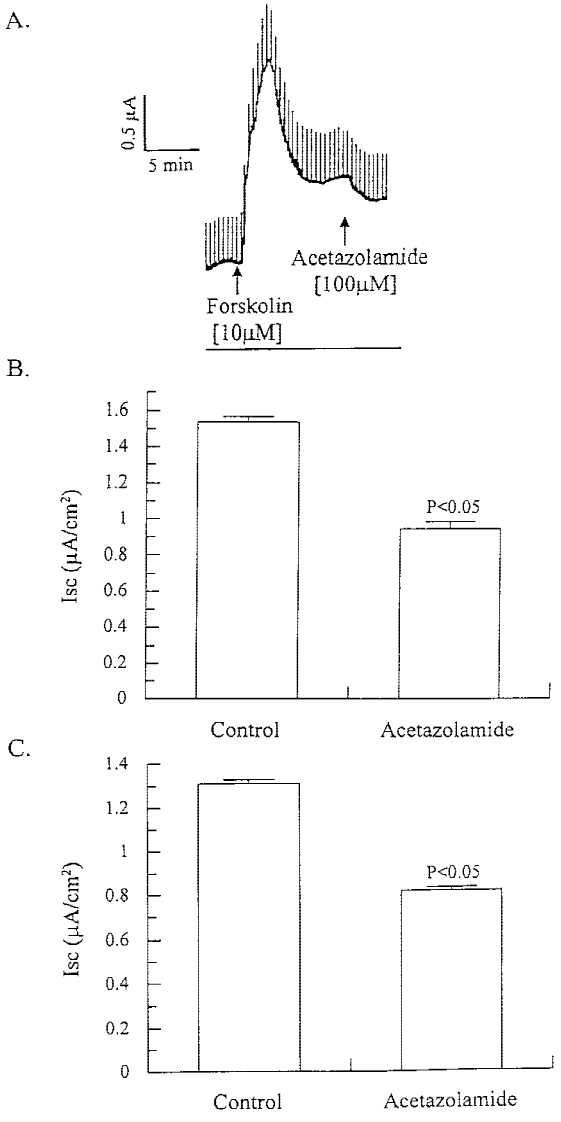
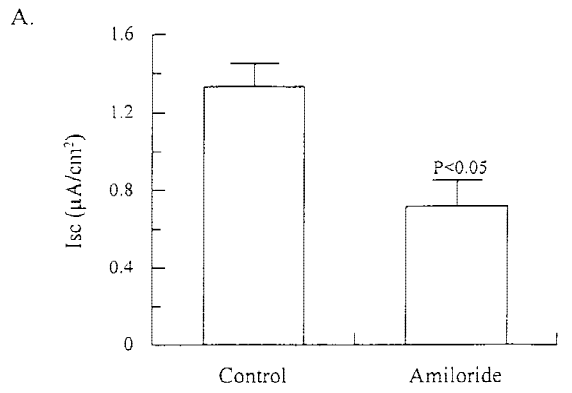


Fig 6. Effect of carbonic anhydrase inhibitor on the forskolin-induced I_{sc}. (*A*) Representative I_{sc} recording showing activation by forskolin and inhibition by acetazolamide in Cl^-/HCO_3^--containing solution. (*B*) Averaged inhibitory effect of acetazolamide (100 μM, basolateral) on forskolin-induced I_{sc} in Cl^-/HCO_3^--containing solution ($n = 6$). (*C*) Effect of acetazolamide obtained in Cl^--free (HCO_3^--containing) solution. Cultured monolayers were pretreated with acetazolamide (45 μM) before the stimulation by forskolin ($n = 6$).

Distribution of Carbonic Anhydrase in CAPAN-1 Cells

Our data suggested that HCO_3^--dependent forskolin-induced I_{sc} was mediated by CA-dependent mechanism. To confirm this, histochemical studies were performed to examine the distribution of CA in CAPAN-1 cells. Figure 11*A* shows positive staining for CA while acetazolamide-treated monolayer (Fig. 11*B*) exhibited negative staining. It was interesting to find that the distribution of



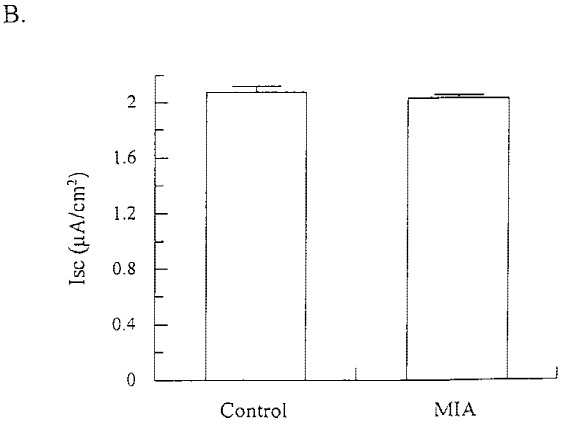


Fig. 7. Effect of Na^+-H^+ exchanger blockers on the forskolin-induced I_{sc}. (*A*) Summary of the effect of amiloride (100 μM, basolateral, $n = 4$). (*B*) Ineffectiveness of a more specific inhibitor of Na^+/H^+ exchanger, MIA (10 μM, $n = 3$) on the I_{sc}. The experiments were performed in Cl^-/HCO_3^--containing solution.

CA staining throughout the culture of CAPAN-1 was discrete, suggesting that not all the cells were involved in HCO_3^--secretion.

Immunolocalization of Vacuolar H^+-ATPase

Positive immunoreactivity of the vacuolar H^+-ATPase was observed in both the apical and basal regions of CAPAN-1 monolayers (Fig. 12*A*). Negative immunostaining was found in the control section in which the primary antibody was omitted (Fig. 12*B*).

Discussion

The present study has demonstrated for the first time the anion secretory mechanisms in the pancreatic duct cells of human origin. The present study has provided evidence showing that reconstituted cultured monolayers of

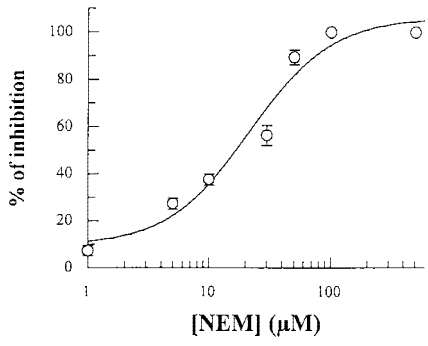
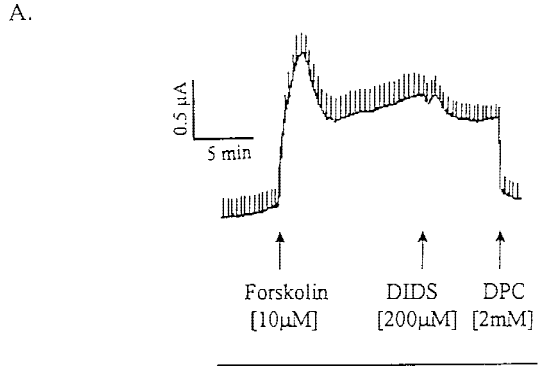


Fig. 8. Concentration-response curve of H^+-ATPase inhibitor, NEM. Percentage of I_{sc} inhibition was plotted against various NEM concentrations added basolaterally. The IC_{50} was about 22 μM.



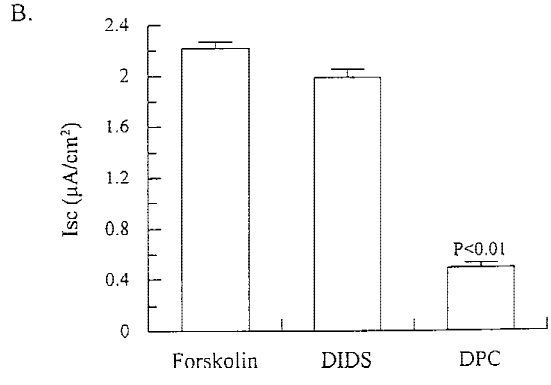


Fig. 9. Comparison of the effects of DIDS and DPC on the I_{sc}. (*A*) I_{sc} recording showing the effect of DPC (2 mM) and DIDS (100 μM) on the forskolin-stimulated I_{sc}. (*B*) Summary of the results of blockers showing that DPC but not DIDS had a prominent inhibitory effect ($n = 10$).

CAPAN-1 cells are capable of secreting Cl^- as well as HCO_3^-. The observations that the forskolin-induced I_{sc} was dependent on external Cl^- and HCO_3^-/CO_2, and inhibitable by agents with known effects on either Cl^- secretion (e.g., bumetanide, Ba^{2+}) or HCO_3^- secretion (e.g., acetazolamide) suggest that CAPAN-1 cells secrete both of these anions under the stimulation of cAMP.

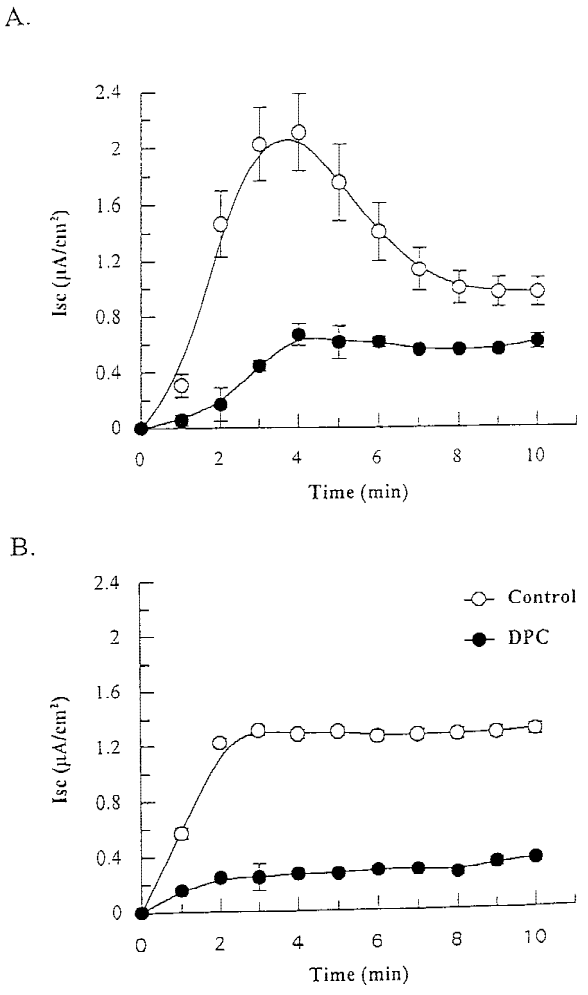


Fig. 10. The inhibitory effect of DPC on Cl^- and HCO_3^--dependent I_{sc} stimulated by forskolin. Averaged forskolin-induced I_{sc} obtained from control and monolayers pretreated with DPC (1 mM) in Cl^-/HCO_3^--containing solution (*A*) and HCO_3^--containing (*B*), solution.

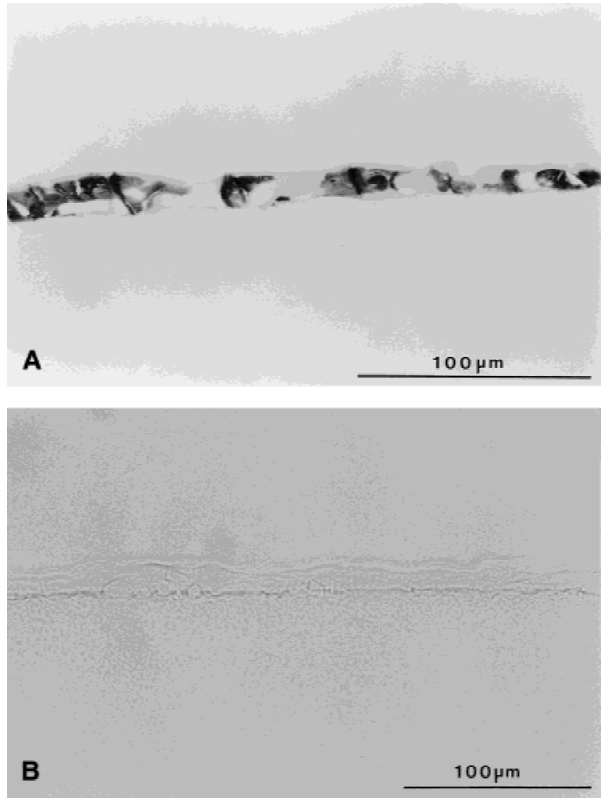


Fig. 11. Histochemical staining for carbonic anhydrase. (*A*) Light micrograph of CAPAN-1 monolayer grown on permeable support for 5 days showing positive reaction of carbonic anhydrase activity (magnification: ×470). There are also cells which do not exhibit this enzyme. (*B*) Control monolayer showing negative staining (magnification: ×370).

The fact that the I_{sc} can be stimulated by secretin, an important physiological regulator of pancreatic secretion, further indicates the conservation of ductal secretory properties in CAPAN-1 cells. A comparison of charge transfer across the epithelium under Cl^--free or HCO_3^--free condition, calculated from areas under curves, suggests that over 80% of the forskolin-induced I_{sc} was HCO_3^--dependent. This is also consistent with the previous finding that cAMP stimulates HCO_3^--rich fluid in the pancreatic ducts (reviewed by Argent & Case, 1994). These results indicate that CAPAN-1 cells may be a useful model for the study of secretory mechanisms of the pancreatic ducts.

The results of the present study suggest that conversion of CO_2 into carbonic acid through carbonic anhydrase appears to be a crucial step in HCO_3^--production by CAPAN-1 cells since the forskolin-induced I_{sc} was inhibitable by an inhibitor of carbonic anhydrase, acetazolamide. The presence of carbonic anhydrase in CAPAN-1 cells was also confirmed by histochemical studies. Although a Na^+-HCO_3^- cotransporter has been recently implicated in HCO_3^- cotransporter has been recently implicated in HCO_3^- accumulation in the pancreatic ducts of the rat (Zhao et al., 1994) and guinea-pig (Ishiguro et al., 1996*a*,*b*), the preset study does not seem to indicate its involvement in CAPAN-1 cells. This is based on the present observations that H_2DIDS, an inhibitor of Na^+-HCO_3^- cotransporter, was ineffective in blocking the forskolin-induced I_{sc} and that removal of external Na^+ did not seem to affect the HCO_3^--dependent I_{sc}.

In the present study the forskolin-induced I_{sc} was sensitive to an H^+-ATPase inhibitor, NEM, as well as an Na^+-H^+ exchanger inhibitor, amiloride, indicating a possible role of these transporters in HCO_3^- secretion. While amiloride could inhibit the I_{sc} at 100 μM as reported by many previously studies, this could be due to its inhibitory effect on cellular metabolism as previously shown for amiloride analogues (Soltoff, Cragoe & Mandel, 1986). In the present study, a more specific Na^+-H^+ ex-

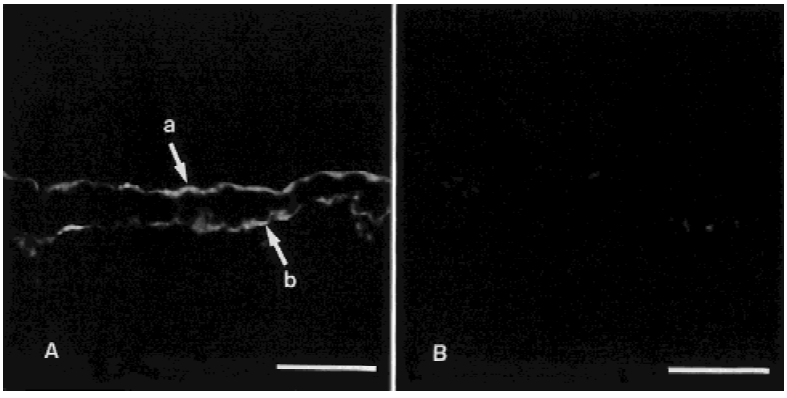


Fig. 12. Immunofluorescence staining of H^+-ATPase in cryosections of the human CAPAN-1 cell. (*A*) Distinct immunoreactivity was observed in the apical (a) and basal (b) regions of the CAPAN-1 cells grown on the filters. (*B*) Immunoreactivity was not observed in the control experiment when the primary antibody was omitted. Scale bar = 50 μm.

changer inhibitor, MIA, was found to be ineffective in blocking the forskolin-induced I_{sc}, indicating the absence of this exchanger in CAPAN-1 cells. Another line of evidence excluding the involvement of the Na^+-H^+ exchanger is that the HCO_3^--dependent I_{sc}, e.g, the plateau phase, was not affected by the removal of external Na^+. On the other hand, our data suggest that the H^+-ATPase may be involved in HCO_3^- secretion. Although one can argue that NEM, like other inhibitors of proton pumps, may have nonspecific effects on other ATPases, the observed IC_{50} value in the present study is in close agreement with that reported to block a vacuolar-type of H^+-ATPase in the rat kidney (Ait-Mohamed et al., 1986). Another important piece of evidence came from the seemingly surprising stimulatory effect of Ba^{2+} observed in Cl^--free solution. Like bumetanide, we expected that Ba^{2+} would not have an inhibitory effect on the forskolin-induced I_{sc} in the absence of Cl^-, however, we did not anticipate a rise in the I_{sc} upon addition of Ba^{2+}. This observation could be explained if a voltage-sensitive H^+-ATPase was placed in the basolateral membrane. When basolateral K^+ channels are blocked by Ba^{2+}, this would result in a depolarization of the membrane that could then drive a highly voltage-sensitive pump as shown previously for a vacuolar-type of H^+-ATPase in *Rana Esculenta* skin (Ehrenfeld et al., 1985). Thus, the observed rise in the HCO_3^--dependent forskolin-induced I_{sc} upon addition of Ba^{2+} may be secondary to a depolarization-induced activation of H^+-ATPase. Taken together, our data suggest that a H^+-ATPase, rather than the Na^+-H^+ exchanger, appears to be involved in HCO_3^- secretion in CAPAN-1 cells. It should be noted that several lines of evidence in support of the involvement of a H^+-ATPase in pancreatic duct secretion have been reported (reviewed by Raeder, 1992). Our immunohistochemical studies also confirm the presence of a vacuolar-type H^+-ATPase in CAPAN-1 cells. However, to our surprise, it is localized to the apical as well as the basolateral membrane. The function of the apically located H^+-ATPase is not immediately apparent.

In contrast to the current model of pancreatic HCO_3^- secretion, which relies on the entry of luminal Cl^- through the Cl^--HCO_3^- exchanger and recycling of Cl^- through apical Cl^- channels, the present results suggest the segregation of the apical Cl^- channel and the Cl^- HCO_3^- exchanger if it is indeed present in CAPAN-1 cells at all. First, in the absence of external Cl^-, under which condition the Cl^--HCO_3^- exchanger would have been disable, over 80% of the forskolin-induced I_{sc} was still observed, indicating that the majority of the HCO_3^- secreted is not dependent on the operation of the Cl^-/ HCO_3^- exchanger. Second, SITS and DIDS did not seem to have a significant effect on the forskolin-induced I_{sc} while DPC inhibited most of the HCO_3^--dependent as well Cl^--dependent I_{sc}, indicating the dependence of both HCO_3^- and Cl^- secretion on the apical Cl^- channel. Our data are consistent with the previous finding of an apical cAMP-dependent Cl^- channel in the pancreatic duct (Novak & Greger, 1988*b;* Gray, Greenwell & Argent, 1988; Gray et al., 1990*c*). In addition, these data suggest that not only could Cl^- but also HCO_3^- be secreted through the apical DPC-sensitive pathway, most likely, the apical Cl^- channel. This notion is supported by patch-clamp studies showing measurable HCO_3^- permeability through Cl^- channels in pancreatic duct cells (Gray et al., 1990*c;* Gray et al., 1990*a*). HCO_3^- conductance as well as pH regulatory capability has also been demonstrated in cells transfected with cystic fibrosis transmembrane conductance regulator (CFTR) (Poulsen et al., 1994), a cAMP-dependent Cl^- channel itself (reviewed by Welsh, 1996). The presently observed sensitivity of the HCO_3^--dependent, as well as Cl^--dependent, forskolin-induced I_{sc} to DPC, a Cl^- channel blocker with known effect on CFTR, suggests that the reduced HCO_3^--secretion observed in CF may be directly due to reduced permeability of HCO_3^- through defect CFTR rather than an indirect effect secondary to the reduced Cl^- permeability as suggested by the current model.

As far as we are aware no active transport mechanism for Cl^- accumulation in the pancreatic duct has been suggested. The present results indicate a secondary active transport mechanism for Cl^- secretion in CA-

PAN-1 cells. The supporting evidence includes: (i) portion of the forskolin-induced I_{sc} was Na^+ and Cl^--dependent; (ii) Na^+-K^+-ATPase inhibitor, ouabain, blocked the forskolin-induced I_{sc}; and (iii) bumetanide and Ba^{2+} inhibited the I_{sc} but not when external Na^+ and Cl^- was removed, indicating the involvement of basolaterally located Na^+-K^+-$2Cl^-$ cotransporter and K^+ channels. Together with the apical Cl^- channels (*see above*), the above transporters could mediate the cAMP-dependent Cl^- secretion in CAPAN-1 cells via the secondary active transport mechanism well documented in many other epithelia (Welsh, 1983; Case et al., 1984; Sato & Sato, 1987, Wong, 1988). While Na^+-K^+-ATPase has been demonstrated in the cat and rat (Bundgaard, Moller & Poulsen, 1981; Madden & Sarras, 1987), K^+ channels are also found in ducts of the rat (Novak & Greger, 1988*a;* Gray et al., 1989; Gray et al., 1990*b*). However, the bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransporter has not been demonstrated in the pancreatic ducts of any species. The presently observed effect of bumetanide could also be attributed to a Na^+-Cl^- cotransporter (Hass, 1994).

Although the current model for pancreatic secretion does not describe Cl^- secretion, impaired Cl^- secretion as well as HCO_3^- secretion, has been observed in the pancreas of patients with cystic fibrosis (Gaskin et al., 1982; Kopelman et al., 1988), thus indicating a role of Cl^- secretion in the normal function of the pancreas. The present study is the first to describe an active Cl^- accumulation mechanism in pancreatic duct cells. This mechanism may be responsible for a Cl^--rich fluid secreted by the rat ducts under stimulation by Ca^{2+}-evoking agents, e.g., acetylcholine (Ashton et al., 1993) and the ATP and angiotensin II-activated HCO_3^- Cl^- secretion by the CF pancreatic duct cells, CAPAN-1 (Chan et al., 1996, 1997). This mechanism could also allow active Cl^- accumulation required to drive HCO_3^- secretion at high concentrations which cannot be supported by the current model (Sohma et al., 1996). Our data do suggest possible dependence of HCO_3^- secretion on Cl^- accumulation. Note that in the present study the Cl^--dependent forskolin-induced I_{sc} (in the absence of HCO_3^-/CO_2) was only about 20% of that observed in Cl^-/HCO_3^--containing solution. However, bumetanide and Ba^{2+} inhibited the I_{sc} by more than 60% in the presence of both Cl^- and HCO_3^-. This can either be explained by a much greater proportion of Cl^- secretion in the presence of HCO_3^- or a dependence of a portion of the HCO_3^- secretion on Cl^- accumulation. The former seems unlikely since cAMP is known to stimulate HCO_3^--rich fluid. This leaves the only alternative that a portion of the HCO_3^- secretion relies on the active Cl^- accumulation; e.g., when Cl^- accumulation is blocked, a portion of the HCO_3^- secretion is also inhibited. The apparent inconsistency in the proportion of HCO_3^--dependent I_{sc} observed in the absence of Cl^- (over 80%) and that of the presumed HCO_3^--dependent I_{sc} (<40%) observed in the presence of both Cl^- and HCO_3^- after treatment with the inhibitors for Cl^- accumulation suggests that the secretory mechanism in the presence of both Cl^- and HCO_3^- may be more complicated. The detail of the mechanism of how HCO_3^- secretion may be dependent on Cl^- remains to be elucidated.

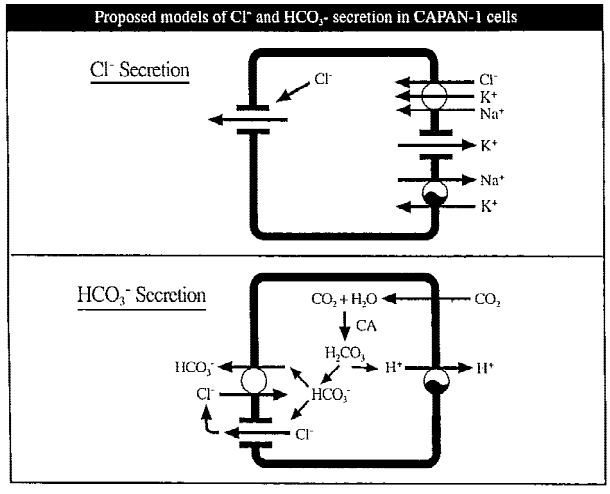


Fig. 13. Proposed model of Cl^- and HCO_3^--secretion in CAPAN-1 cells. The cellular mechanisms for Cl^- and HCO_3^- secretion are described separately as suggested by the present results.

Although there might be interaction between Cl^- and HCO_3^-, the present study has clearly demonstrated that Cl^- and HCO_3^- secretion in CAPAN-1 cells may occur independently since forskolin could stimulate I_{sc} in the absence of either Cl^- or HCO_3^-. Our data also indicate that some of the mechanisms involved in Cl^- secretion do not seem to affect the HCO_3^--dependent forskolin-induced I_{sc} in the absence of Cl^-, and *vice versa,* as though the two processes were entirely separate. The discrete distribution of CA in CAPAN-1 cells indicates possible segregation of HCO_3^- and Cl^- secretions at the cellular level. Based on these findings, we propose a secretory model consisting of two separate transport mechanisms for HCO_3^- and Cl^- secretion in CAPAN-1 cells (Fig. 13). The notion that different secretions, e.g., Cl^--rich *vs.* HCO_3^--rich, may be mediated by different cells in different regions of the pancreas has long been suggested (Mangos & McSherry, 1971; Swanson & Solomon, 1973; Lightwood & Reber, 1977). Although this could explain readily the independent HCO_3^- and Cl^- secretions observed in the present study, it would be difficult to account for any interaction which might occur between these secretory processes as observed in the present study if their cellular mechanisms were localized to different cell populations.

In summary, the present study is the first attempt to study secretory mechanisms in the pancreatic duct cells

of human origin. The results show that CAPAN-1 cells are capable of secreting both Cl^- and HCO_3^- concurrently and independently. The results of the present study indicate that the currently accepted model of pancreatic secretion may not apply to the pancreatic duct of humans, or that the current model requires modification as suggested by the growing body of evidence obtained recently from a number of animal species.

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References

Ait-Mohamed, A.K., Marsy, S., Barlet, C., Khadouri, C., Doucet, A. 1986. Characterization of N-Ethylmaleimide-sensitive protein pump in the rat kidney. *J. Biol. Chem.* **261:**12526–12533

Argent, B.E., Case, R.M. 1994. Pancreatic ducts — cellular mechanism and control of bicarbonate secretion. *In:* Physiology of the Gastrointestinal Tract. L.R. Johnson, editor. 3rd edition, pp. 1473–1397. Raven Press, New York

Ashton, N., Evans, R.L., Elliott, A.C., Green, R., Argent, B.E. 1993. Regulation of fluid secretion and intracellular messengers in isolated rat pancreatic ducts by acetylcholine. *J. Physiol.* **471:**549–562

Becq, F., Fanjul, M., Mahieu, I., Berger, Z., Gola, M., Hollande, E. 1992. Anion channels in a human pancreatic cancer cell line (CAPAN-1) of ductal origin. *Pfluegers Arch.* **420:**46–53

Becq, F., Hollande, E., Gola, M. 1993. Phosphorylation-regulated low-conductance Cl^- channels in a human pancreatic duct cell line. *Pfluegers Arch.* **425:**1–8

Bundgarrd, M., Moller, M., Poulsen, J.H. 1981. Localization of sodium pumps in cat pancreas. *J. Physiol.* **313:**405–414

Case, R.M., Hunter, M., Novak, I., Young, J.A. 1984. The anionic basis of fluid secretion by the rabbit mandibular salivary gland. *J. Physiol.* **349:**619–630

Chan, H.C., Cheung, W.T., Leung, P.Y., Wu, L.J., Cheng Chew, S.B., Ko, W.H., Wong, P.Y.D. 1996. Purinergic regulation of anion secretion by CF pancreatic duct cells. *Am. J. Physiol.* **271:**C469–C477

Chan, H.C., Law, S.H., Leung, P.S., Wong, P.Y.D. 1997. Angiotensin II receptor type I-regulated anion secretion in cystic fibrosis pancreatic duct cells. *J. Membrane Biol.* **156:**241–249

Cheng, H.S., Chan, H.C. 1997. Independent Cl^- and HCO_3^- secretion in human pancreatic duct cells. *FASEB J.* **11:**A305

Ehrenfeld, J., Garcia-Romeu, F., Harvey, B.J. 1985. Electrogenic active proton pump in *Rana Esculenta* skin and its role in sodium ion transport. *J. Physiol.* **359:**331–355

Gaskin, K.J., Durie, P.R., Corey, M., Wei, P., Forstner, G.G. 1982. Evidence for a primary defect of pancreatic HCO_3^- secretion in Cystic Fibrosis. *Pediat. Res.* **16:**554–557.

Gray, M.A., Greenwell, J.R., Argent, B.E. 1988. Secretin-regulated Cl^- channel on the apical plasma membrane of pancreatic duct cells. *J. Membrane Biol.* **105:**131–142

Gray, M.A., Greenwell, J.R., Argent, B.E. 1990*a*. The role of ion channels in the mechanism of pancreatic bicarbonate secretion. *In:* Epithelial Secretion of Water and Electrolytes. J.A. Young, and P.Y.D. Wong, editors. pp. 254–265. Springer-Verlag, Berlin

Gray, M.A., Greenwell, J.R., Garton, A.J., Argent, B.E. 1990*b*). Regulation of Maxi-K^+ channels on pancreatic duct cells by cAMP-dependent phosphorylation. *J. Membrane Biol.* **115:**203–215

Gray, M.A., Harris, A., Coleman, L., Greenwell, J.R., Argent, B.E. 1989. 2 types of Cl^- channel on duct cells cultured from human fetal pancreas. *Am. J. Physiol.* **257:**C240–C251

Gray, M.A., Pollard, C.E., Harris, A., Coleman, L., Greenwell, J.R., Argent, B.E. 1990*c*. Anion selectivity and block of the small conductance Cl^- channel on pancreatic duct cells. *Am. J. Physiol.* **259:**C752–C761

Hass, M. 1994. The Na-K-Cl cotransporters. *Am. J. Physiol.* **267:** C869–C885

Ishiguro, H., Steward, M.C., Lindsay, A.R.G., Case, R.M. 1996*a*. Accumulation of intracellular HCO_3^- by Na^+-HCO_3^- cotransporter in interlobular ducts from guinea-pig pancreas. *J. Physiol.* **495:**69–178

Ishiguor, H., Steward, M.C., Wilson, M.C., Case, R.M. 1996*b*. HCO_3^- secretion in interlobular ducts from guinea-pig pancreas. *J. Physiol.* **495:**179–191

Kopelman, H., Corey, M., Gaskin, K., Durie, P., Weizman, Z.V.I., Forstner, G. 1988. Impaired chloride secretion, as well as bicarbonate secretion, underlies the fluid secretory defect in the Cystic Fibrosis pancreas. *Gastroenterology* **95:**349–355

Kyriazis, A.P., Kyriazis, A.A., Scarpelli, D.G., Rao, M.S., Fogh, J., Lepera, R. 1982. Human pancreatic adenocarcinoma line capan-1 in tissue culture and the nude mouse. *Am. J. Pathol.* **106:**250–260

Levrat, J.H., Palevody, C., Daumas, M., Ratovo, G., Hollande, E. 1988. Differentiation of the human pancreatic adenocarcinoma cell line (CAPAN-1) in culture and co-culture with fibroblasts dome formation. *Int. J. Cancer* **42:**615–621

Lightwood, R., Reber, H.A. 1977. Micropuncture study of pancreatic secretion in the cat. *Gastroenterology* **72:**61–66

Madden, M.E., Sarras, M.P. 1987. Distribution of Na^+,K^+-ATPase in rat exocrine pancreas as monitored by K^+-NPPase cytochemistry and [^{3}H]-ouabain binding: a plasma membrane protein found primarily to be ductal cell associated. *J. Histochem. Cytochem.* **35:**1365–1374

Mangos, J.A., McSherry, N.R. 1971. Micropuncture study of excretion of water and electrolytes by the pancreas. *Am. J. Physiol.* **221:**496–503

Novak, I., Greger, R. 1988*a*. Electrophysiological study of transport systems in isolated perfused pancreatic ducts: properties of the basolateral membrane. *Pfluegers Arch.* **411:**58–68

Novak, I. 1990. Electrolyte transport in pancreatic ducts. *In:* Epithelial Secretion of Water and Electrolytes. J.A. Young and P.Y.D. Wong, editors. pp. 240–252. Springer-Verlag, Berlin

Novak, I., Greger, R. 1988*b*. Properties of the luminal membrane of isolated perfused rat pancreatic ducts — effect of cyclic AMP and blockers of chloride transport. *Pfluegers Arch.* **411:**546–553

Poulsen, J.H., Fischer, H., Illek, B., Machen, T.E. 1994. Bicarbonate conductance and pH regulatory capability of cystic fibrosis transmembrane conductance regulator. *Proc. Natl. Acad. Sci. USA* **91:**5340–5344

Raeder, M.G. 1992. The origin of and subcellular mechanisms causing pancreatic bicarbonate secretion. *Gastroenterology* **103:**1674–1684

Rosen, S. 1970. Localization of carbonic anhydrase activity in transporting urinary epithelia. *J. Histochem. Cytochem.* **18:**668–670

Sato, F., Sato, K. 1987. Effect of periglandular ionic composition and transport inhibitors on rhesus monkey eccrine sweat gland function in vitro. *J. Physiol.* **393:**195–212

Sohma, Y., Gray, M.A., Imai, Y., Argent, B.E. 1996. A mathematical model of the pancreatic ductal epithelium. *J. Membrane Biol.* **154:**53–67

Soltoff, S. P., Cragoe, E.J., Mandel, L.J. 1986. Amiloride analogues

inhibit proximal tubule metabolism. *Am. J. Physiol.* **250:**C744–C747

Swanson, C.H., Solomon, A.K. 1973. A micropunture investigation of the whole tissue mechanism of electrolyte secretion by the in vitro rabbit pancreas. *J. Gen. Physiol.* **62:**407–429

Ussing, H.H., Zerahn, K. 1951. Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. *Acta Physiol. Scand.* **23:**110–127

Welsh, M.J. 1983. Inhibition of chloride secretion by furosemide in canine tracheal epithelium. *J. Membrane Biol.* **71:**219–226

Welsh, M.J. 1996. Cystic fibrosis. *In:* Molecular Biology of Membrane Transport Disorders. S.G. Schultz, T.E. Andreoli, A.M. Brown, D.M. Fambrough, J.F. Hoffman, and M.J. Welsh, editors. pp. 605–623. Plenum Press, New York and London

Wong, P.Y. 1988. Mechanisms of adrenergic stimulation of anion secretion in cultured epididymal epithelium. *Am. J. Physiol.* **254:** F121–F133

Zhao, H., Star, R.A., Muallem, S. 1994. Membrane localization of H^+ and HCO_3^- transporters in the rat pancreatic duct. *J. Gen. Physiol.* **104:**57–85