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# Vectorial Bicarbonate Transport by Capan-1 Cells: a Model for Human Pancreatic Ductal Secretion

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**Key Words**

Bicarbonate secretion • Exocrine pancreas • Secretin • Vasoactive intestinal peptide • Purinoceptors

**Abstract**

Human pancreatic ducts secrete a bicarbonate-rich fluid but our knowledge of the secretory process is based mainly on studies of animal models. Our aim was to determine whether the $HCO_3^-$ transport mechanisms in a human ductal cell line are similar to those previously identified in guinea-pig pancreatic ducts. Intracellular pH was measured by microfluorometry in Capan-1 cell monolayers grown on permeable filters and loaded with BCECF. Epithelial polarization was assessed by immunolocalization of occludin. Expression of mRNA for key electrolyte transporters and receptors was evaluated by RT-PCR. Capan-1 cells grown on permeable supports formed confluent, polarized monolayers with well developed tight junctions. The recovery of $pH_i$ from an acid load, induced by a short $NH_4^+$ pulse, was mediated by $Na^+$-dependent transporters located exclusively at the basolateral membrane. One was independent of $HCO_3^-$ and blocked by EIPA (probably NHE1) while the other was $HCO_3^-$-dependent and blocked by $H_2$DIDS (probably pNBC1). Changes in $pH_i$ following blockade of basolateral $HCO_3^-$ accumulation confirmed that the cells achieve vectorial $HCO_3^-$ secretion. Dose-dependent increases in $HCO_3^-$ secretion were observed in response to stimulation of both secretin and VPAC receptors. ATP and UTP applied to the apical membrane stimulated $HCO_3^-$ secretion but were inhibitory when applied to the basolateral membrane. $HCO_3^-$ secretion in guinea-pig ducts and Capan-1 cell monolayers share many common features, suggesting that the latter is an excellent model for studies of human pancreatic $HCO_3^-$ secretion.



## Introduction

The principal physiological role of pancreatic ducts is to secrete a bicarbonate-rich, isotonic fluid. When this secretory mechanism fails, disease follows - certainly in the case of cystic fibrosis, most likely in the case of chronic pancreatitis, and perhaps even in acute pancreatitis.

The advent of modern molecular biology and the introduction of sophisticated techniques in experimental cell physiology have facilitated considerable progress in

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our knowledge of the various transport mechanisms responsible for ductal $HCO_3^-$ secretion [1]. However, most of this progress has been as a result of experiments on animal tissues. Unfortunately, the extent to which different transport pathways are involved in secretion varies significantly between species. This explains why the maximum pancreatic bicarbonate secretion in the rat (and probably also the mouse) is around 70 mM compared with around 140 mM in the human and in laboratory species such as the guinea pig [2, 3] and why, therefore, rat and mouse pancreatic ducts represent poor models of human duct cell function [4].

Although difficult, it is possible to obtain sufficient human pancreatic tissue for both molecular biological and immunohistochemical studies. This has allowed identification and localisation of some of the key membrane components involved in human duct cell secretion and its regulation. For example, using these approaches, we have determined the presence and distribution of aquaporin water channels [5] and the extracellular $Ca^{2+}$-sensing receptor [6] in human pancreatic ducts. Unfortunately, it is virtually impossible to obtain sufficient human pancreatic tissue on a regular basis to permit functional studies. However, cell lines derived from human pancreatic duct cells should permit such studies. Of these, the well-differentiated Capan-1 cell line, derived from a human pancreatic adenocarcinoma of ductal origin [7, 8], seems potentially to be a good model for studying ductal transport [9-11].

Accordingly we have devised methods for subjecting polarized monolayers of Capan-1 cells to the same measurements and experimental manoeuvres that we have previously performed on isolated guinea-pig pancreatic ducts [12, 13]. Our aim was to determine whether the transport events that we have characterized in the guinea pig are duplicated in humans. For the most part the answer is yes: the transport and regulatory mechanisms responsible for pancreatic $HCO_3^-$ secretion in guinea pigs are also responsible for $HCO_3^-$ secretion by Capan-1 cells. By extrapolation, we propose that these are also likely to be the dominant mechanisms in the ducts of the normal human pancreas.

## Materials and Methods

*Solutions*

The $HCO_3^-$-free, Hepes-buffered solution used in these experiments contained (in mM) 130 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose and 10 Hepes, and was equilibrated with 100% $O_2$. The $HCO_3^-$-buffered solution contained (in mM) 115 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose and 25 $NaHCO_3$, and was equilibrated with 5% $CO_2$/95% $O_2$. The $Na^+$-free solutions contained N-methyl-D-glucamine ($NMDG^+$) in place of $Na^+$. In solutions containing 20 mM $NH_4^+$, the concentration of $Na^+$ was reduced by the same amount to maintain the osmolarity. All solutions were adjusted to pH 7.4 at 37°C. Unless otherwise indicated, all reagents were from Sigma.

*Cell culture*

Capan-1 cells were obtained at passage 18 from the American Type Culture Collection (HTB-79, ATCC, Manassas, VA) and used for experiments after a further 3-15 passages. Cells were grown in Iscove's Modified Dulbecco's Medium with glutamine and HEPES (IMDM, Gibco) supplemented with 10% fetal bovine serum (Gibco), 50 U/ml penicillin and 50 µg/ml streptomycin. For the microfluorometry experiments, 5 x $10^5$ cells were seeded onto collagen-coated PTFE culture inserts (12 mm diameter, 0.4 µm pore, Transwell-COL, Corning, NY). Experiments were performed after 3-5 days when the transepithelial electrical resistance of the monolayer had increased to at least 50 Ω $cm^2$ (after subtraction of the filter resistance) as judged by measurements with an EVOM epithelial volt-ohm-meter (World Precision Instruments, Hamden, CT).

*Reverse transcriptase-polymerase chain reaction (RT-PCR)*

Total RNA was isolated from Capan-1 cells using the TRI-reagent (Sigma). Total RNA from human pancreas, brain and kidney (Stratagene, La Jolla, CA) was used for positive controls. 5 µg of total RNA from each sample was reverse transcribed using SuperScript II (Life Technologies, San Diego) with an oligo(dT) primer. First-strand cDNA was amplified with PCR primers designed, using Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA), to be specific for selected transporters and receptors (online supplementary material). PCR was performed using *Taq* polymerase (Promega, Madison, WI). Following an initial denaturation at 95°C for 2 minutes and 35 cycles of amplification, samples were incubated at 72°C for a further 5 minutes. The PCR products were resolved on agarose gels. As an internal concentration reference for the PCR experiments, we performed 19 cycles of amplification with primers for the acidic ribosomal phosphoprotein (XS13) [14]. The identities of the PCR products were confirmed by sequencing using an ABI Prism BigDye® Terminator v3.1Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

*Immunocytochemistry*

After 10-14 days of culture on Transwell filters, the cell monolayers were fixed for 10 min at room temperature (22°C) with 4% (w/v) paraformaldehyde. After fixation, they were washed three times in Tris-buffered saline (TBS), permeabilised with 0.5% Triton-X-100 in TBS for 7 min and washed again in TBS. The monolayers were blocked in 2% normal goat serum and 2% BSA for 1 h at room temperature and then incubated overnight with the primary antibody at 4°C. The mouse monoclonal anti-occludin (Zymed Laboratories, San Francisco, CA) was used at 1:50 dilution and detected with an anti-mouse

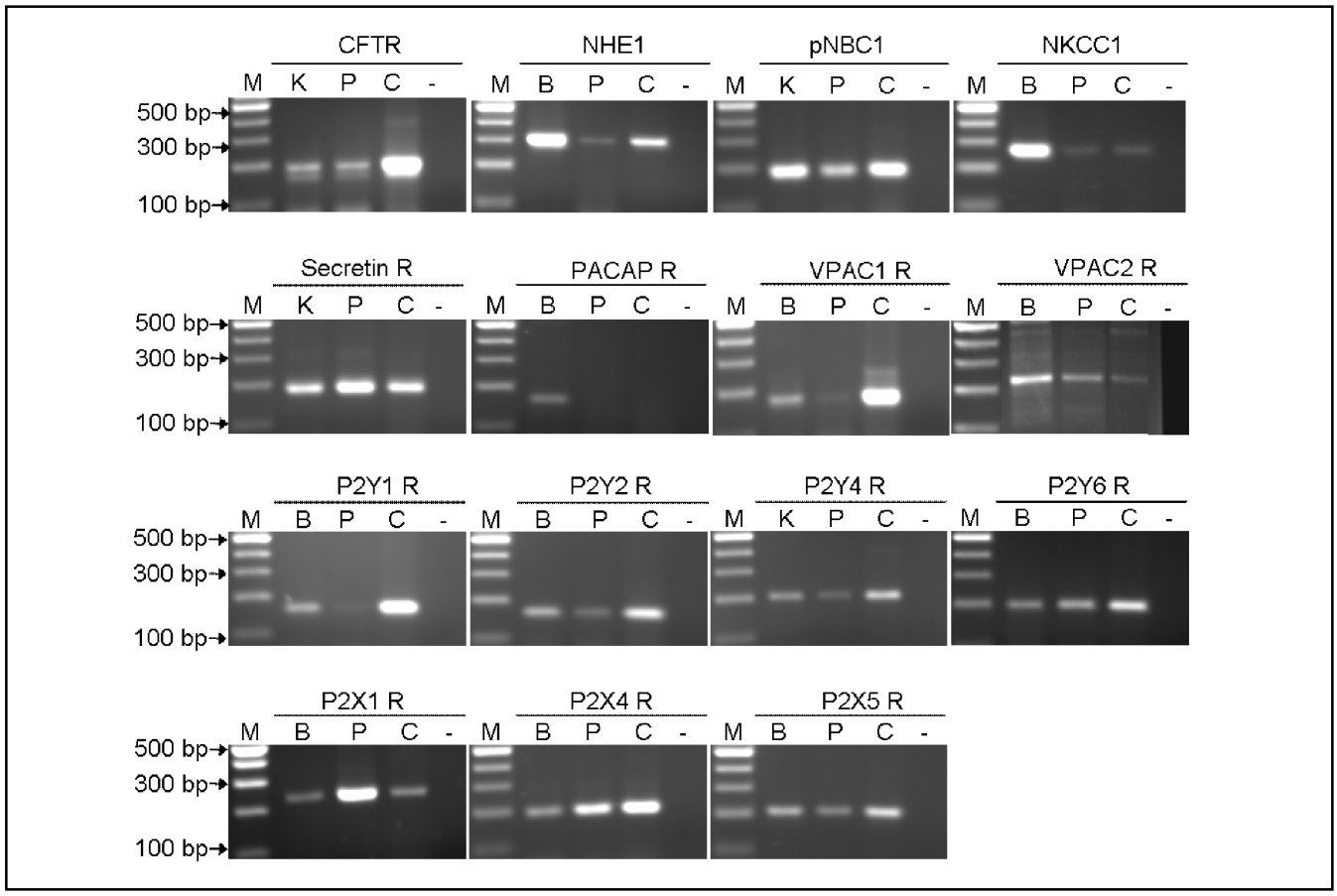


**Fig. 1.** Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of transporter and receptor expression in Capan-1 cells. Agarose gels stained with ethidium bromide show PCR products obtained with RNA extracted from Capan-1 cells (C), normal human pancreas (P) and human control tissues (K, kidney; B, brain) using specific primers for selected electrolyte transporters (CFTR, NHE1, pNBC1 and NKCC1), peptide receptors (secretin, PACAP, $VPAC_1$ and $VPAC_2$) and purinoceptors ($P2Y_{1,2,4,6}$ and $P2X_{1,4,5}$). M, markers; -, RT negative control.

secondary antibody conjugated to FITC (Stratech Laboratories, Cambridge, UK). The filters were washed in TBS and mounted in Vectashield mounting medium containing propidium iodide as a nuclear counterstain (Vector Laboratories, Ltd., Peterborough, England). Images were captured by confocal laser scanning microscopy (Biorad MRC 1024MP mounted on a Nikon Eclipse TE300 microscope). A gallery of 40-50 optical sections (0.5 µm thick) through the *z*-plane was obtained and the composite images were processed using Confocal Assistant software (Bio-Rad, Hercules, CA).

*Measurement of intracellular pH*

Intracellular pH ($pH_i$) was measured by standard microfluorometric techniques in Capan-1 monolayers superfused with separate apical and basolateral solutions. For these experiments, Transwell inserts were mounted in a purpose-built, temperature-controlled chamber mounted on the stage of a Nikon Eclipse TE200 inverted microscope. The cells were loaded with the pH-sensitive fluoroprobe BCECF (2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein) by incubation in Hepes-buffered solution containing the acetoxymethyl ester BCECF-AM (2 µM, Molecular Probes, Leiden) for 20-30 min at room temperature. The monolayers were superfused on both sides at 2 ml/min with either Hepes- or $HCO_3^-$-buffered solution at 37°C. A small region of the epithelium was illuminated alternately at 440 and 490 nm, and the fluorescence intensities ($F_{440}$ and $F_{490}$ respectively) were measured at 530 nm. Intracellular pH was calculated from the $F_{440}/F_{490}$ ratio using calibration data obtained *in situ* by the nigericin-$K^+$ method [15].

*Statistics*

Data are presented as the means ± S.E.M. For statistical comparisons, analysis of variance was followed by a Bonferroni post-hoc test (Instat, GraphPad Software). Rates of change of $pH_i$ were determined by linear regression, and dose-response data were fitted with sigmoid curves in order to estimate the $EC_{50}$ (Prism, GraphPad Software).

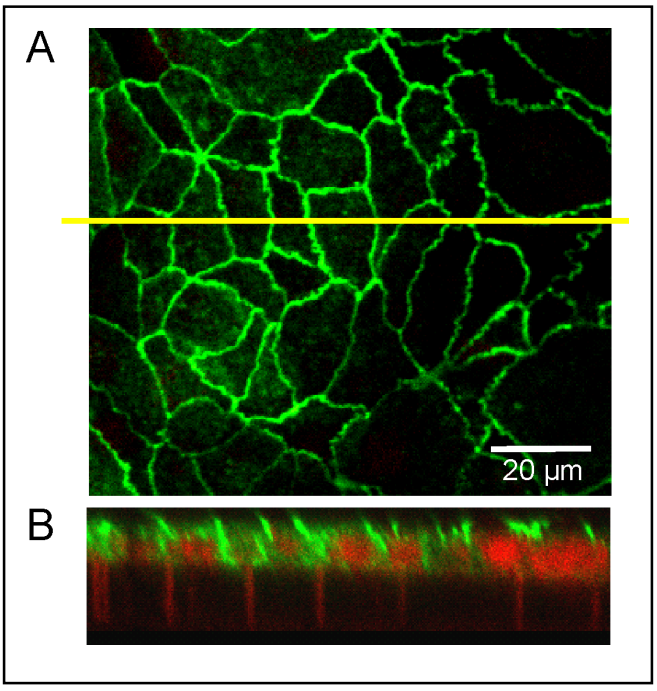


**Fig. 2.** Localisation of the tight-junction protein occludin in Capan-1 cell monolayers by confocal immunofluorescence microscopy. Capan-1 cells were grown to confluence on permeable supports then fixed and labelled with a monoclonal antibody for occludin (green) and counterstained with propidium iodide (red nuclei). A. *xy* section at the level of the tight junctions showing the characteristic chicken-wire pattern of occludin labelling. B. *xz* profile obtained in the plane indicated by the yellow line in panel A.

## Results

*RT-PCR analysis of transporter and receptor expression*

The expression of mRNA for key electrolyte transporters was evaluated by PCR using cDNAs obtained from normal human pancreas and Capan-1 cells. As illustrated in Fig. 1, transcripts of the expected size were detected for CFTR (cystic fibrosis transmembrane conductance regulator), NHE1 (but not NHE3), pNBC1 and NKCC1 (but not NKCC2) in both normal pancreas and in the cell line. Transcripts were also detected for secretin, VIP/PACAP ($VPAC_1$ and $VPAC_2$) receptors, but not for PACAP (pituitary adenylate cyclase-activating polypeptide) receptors. Of the purinoceptors examined, positive results were obtained for $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$, and for $P2X_1$, $P2X_4$ and $P2X_5$, but not $P2X_2$, $P2X_6$ or $P2X_7$. Identities of all PCR products were confirmed by sequencing and/or restriction enzyme digest.

In general the expression patterns were very similar in Capan-1 cells and normal human pancreas. However, it should be remembered that around 90% of the mRNA from whole pancreas derives from cell types other than duct cells. This could explain why some of the signals (e.g. $VPAC_1$ and $P2Y_1$ receptors) were weaker in whole pancreas than in the Capan-1 cells.

*Polarization of Capan-1 monolayers grown on permeable supports*

To confirm that the Capan-1 cells form polarized monolayers when grown on permeable supports, we examined the morphological distribution of the tight-junction protein occludin by confocal immunofluorescence microscopy. Occludin labelling (Fig. 2) was localised to the plasma membrane and showed a characteristic 'chicken wire' pattern at the apical border of the cells, consistent with its expression at the tight junctions. Measurements of transepithelial electrical resistance during the culture period showed a steady increase to a plateau value of between 50 and 150 Ω $cm^2$ after 8-12 days, consistent with the relatively leaky phenotype previously described [9].

*Evidence for basolateral $Na^+/H^+$ exchange*

In order to identify and localize the transporters responsible for $H^+$ extrusion in Capan-1 cells in the absence of $HCO_3^-/CO_2$, monolayers bathed with the Hepes-buffered solution were subjected bilaterally to 20 mM $NH_4^+$ for a period of 3 min followed immediately by bilateral substitution of $Na^+$ with $NMDG^+$ (Fig. 3A). This protocol had the effect of acid loading the cells and it was clear that there was no subsequent recovery of $pH_i$ in the absence of extracellular $Na^+$. Thus there was no evidence for any $Na^+$-independent mechanism, such as an $H^+$-ATPase, under the conditions of these experiments. Furthermore there was no recovery of $pH_i$ when $Na^+$ was restored to the apical solution, thus indicating the absence of a $Na^+$-dependent $H^+$ extruder at the apical membrane.

When $Na^+$ was restored to the basolateral bathing fluid, however, there was a swift recovery of $pH_i$, consistent with the presence of a $Na^+$-dependent $H^+$ extruder at the basolateral membrane. Since the recovery of $pH_i$ was completely abolished by 3 µM EIPA (5-(*N*-ethyl-*N*-isopropyl)-amiloride) (Fig. 3B), the basolateral extrusion of $H^+$ is most likely mediated by an $Na^+/H^+$ exchanger, probably the ubiquitous NHE1.

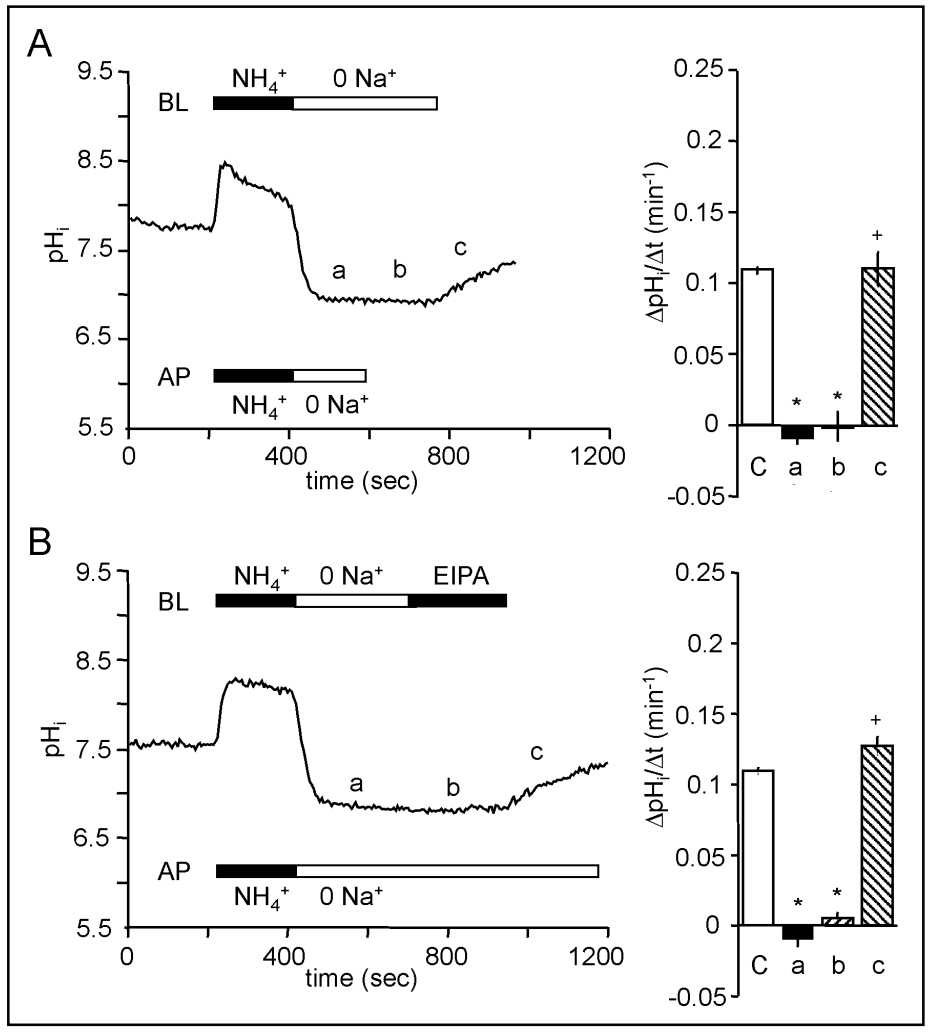


**Fig. 3.** Recovery of $pH_i$ from an acid load in the absence of $HCO_3^-/CO_2$. Unstimulated Capan-1 cell monolayers were exposed bilaterally to 20 mM $NH_4^+$ for 3 min followed by bilateral substitution of $Na^+$ with $NMDG^+$. A. Effects of restoring $Na^+$ first to the apical solution (AP, lower bar) and then to the basolateral solution (BL, upper bar). Mean rates of recovery of $pH_i$ at the time points indicated are shown on the right (n = 4); C represents the recovery rate in control experiments where $Na^+$ was present after the $NH_4^+$ pulse (n = 4). B. Effect of restoring basolateral $Na^+$ in the presence of EIPA (3 μM) to inhibit $Na^+/H^+$ exchange (n = 5). * $P < 0.001$ compared with C; + $P < 0.001$ compared with b.

*Evidence for basolateral $Na^+$-$HCO_3^-$ cotransport*

When similar experiments were performed in the presence of $HCO_3^-/CO_2$, a very slow but significant recovery of $pH_i$ was observed after the $NH_4^+$ pulse despite the absence of extracellular $Na^+$ (Fig. 4A). This slow increase in $pH_i$ was not inhibited by 1 μM bafilomycin $A_1$ (Fig. 4B), suggesting that it was probably not due to $H^+$ extrusion by a vesicular-type $H^+$-ATPase (V-ATPase). The identity of the transporter responsible for this phenomenon remains to be determined.

Restoring $Na^+$ to the apical bathing solution had no significant effect on the recovery of $pH_i$ (Fig. 4A). From this we can conclude that there are no $Na^+$-dependent $H^+$ extruders or $HCO_3^-$ loaders at the apical membrane. In contrast, restoring $Na^+$ to the basolateral bathing solution led to a rapid recovery of $pH_i$ (Fig. 4A). This was abolished by the combined basolateral application of 3 μM EIPA and 500 μM $H_2$DIDS (dihydro-4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid) (Fig. 4C). However, after withdrawal of the $H_2$DIDS, but in the continued presence of EIPA, there was a marked increase in $pH_i$. This shows that, in addition to the NHE activity already demonstrated, there is an $H_2$DIDS-sensitive, $Na^+$-dependent $HCO_3^-$ uptake mechanism at the basolateral membrane, most probably the $Na^+$-$HCO_3^-$ cotransporter NBC1 that was detected by RT-PCR (see above).

*Evidence for basolateral $Na^+$-$K^+$-$2Cl^-$ cotransport*

One factor that may contribute to the partial recovery of $pH_i$ from the alkalinization that occurs during the $NH_4^+$ pulse is the acidifying effect of $NH_4^+$ uptake. This could be mediated by an $Na^+$-$K^+$-$2Cl^-$ cotransporter (NKCC1) which will transport $NH_4^+$ in place of $K^+$ [16]. Fig. 5 shows that basolateral application of 500 μM bumetanide, an NKCC1 inhibitor [17], slowed the re-acidification that occurred during the $NH_4^+$ pulse by about 60%. This suggests that there may be some NKCC1 activity in these cells, which would be consistent with the RT-PCR data described above.

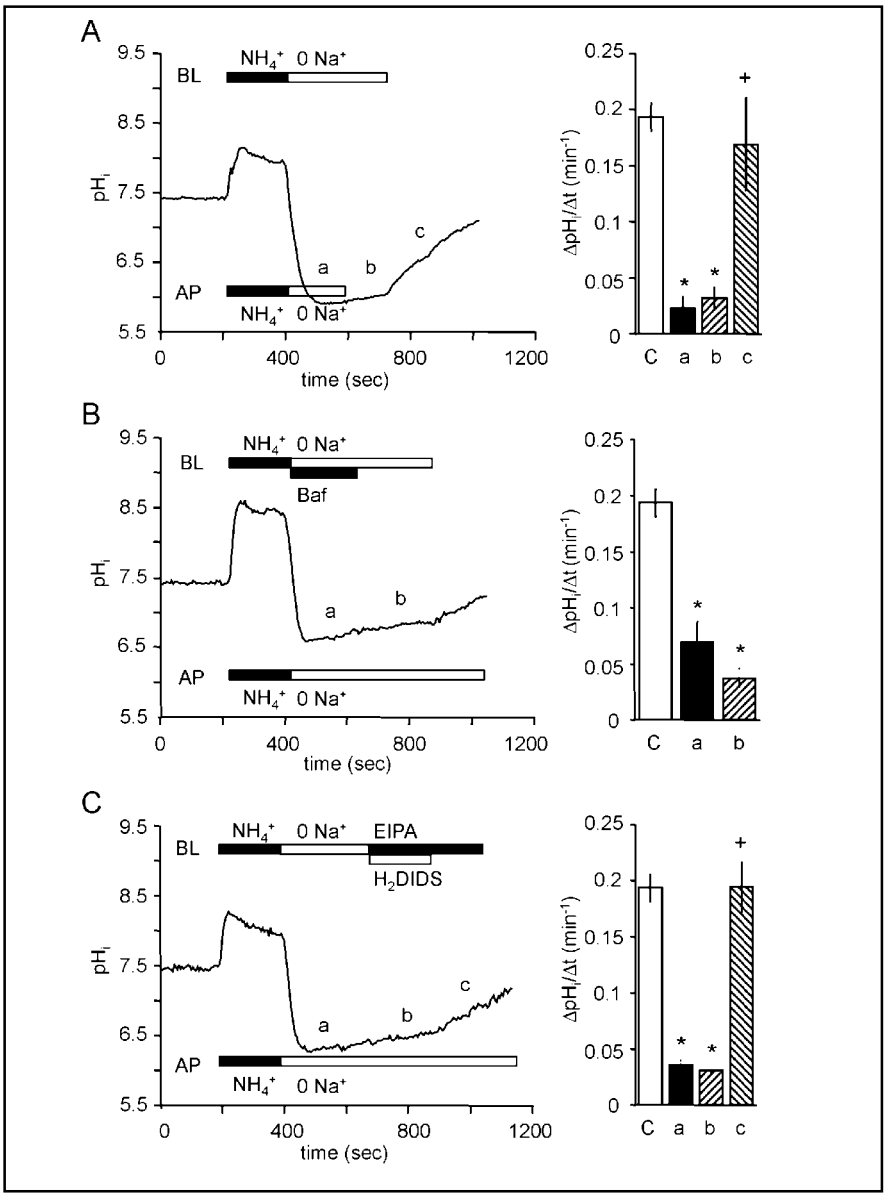


**Fig. 4.** Recovery of $pH_i$ from an acid load in the presence of $HCO_3^-/CO_2$. Unstimulated Capan-1 cell monolayers were exposed bilaterally to 20 mM $NH_4^+$ for 3 min followed by bilateral substitution of $Na^+$ with $NMDG^+$. A. Effects of restoring $Na^+$ first to the apical solution (AP) and then to the basolateral solution (BL). Mean rates of recovery of $pH_i$ at the time points indicated are shown on the right (n = 4); C represents the recovery rate in control experiments where $Na^+$ was present after the $NH_4^+$ pulse (n = 5). B. Effect of the V-ATPase inhibitor bafilomycin $A_1$ (Baf, 1 µM) on the recovery of $pH_i$ in the absence of $Na^+$ (n = 3). C. Effect of restoring basolateral $Na^+$ in the presence of EIPA (3 µM) and $H_2DIDS$ (500 µM) to inhibit $Na^+/H^+$ exchange and $Na^+$-$HCO_3^-$ cotransport respectively (n = 4). * $P < 0.001$ compared with C; + $P < 0.001$ compared with b.

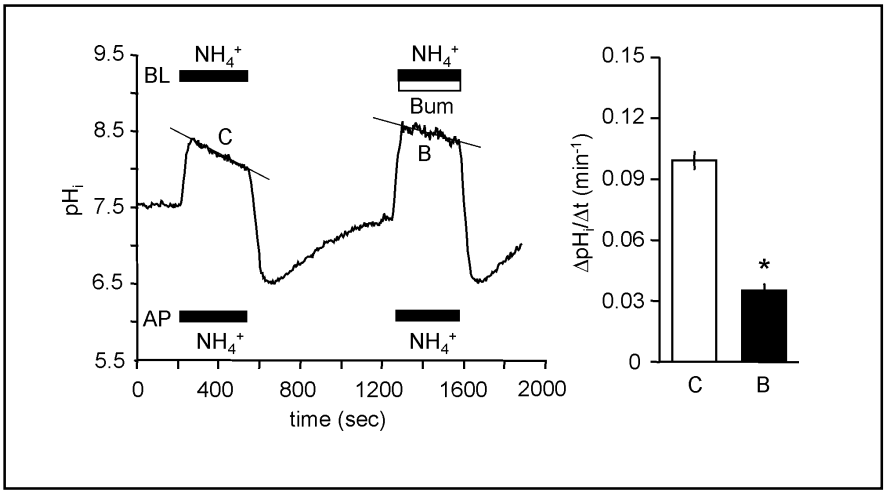


**Fig. 5.** Evidence for basolateral $NH_4^+$ uptake via NKCC1. Unstimulated Capan-1 cell monolayers, superfused with the standard $HCO_3^-$-buffered solution, were exposed bilaterally to 20 mM $NH_4^+$ for 3 min and then the treatment was repeated with simultaneous basolateral application of bumetanide (Bum, 500 µM) to block $NH_4^+$ uptake via NKCC1. Mean rates of recovery of $pH_i$ in the controls (C) and in the presence of bumetanide

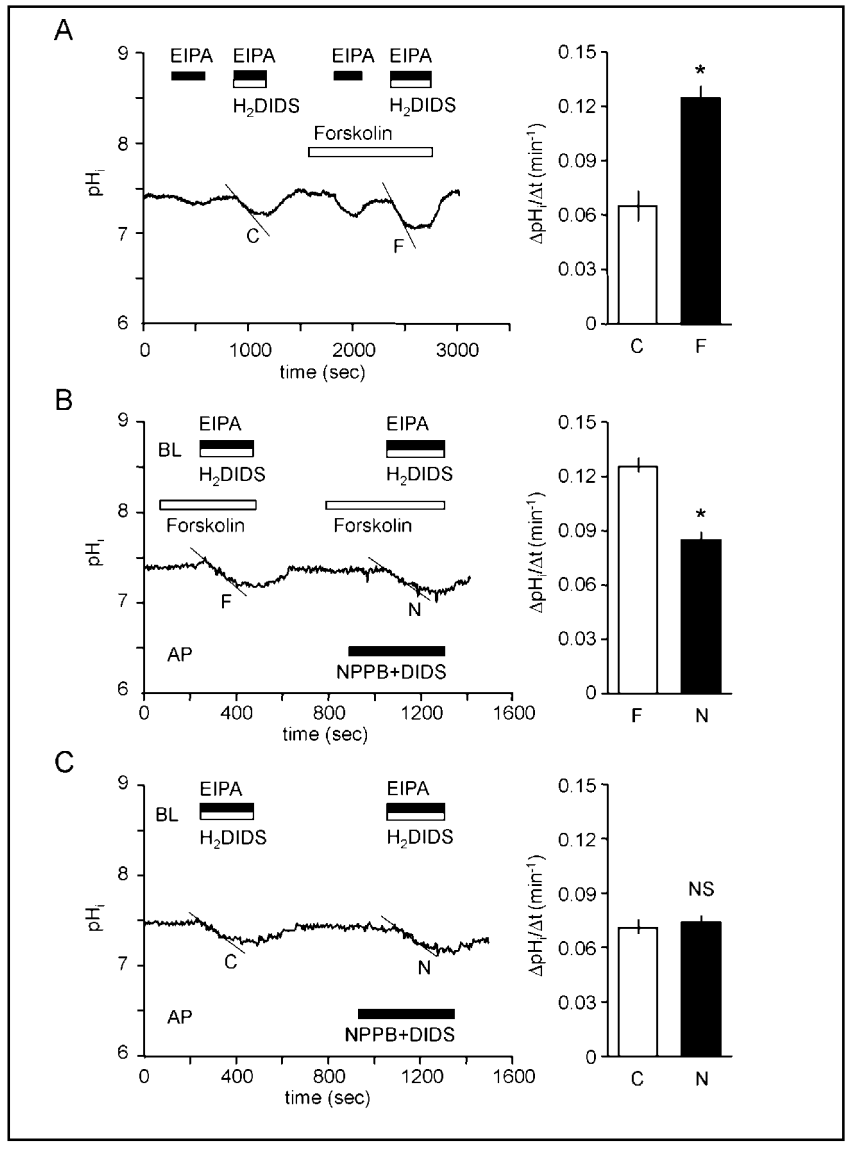


**Fig. 6.** Measurements of $HCO_3^-$ secretion in Capan-1 cell monolayers by blockade of basolateral $HCO_3^-$ uptake. A. Changes in $pH_i$ induced by EIPA (3 μM) alone and in combination with $H_2$DIDS (500 μM) before and during stimulation with forskolin (10 μM). Mean rates of decrease in $pH_i$ induced by combined application of EIPA and $H_2$DIDS in unstimulated controls (C) and in forskolin-stimulated cells (F) are shown on the right (n = 5, * P < 0.001 compared with C). B. Changes in $pH_i$ induced by EIPA and $H_2$DIDS during stimulation with forskolin (10 μM) in the absence (F) and presence (N) of apical NPPB and DIDS (both 100 μM). Mean acidification rates are shown on the right (n = 4, * P < 0.001 compared with F). C. Changes in $pH_i$ induced by EIPA and $H_2$DIDS in unstimulated Capan-1 cells in the absence (C) and presence (N) of apical NPPB and DIDS. Mean acidification rates are shown on the right (n = 4, NS, not significant compared with C).

*Measurement of transepithelial $HCO_3^-$ secretion*

Intracellular pH is maintained during $HCO_3^-$ secretion by a balance between the alkalinizing effect of basolateral $HCO_3^-$ uptake (and $H^+$ extrusion) and the acidifying effect of $HCO_3^-$ efflux across the apical membrane. It is therefore possible to quantify the instantaneous $HCO_3^-$ efflux across the apical membrane by recording the change in $pH_i$ when the basolateral transporters are suddenly inhibited by the combined application of 3 μM EIPA and 500 μM $H_2$DIDS. Previous studies have shown that, when basolateral $HCO_3^-$ uptake is blocked in this way, the continuing efflux of $HCO_3^-$ across the apical membrane leads to a fall in $pH_i$ [12]. The initial rate of decrease in $pH_i$ is therefore believed to be a good indirect measure of apical $HCO_3^-$ secretion [13, 18].

We have used this measurement to investigate the physiological regulation of vectorial $HCO_3^-$ secretion in Capan-1 cell monolayers. The experiment shown in Fig. 6A shows the effects of basolateral application of 3 μM EIPA and 500 μM $H_2$DIDS, before and after the application of 10 μM forskolin to stimulate secretion by elevating intracellular cAMP. Application of forskolin did not in itself cause any change in $pH_i$, but when EIPA and $H_2$DIDS were subsequently applied, the rate of decrease in $pH_i$ was approximately doubled when compared with the corresponding values in unstimulated cells. Thus, forskolin clearly accelerates the efflux of $HCO_3^-$ from Capan-1 cells.

To establish whether the increase in $HCO_3^-$ efflux represents apical $HCO_3^-$ secretion, we examined the effect of blocking the $HCO_3^-$ efflux pathways believed to be activated by cAMP at the apical membrane of these cells, namely the CFTR $Cl^-$ channel and a $Cl^-/HCO_3^-$ exchanger. Application of 100 μM NPPB and 100 μM DIDS

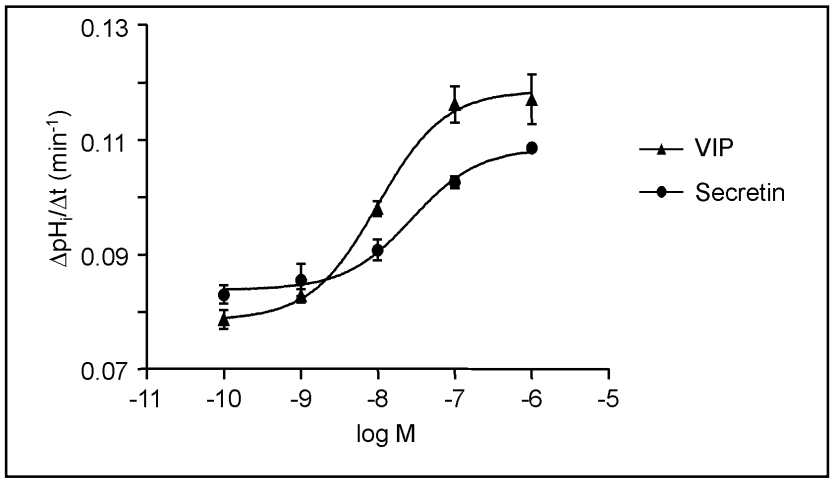


**Fig. 7.** Concentration-response curves for secretin- and VIP-stimulated $HCO_3^-$ secretion. Data are mean values of the initial rates of change of $pH_i$ (secretin, n = 4-5; VIP, n = 4-5) induced by combined application of EIPA and $H_2$DIDS. $EC_{50}$ values were obtained from the fitted sigmoid curves.

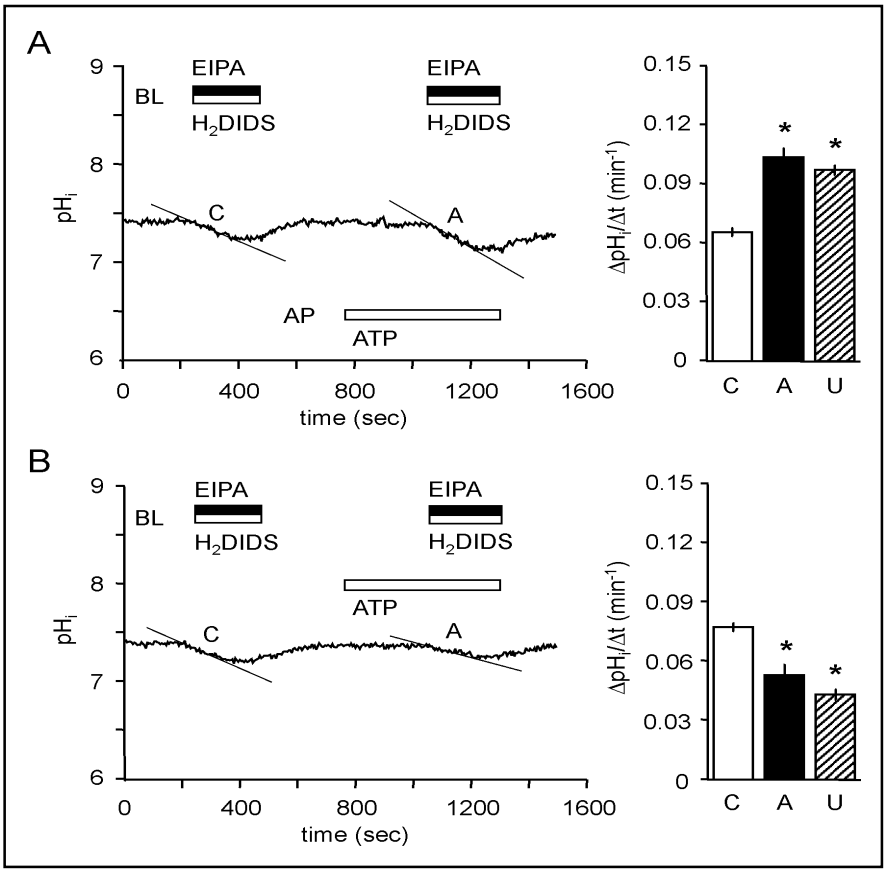


**Fig. 8.** Effects of luminal and basolateral ATP and UTP on $HCO_3^-$ secretion by Capan-1 cell monolayers. A. Changes in $pH_i$ induced by EIPA (3 μM) and $H_2$DIDS (500 μM) before and during apical stimulation with ATP (1 μM). Mean acidification rates in unstimulated controls (C) and in cells stimulated with ATP (A, n = 4) and with 1 μM UTP (U, n = 4) are shown on the right. B. Changes in $pH_i$ induced by EIPA and $H_2$DIDS before and during basolateral administration of ATP (1 μM). Mean acidification rates are shown on the right (A, n = 5; U, n = 5). * $P < 0.05$ compared with C.

to the apical membrane markedly slowed the fall in $pH_i$ in response to the basolateral inhibitors during stimulation with forskolin (Fig. 6B). The acidification rate under these conditions was very similar to that in the unstimulated cells. Furthermore, the acidification rate in the unstimulated cells was not significantly affected by apical NPPB and DIDS (Fig. 6C). If we assume that NPPB and DIDS are reasonably effective in blocking the apical CFTR and anion exchanger respectively [13], these results confirm that forskolin stimulates $HCO_3^-$ secretion across the apical membrane.

*Effects of secretin and vasoactive intestinal peptide (VIP) on transepithelial $HCO_3^-$ secretion*

As observed with forskolin, the application of secretin did not in itself change $pH_i$. However, the intracellular acidification resulting from subsequent application of EIPA and $H_2$DIDS was accelerated by secretin in a

dose-dependent manner. Using the initial rate of change of $pH_i$ as an index of $HCO_3^-$ secretion, Fig. 7 shows the dose-response relationships for both secretin and for VIP, which is known to stimulate $HCO_3^-$ secretion in guinea-pig pancreatic ducts. The two peptides had similar potencies, both inducing 50% of maximal stimulation in the 10 nM range. Secretin was approximately three times less potent than VIP with the $logEC_{50}$ values estimated to be 7.56 ± 0.20 and 8.00 ± 0.15 respectively.

*Effects of ATP and UTP on transepithelial $HCO_3^-$ secretion*

Since ATP is present in human pancreatic juice [19] and luminal nucleotides are known to evoke bicarbonate secretion in guinea-pig pancreatic ducts [20], we examined whether ATP and UTP have a similar effect on Capan-1 cells. Apical application of either 1 μM ATP or UTP had no direct effect on the $pH_i$ but the acidification evoked by the subsequent application of EIPA and $H_2$DIDS was significantly accelerated when compared with the unstimulated controls (Fig. 8A). Applied to the basolateral side, however, ATP and UTP both reduced the rate of intracellular acidification evoked by EIPA and $H_2$DIDS (Fig. 8B). These results suggest that, as in the guinea-pig, apical application of ATP and UTP stimulates $HCO_3^-$ secretion by Capan-1 cells, while basolateral application inhibits $HCO_3^-$ secretion.

## Discussion

Pancreatic juice $HCO_3^-$ concentrations approach 140 mM in both humans [21] and guinea-pigs [22]. During the past ten years, the transport events responsible for $HCO_3^-$ secretion have been analysed in detail in ducts isolated from guinea-pig pancreas such that we now have a fairly clear idea of the mechanisms involved [1, 3]. The question we now seek to answer is whether the same mechanisms are responsible for $HCO_3^-$ secretion by human pancreatic ducts.

A number of stable cell lines derived from human pancreatic adenocarcinomas, including Capan-1, CFPAC-1, Panc-1 and HPAF, have been used as experimental models for human ductal secretion [1]. However, only Capan-1 cells and Panc-1 cells express functional CFTR [23, 24] and, of these, only Capan-1 cells form confluent, polarized monolayers on permeable supports [9, 24, 25]. Although CFPAC-1 cells transfected with wild-type CFTR (CFPAC-WT) have been used successfully [10], the Capan-1 cell line has the advantage that it expresses receptors for a range of physiological stimuli, including secretin [9, 25], VIP [26, 27], ATP [28], angiotensin II [29] and extracellular $Ca^{2+}$ [6]. We have therefore begun a systematic study of the mechanisms and regulation of $HCO_3^-$ transport in these cells as a candidate model for the human pancreatic duct epithelium.

Previous studies using Capan-1 cells grown on permeable filters have reported widely divergent values for the transepithelial electrical resistance. Rotoli *et al.* [25] obtained baseline values in excess of 3000 Ω $cm^2$ while others [9, 24] have described a much 'leakier' phenotype with values of around 100-300 Ω $cm^2$. The reason for this discrepancy is not clear. However, our data are more in line with the latter range and are also consistent with previous evidence that native pancreatic ducts have a substantial paracellular permeability [30]. Despite the low resistance of the Capan-1 monolayers, immunolabelling with an occludin antibody shaw that the cells form polarized monolayers with well developed tight junctions.

Functional polarization of the Capan-1 cells was confirmed by experimental manipulation of $pH_i$. In the absence of $HCO_3^-/CO_2$, the recovery of $pH_i$ following an acid load was dependent on basolateral, but not apical, $Na^+$ and was completely blocked by basolateral application of EIPA. The presence of a basolateral $Na^+/H^+$ exchanger, usually NHE1, is an almost universal feature of mammalian epithelia. The absence of apical NHE activity, which has been reported in the larger ducts of some species [31], suggests that the Capan-1 cells do not reabsorb $HCO_3^-$ but rather have a predominantly secretory phenotype more typical of smaller ducts.

The presence of a basolateral $Na^+$-$HCO_3^-$ cotransporter was revealed in acid loading experiments performed in the presence of $HCO_3^-/CO_2$. This is consistent both with our RT-PCR evidence for pNBC1 expression and with a previous report of $Na^+$-$HCO_3^-$ cotransporter activity in Capan-1 cells [10]. However, this is the first demonstration of its basolateral localization in these cells - a finding that is consistent with previous observations in rat [32] and guinea-pig [33] ducts and in CFPAC-WT cells [10]. The absence of an apical $Na^+$-$HCO_3^-$ cotransporter in the Capan-1 cells, such as the electroneutral isoform thought to be present in the main ducts of mouse pancreas [31], again argues against these cells having any significant capacity for $HCO_3^-$ reabsorption.

The $pH_i$ changes that occured during $NH_4^+$ pulse experiments also revealed the likely presence of an $Na^+$-$K^+$-$2Cl^-$ cotransporter. Although difficult to quantify, this

would be consistent with the previous observation that a bumetanide-sensitive mechanism is responsible for much of the secretin-evoked short-circuit current in Capan-1 cells [9]. The presence of NKCC1 in these cells is somewhat surprising given that $Na^+$-coupled $Cl^-$ uptake at the basolateral membrane would provide a driving force for apical secretion of $Cl^-$ and this is thought to be incompatible with the secretion of a $HCO_3^-$-rich juice [34]. Its presence in Capan-1 cells may, however, reflect the origin of these cells in the larger ducts of the human pancreas [35], where the expression levels of the basolateral transporters may differ from those in the smaller intercalated and intralobular ducts that are thought to secrete most of the $HCO_3^-$-rich fluid [5, 36].

As far as $HCO_3^-$ secretion is concerned, we have shown that in Capan-1 cells the basolateral NHE and NBC are the main transporters involved in the accumulation of intracellular $HCO_3^-$ for subsequent secretion across the apical membrane. However, our results appear to contradict the findings of Cheng *et al.* [9] who reported a negligible role for the NBC in $HCO_3^-$ accumulation in Capan-1 cells. This was based on the observations that $H_2$DIDS was ineffective in blocking the forskolin-stimulated short-circuit current. However, in that study $H_2$DIDS was applied at a concentration of 150 μM which in our hands has relatively little effect on NBC activity (results not shown).

Although the stimulatory effects of other agents know to raise intracellular cAMP in pancreatic duct cells, such as secretin, VIP and PACAP, are well known [37-39], the receptors responsible for these effects have not been firmly identified. Previous work has suggested that both secretin and VIP receptors [40, 41] may be expressed in Capan-1 cells. Consistent with this we have found evidence for the expression of secretin, $VPAC_1$ and $VPAC_2$ receptors by RT-PCR, and our functional studies also indicate that both secretin and VIP receptors are active in these cells. Although the two peptides clearly stimulated $HCO_3^-$ secretion, secretin had a somewhat lower potency and efficacy than VIP. The potency of secretin in the Capan-1 cells was also less than that previously reported for rat and guinea-pig pancreatic acini and ducts [13, 42, 43].

In microperfused guinea-pig ducts, application of ATP and UTP to the luminal and basolateral membranes evoked opposite effects: luminal nucleotides stimulated $HCO_3^-$ secretion while basolateral nucleotides were inhibitory [20]. In the present study we have observed similar effects in Capan-1 cells. Although our RT-PCR analysis showed that Capan-1 cells express mRNAs for P2Y and P2X receptors, the similarity of the responses to ATP and UTP suggests that purinergic activation at both apical and basolateral locations is mediated by P2Y receptors, which are equally responsive to ATP and UTP [44].

Previous studies on Capan-1 cells [11] have shown that ATP stimulates $Cl^-/HCO_3^-$ exchange at the apical membrane by a $Ca^{2+}$-dependent process that is also dependent on the presence of CFTR. Although the mechanism is not fully understood, it could help to explain the stimulatory effect of apical ATP on $HCO_3^-$ secretion that we have observed here. Furthermore, our finding that human pancreatic juice contains significant amounts of ATP [19] supports the notion that luminal ATP may be an important paracrine signal in the pancreatic ductal system [45].

The inhibitory effect of ATP applied to the basolateral membrane is more difficult to explain, since it too raises intracellular $Ca^{2+}$ in Capan-1 cells [11] as it does in guinea-pig pancreatic ducts [20]. One possible clue is the observation that, in rat pancreatic duct cells, stimulation of $P2Y_2$ and/or $P2Y_4$ receptors raises intracellular $Ca^{2+}$ but at the same time reduces the whole-cell $K^+$ conductance, which would depolarize the cells and thereby inhibit $HCO_3^-$ secretion [46]. Furthermore, coexpression studies in *Xenopus* oocytes suggest that $P2Y_2$ and $P2Y_4$ receptors can have opposite effects on the different $Ca^{2+}$-activated $K^+$ channels that are found in rat ducts [47].

In summary, our data show that Capan-1 cells grown as monolayers on permeable filters achieve vectorial $HCO_3^-$ transport in a regulated manner. At the basolateral membrane, an $Na^+/H^+$ exchanger (probably NHE1) and an $Na^+$-$HCO_3^-$ cotransporter (probably pNBC1) both contribute to the cellular accumulation of $HCO_3^-$. There is also an $Na^+$-$K^+$-$2Cl^-$ cotransporter (probably NKCC1) which may generate a driving force for $Cl^-$ secretion. Secretin, VIP and forskolin all stimulate $HCO_3^-$ secretion across the apical membrane. We have also shown that both apical and basolateral purinoceptors regulate $HCO_3^-$ secretion, but with opposite effects. With the possible exception of the presence of NKCC1, all of these features are also characteristic of guinea-pig ducts. This suggests that the mechanisms and regulation of $HCO_3^-$ secretion in guinea-pig ducts and Capan-1 monolayers are similar and that the latter are a good model for studies of human pancreatic $HCO_3^-$ secretion.

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