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Background

Pancreatic duct cells are responsible for **secreting** an alkaline, **HCO_3^- rich isotonic fluid**, which serves two important functions: to flush digestive enzymes down the ductal tree and to help neutralize gastric acid (Steward et al. 2005; Argent et al. 2006). The initial step of HCO_3^- secretion is the accumulation of HCO_3^- within the duct cell. This can occur by two mechanisms: (1) the forward transport of HCO_3^- by the Na^+/HCO_3^- co-transporter (NBC), and (2) diffusion of CO_2 into the duct cell which is then hydrated to carbonic acid by carbonic anhydrase, followed by the backward transport of protons via the Na^+/H^+ exchangers (NHEs) and H^+-pumps. The HCO_3^- ions are then secreted across the apical membrane via Cl^-/HCO_3^- exchangers (**SLC26A3, DRA** and **SLC26A6, PAT1**) and/or **cystic fibrosis transmembrane conductance regulator (CFTR)** Cl^- channels, which exhibit a finite permeability to HCO_3^-. The exact mechanism how the SLC26 exchangers and apical Cl^- channels produce a high HCO_3^- secretion is controversial. Nevertheless, the key role of CFTR in HCO_3^- secretion is emphasized by the fact that the severity of the pancreatic phenotype in cystic fibrosis (CF) correlates best with the ability of mutant CFTRs to activate SLC26 exchangers, rather than their ability to conduct Cl^- ions.

Our current understanding of pancreatic ductal HCO_3^- secretion is mainly based on experiments performed on animal tissues. Studies on human tissue have been largely confined to adenocarcinoma duct cell lines; however, with the exception of CAPAN-1, human duct cell lines do not express CFTR to any significant degree. An alternative approach is to use CFPAC-1 cells, which were derived from a 26-year-old CF patient with the F508del CFTR mutation (Schoumacher et al., 1990). Anion transport in CFPAC-1 cells can be increased by Ca^{2+} ionophores but not by forskolin, cyclic adenosine monophosphate (cAMP) analogues or phosphodiesterase inhibitors, though cAMP production or protein kinase activity is not decreased in response to forskolin. The utility of **CFPAC-1 cells is that they allow us to examine HCO_3^- transport** not only in the **diseased** state but also in the **corrected state** by transducting the cells with wild-type CFTR. Stable transfection methods have been used successfully in the past to correct the CFTR defect in CFPAC-1 cells (Greeley et al., 2001). Our work was prompted by the fact that DNA-based therapies are currently being explored as treatment strategies for other pancreatic diseases, such as pancreatic cancer (for a review see Tamada et al. 2005). Moreover, pancreatic sufficiency (as judged by the absence of steatorrhoea) can be maintained with only 10 % of normal pancreatic function (DiMagno et al., 1973), so a gene therapy protocol that provide even a small improvement in pancreatic function might be beneficial to CF patients.

Results and discussion

1. Setup of polarised human pancreatic duct cell model system.

During the tenure of my Wellcome Trust Travelling Fellowship (2003-2005) at the University of Newcastle upon Tyne, I have successfully developed an *in vitro*, polarised, **human pancreatic duct cell (CFPAC-1) model system to study HCO_3^- secretion**. In the first year of my OTKA postdoctoral fellowship, I've managed to set up a similar system at the University of Szeged so I could continue my research project on pancreatic duct cells. The advantage of this model is that we could simultaneously perfuse the basolateral and apical membranes of the cells with different solutions (allowing us to differentially investigate the transporters). Furthermore, we have a cystic fibrosis (CF) cell line –in which CFTR and HCO_3^- secretion are defective- but that can be converted to a non-CF phenotype by CFTR gene transfer.

2. Transduction with recombinant Sendai virus.

Confluent CFPAC-1 cells grown on polyester Transwells and were infected from the apical or basolateral side with 6 x 10^5 or 3 x 10^6 plaque forming units (multiplicity of infection, MOI=3 or 15, respectively) of Sendai virus containing the wild-type CFTR (**SeV-CFTR**) or β-galactosidase (**SeV-LacZ**, nuclear localized) gene 3 days after seeding at high density.

To estimate the efficiency of gene transfer, SeV-LacZ was added to either the basolateral or apical membrane of polarized CFPAC-1 monolayers for 1 h. LacZ activity was measured between 48 and 96 h after transduction. Initial experiments showed that very little gene transfer occurred following basolateral exposure to SeV-LacZ. However, following application of SeV-LacZ to the apical membrane at MOI=3, strong, homogenous LacZ activity was observed in 32 ± 2% of cells. At the higher MOI=15, 68 ± 3% of the cells were stained. Thus, **transduction with SeV vector was more efficient via the apical membrane** of CFPAC-1 cells **and** the proportion of cells infected **was clearly dose-related**.

3. CFTR expression in CFPAC-1 pancreatic duct cells.

Untransduced and SeV-LacZ infected CFPAC-1 cells exhibited very little CFTR protein expression as judged by Western blotting. In these cells only immature CFTR (i.e., core-glycosylated or band B) could be detected, as a very faint band. However, both **immature and mature** (i.e., fully-glycosylated, processed or band C) **forms of CFTR were detected in SeV-CFTR** transduced CFPAC-1 cells.

As CFTR overexpression has previously been shown to result in mislocalisation of the protein (Farmen et al., 2005), we also wanted to make sure that CFTR was correctly localized to the apical membrane. Therefore, we also used immunocytochemistry to clearly demonstrate **apical localization of CFTR in SeV-CFTR** (MOI=3) **transduced CFPAC-1** cells. In accordance with our LacZ staining results, at MOI=3 about 30% of the SeV-CFTR CFPAC-1 cells expressed CFTR. However, this relatively low rate of CFTR transduction was sufficient to upregulate apical Cl^-/HCO_3^- exchange activity in the human duct cells (see below).

Functional expression of CFTR was assessed by the iodide efflux assay. Increases in intracellular cAMP had no effect on iodide efflux in SeV-LacZ (MOI=3) or in SeV-CFTR (MOI=15) cells. In contrast, cAMP stimulated iodide efflux in 4 out of 5 experiments (80%) with SeV-CFTR (MOI=3) cells. We also showed that the effect of cAMP on iodide efflux was completely blocked by the CFTR Cl^- channel inhibitor 10 µM $CFTR_{inh}$-172.

Taken together, we've demonstared that the **transduction** of CFPAC-1 cells **with SeV-CFTR resulted in the expression of functional CFTR** Cl^- channels **on the apical membrane**.

4. Effect of SeV vector-mediated transduction on the integrity of CFPAC-1 cell monolayer.

4.1. Structural integrity

CFPAC-1 cells grown on polyester Transwells became confluent 2-3 days after seeding, as judged by visual observation. **Transepithelial resistance** (R_T) can be used as an indicator of the structural integrity of an epithelial sheet, because electrical resistance is largely determined by the 'leakiness' of the tight junctions. The presence of tigth junctions was confirmed in untransduced and SeV-transduced cell lines by expression of the tight junction protein, **ZO-1**. R_T increased steadily over 4-5 days up to a maximum of 199 ± 10 ohm x cm^2 in the untransduced cells. R_T was significantly higher in the SeV-LacZ (MOI= 3: 400 ± 15 ohm x cm^2, MOI= 15: 314 ± 13 ohm x cm^2) and in the SeV-CFTR (MOI= 3: 243 ± 5 ohm x cm^2, MOI= 15: 268 ± 9 ohm x cm^2) infected groups compared to the untransduced

cells (n = 9-69). These data show that transduction with **SeV vector did not disrupt the structural integrity of the CFPAC-1 epithelium**; if anything the epithelium became slightly 'tighter' after exposure to the virus.

4.2. Functional integrity

4.2.1 Resting intracellular pH, buffering capacity

As HCO_3^- is a component of a buffer system, intracellular pH (pH_i) and buffering capacity (β_I) are crucial parameters in a HCO_3^--secreting epithelial cell. The resting pH_i of CFPAC-1 cells bathed in the standard HEPES solution was 7.11 ± 0.08 (n=6) and was not significantly different in SeV-CFTR (MOI=3) transduced cells (7.09 ± 0.10, n=6). However, the resting pH_i value of SeV-LacZ cells (MOI=15) was significantly increased (7.26 ± 0.01) compared to the SeV-CFTR cells (7.20 ± 0.02) in the standard HCO_3^-/CO_2 solution (n=12). We have previously reported that the intrinsic buffering capacity (β_i) is quite variable in different CFPAC-1 monolayers (see Fig. 1 in Rakonczay et al., 2006). In a previous study, we found that uninfected CFPAC-1 cells had a β_i of 34 ± 8 mM/pH over the pH_i range 7.0 - 7.2. The respective β_i values for SeV-CFTR and SeV-LacZ (MOI=15) transduced cells (n = 9-11) at this pH_i range were 46 ± 7 and 46 ± 6 mM/pH. These β_i values are not statistically different. Taken together, our results indicate that **SeV vector infection has no obvious effect on pH_i regulatory mechanisms or β_i** in CFPAC-1 cells.

4.2.2. Differential permeability to HCO_3^-/CO_2

We have previously shown that the apical and basolateral membranes of **CFPAC-1 cell monolayers exhibit marked differences in their relative CO_2 and HCO_3^- permeabilities** (Rakonczay et al., 2006). Exposing the basolateral side to a solution containing HCO_3^-/CO_2 causes pH_i to alkalinize rapidly, suggesting that HCO_3^- permeates the basolateral membrane rather faster than CO_2 (Rakonczay et al., 2006), consistent with presence of base loaders (principally a $NaHCO_3$ cotransporter) on the basolateral membrane of the duct cell. In contrast, exposing the apical membrane to HCO_3^-/CO_2 causes pH_i to acidify rapidly, consistent with faster diffusion of CO_2 from lumen to cell compared with HCO_3^- (Rakonczay et al., 2006). That the apical membrane of pancreatic duct cells resists back diffusion of HCO_3^- from the lumen is likely to be of physiological importance, since it will favour retention of secreted HCO_3^- in the duct lumen. We therefore checked whether the functional polarity of CFPAC-1 cells was disrupted by SeV vector transduction by exposing the apical and basolateral membranes of SeV-LacZ and SeV-CFTR-transduced cells to HCO_3^-/CO_2.

To check the functional polarity of CFPAC-1 cells, initially, the apical and basolateral membranes were perfused with the standard HEPES solution and then the apical solution was switched to HCO_3^-/CO_2. This caused the expected rapid acidification of pH_i, most likely due to CO_2 diffusion into the cells. In 24 similar experiments the ΔpH_i and base efflux ($-J_B$) following apical HCO_3^-/CO_2 addition were -0.20 ± 0.01 and -23.7 ± 0.9 mM B/min respectively. After the rapid acidification, pH_i remained stable at the new level. Finally, switching the basolateral solution to HCO_3^-/CO_2 caused a rapid alkalinization of pH_i, most likely due to rapid HCO_3^- uptake into the cells (Rakonczay et al., 2006). The associated ΔpH_i and base influx (J_B) were 0.37 ± 0.01 and 17.14 ± 0.74 mM B/min respectively.

In SeV-CFTR (MOI=15) transduced cells (n=12), the fall in pH_i following apical HCO_3^-/CO_2 addition was not significantly different from that observed in the SeV-LacZ cells. However, after the rapid acidification, pH_i continued to rise slowly in about 50% of the experiments (by 0.034 ± 0.006 during 4 minutes, n=8) with SeV-CFTR transduced cells. CFTR does exhibit a finite permeability to HCO_3^- (Gray et al., 1990), so this slow alkalinization might reflect back flux of HCO_3^- from the luminal compartment through CFTR.

Switching the basolateral solution to HCO_3^-/CO_2 caused pH_i to alkalinize in the high titre SeV-CFTR (MOI=15) cells. However, both the rate (J_B was 10.37 ± 0.69 mM B/min) and the degree of the alkalinization (ΔpH_i was 0.27 ± 0.01) of SeV-CFTR cells were significantly reduced compared to SeV-LacZ cells (see J_B and ΔpH_i values in the previous paragraph). In contrast, in the low titre SeV-CFTR (MOI=3) cells, switching the basolateral solution to HCO_3^-/CO_2 caused pH_i changes similar to those observed in SeV-LacZ cells.

These data are consistent with hyperexpression of CFTR in the high titre MOI=15 cells either decreasing the rate at which HCO_3^- enters across the basolateral membrane or increasing the rate of HCO_3^- efflux across the apical membrane. Nevertheless, cells transduced with SeV vector, even at a high vector concentration, **clearly retain the typical differences in apical and basolateral CO_2 and HCO_3^- permeabilities** that we have previously described in untransduced CFPAC-1 cells (Rakonczay et al., 2006).

5. Molecular identities of acid and base transporters in duct cells.

PAT-1, AE2 and **pNBC1 mRNA were constitutively expressed** in uninfected and SeV vector transduced CFPAC-1 cells as determined by real-time RT-PCR. However, we did not detect mRNA for DRA, NHE2 and NHE3. CFTR expression was upregulated in SeV-CFTR transduced cells. **CFTR transduction had no obvious effect on the mRNA levels of** the the investigated **transporters**.

6. Functional expression and regulation of acid and base transporters.

6.1. Effect of SeV vector-mediated transduction and CFTR expression on Cl^-/HCO_3^- exchange activity.

Anion exchange activity can be detected in both the basolateral and apical membranes of pancreatic duct cells. The physiological role of the basolateral anion exchangers is uncertain, as with a normal transmembrane Cl^- gradient they would be expected to cause HCO_3^- efflux and to oppose secretion. In contrast, it is well established that secretion of HCO_3^- across the apical membrane of pancreatic duct cells involves both CFTR and SLC26 family Cl^-/HCO_3^- exchangers, although the quantitative importance of each pathway is controversial. Furthermore, it has been shown that phosphorylation of CFTR, as occurs during stimulation of HCO_3^- secretion, activates SLC26 anion exchangers (Shcheynikov et al., 2006). Our PCR data indicate that PAT 1 (SLC26A6) is the important SLC26 exchanger in CFPAC-1 cells. Given the role of anion exchangers in pancreatic duct cell function, we decided to investigate whether SeV vector transduction and CFTR expression had any effect on their activity.

6.1.1. Basolateral membrane.

Removal of Cl^- from the basolateral HCO_3^-/CO_2 solution caused a clear increase in pH_i, indicating that the SeV-LacZ cells have an anion exchanger on their basolateral membrane. However, when the same experiment was performed on a SeV-CFTR infected monolayer, the alkalinization caused by Cl^- removal was much smaller.

In the SeV-CFTR cells (both MOI=3 and MOI=15) the effects of basolateral Cl^- removal on pH_i and J_B were significantly reduced vs SeV-LacZ cells. Also, increasing intracellular cAMP, by exposing the cells to a cocktail of forskolin (10 μM), IBMX (100 μM) and dbcAMP (100 μM), had no significant effect on the ΔpH_i and J_B observed after Cl^- removal in either the SeV-LacZ or the SeV-CFTR (MOI=3 and MOI=15) cell groups. These data indicate that transduction of CFPAC-1 cells with **CFTR inhibits basolateral Cl^-/HCO_3^- exchange (most likely mediated by AE2)** and that cAMP has no effect on the activity of this exchanger. However, we cannot exclude the fact that the reduced pH_i change following Cl^- removal, in the absence or presence of cAMP stimulation could simply reflect faster exit of

HCO_3^- across the apical membrane, either through the apical anion exchanger or CFTR. Nonetheless physiologically, inhibition of the basolateral AE2 by CFTR expression would tend to favour HCO_3^- secretion and would be beneficial to CF patients.

6.1.2. Apical membrane.

In uninfected CFPAC-1 cells and in SeV-LacZ infected cells, removal of Cl^- from the apical membrane of the monolayers had no effect on pH_i, either in the absence or presence of cAMP. Thus **Cl^-/HCO_3^- exchange activity was not detectable in the apical membrane of CFPAC-1 cells that did not express CFTR**.

In contrast, **CFTR MOI=3 transduced cells exhibited a low, but clearly detectable, level of apical anion exchange activity**. Furthermore, in these cells **cAMP significantly increased ΔpH_i and J_B after apical Cl^- removal by 2.2-fold and 3.4-fold** respectively ($P<0.05$ for both parameters, n=31). In contrast, unstimulated CFTR MOI=15 cells exhibited a much higher level of anion exchange than the unstimulated MOI=3 cells, and the ΔpH_i and J_B values were unaffected by cAMP (n=6). We conclude that transducing CFPAC-1 cells with CFTR at MOI=3 are consistent with an apical Cl^-/HCO_3^- exchange activity. In contrast, **cells transduced with CFTR at the higher virus titre of MOI=15 have a constitutively active apical Cl^-/HCO_3^- exchanger** that cannot be further stimulated by cAMP.

cAMP-stimulated anion exchange activity in the SeV-CFTR MOI=3 cells was completely blocked by the anion exchange inhibitor 500 µM H_2-DIDS; J_B was -0.10 ± 0.26 mM B/min and ΔpH_i was -0.022 ± 0.011 when apical Cl^- was removed in the presence of the inhibitor (control values were 2.45 ± 0.40 mM B/min and 0.051 ± 0.007, respectively, n=5). To test the electrogenicity of the cAMP stimulated anion exchange activity, SeV-CFTR cells were perfused with basolateral K^+-free or high-K^+ HCO_3^-/CO_2 solution 10 minutes before the removal of apical Cl^-. Apical Cl^- withdrawal in the absence of basolateral K^+ did not significantly alter J_B (1.48 ± 0.22 mM B/min) and ΔpH_i (0.066 ± 0.009) vs standard conditions (J_B: 1.54 ± 0.29 mM B/min, ΔpH_i: 0.05 ± 0.008, n=7). In addition, apical Cl^- withdrawal in the presence of basolateral high-K^+ HCO_3^-/CO_2 solution resulted in no alteration of J_B (3.12 ± 0.49 mM B/min), but a significant increase of ΔpH_i (0.12 ± 0.01) compared to the control (2.24 ± 0.45 mM B/min and 0.06 ± 0.008, respectively, n=9). Overall, it seems that the **cAMP stimulated apical Cl^-/HCO_3^- exchange activity is mediated by PAT-1** and is electroneutral.

Finally, the CFTR inhibitor $CFTR_{inh}$-172 had no effect on either the rate or magnitude of the pH_i alkalinization following activation of apical Cl^-/HCO_3^- exchange in cyclic AMP stimulated SeV-CFTR (MOI=3) CFPAC-1 cells (n=7). Thus **Cl^- transport by CFTR is** probably **not required to maintain apical Cl^-/HCO_3^- exchange activity.**

6.1.2.1. Effect of SeV vector-mediated CFTR expression on PKA activity and expression of thePKA catalytic subunit.

Because apical Cl^-/HCO_3^- exchange in the MOI=15 SeV-CFTR transduced cells was constitutively active and did not respond to cAMP, we were concerned that the higher virus titre may have affected the cAMP signalling system. We therefore measured protein kinase-A (PKA) activity, and the amount of the 42kDa PKA catalytic subunit (PKAcat), in the SeV vector transduced CFPAC-1 cells.

PKA activity was similar in the uninfected, and in the SeV-LacZ and SeV-CFTR cells infected at MOI=3. About the same level of PKA activity was also observed in SeV-LacZ cells infected at MOI=15. However, in contrast, **PKA activity was almost undetectable in SeV-CFTR cells infected at the higher titre**.

We used western blotting to measure the amount of PKAcat expressed in the various cell types. The results indicated that all cells groups contained about the same amount of

PKAcat. Taken together, these data suggest that hyperexpression of CFTR inhibits PKA activity, thus providing an explanation for our failure to detect cAMP stimulation of apical Cl^-/HCO_3^- exchange activity in SeV-CFTR MOI=15 cells. Clearly, disabling the cAMP signalling pathway in this way would be a disadvantage in terms of gene therapy and predicts that **over-expression of CFTR in pancreatic duct cells of CF patients will need to be avoided**.

6.2. The effect of SeV vector-mediated CFTR expression on apical Na^+/H^+ exchange activity.

Apical NHE activity has been detected in the main pancreatic duct and may be involved in HCO_3^- scavenging from the duct lumen (Steward et al. 2005; Argent et al. 2006). HCO_3^- scavenging is probably a protective mechanism which acidifies the ductal contents, thereby reducing the chances of pro-enzyme activation when flow rates are low during interdigestive periods. We have recently shown that CFPAC-1 cells express an apical NHE activity (Rakonczay et al., 2006).

The activity of apical NHE was estimated by using the NH_4Cl pulse technique. Exposing SeV-LacZ transduced CFPAC-1 cells to a 20 mM NH_4Cl pulse, administered in the absence of Na^+ on both sides of the monolayer, reduced pH_i to about 6.7. In the continued absence of Na^+, pH_i stabilized at this new value, indicating the absence of any other Na^+-independent pH_i recovery mechanisms such as H^+ pumps. Re-addition of Na^+ to the apical membrane caused pH_i to increase, due to activation of the apical NHE. Similar results were obtained when the SeV-LacZ cells were exposed to the NH_4Cl pulse in the presence of the cAMP cocktail.

The results obtained from the ammonium pulse technique in SeV-CFTR cells were similar to those obtained with the SeV-LacZ cells. However, the effect of re-adding Na^+ on pH_i and J_B was significantly greater in the SeV-CFTR cells as compared to the SeV-LacZ cells (MOI=3: 1.71 ± 0.27 fold, n = 10; MOI=15: 2.12 ± 0.44 fold, $P<0.05$, n=5). Finally, exposure of the SeV-CFTR monolayer to the cAMP cocktail had no significant effect on the J_B observed in response to re-addition of Na^+. These data suggest that the **NHE expressed in the apical membrane of CFPAC-1 cells is up-regulated in the presence of CFTR**, but is unaffected by cAMP stimulation.

7. Small doses of chenodeoxycholate stimulate apical Cl^-/HCO_3^- activity independently of CFTR's Cl^- transport.

Our recent studies on guinea-pig pancreatic ducts have suggested that stimulation of HCO_3^- secretion may represent a defense mechanism against toxic factors, such as viruses (Hegyi et al., 2005) and bile acids (Venglovecz et al., 2008), that have gained access to the ductal tree. Therefore, we also tested the effect of the unconjugated bile acid, chenodeoxycholate (CDC), on the activity of apical Cl^-/HCO_3^- exchange activity in human pancreatic duct cells. Moreover, we wanted to address the specific issue as to whether CFTR was involved in the bile-acid induced increase in HCO_3^- secretion that we have previously reported in guinea-pig ducts.

In SeV-LacZ transduced CFPAC-1 cells, removal of Cl^- from the standard HCO_3^-/CO_2 solution perfusing the apical membrane of the monolayers had no effect on pH_i, either in the absence or presence of 0.1 mM CDC. However, Cl^- removal from the apical membrane of SeV-CFTR transduced cells caused a clear alkalinization of pH_i (J_B, 0.61 ± 0.07 mM B/min and ΔpH_i, 0.018 ± 0.003), which was significantly increased by about three-fold (J_B 1.75 ± 0.08 mM B/min and ΔpH_i, 0.059 ± 0.006) in the presence of 0.1 mM CDC. Overall, CDC stimulation of Cl^-/HCO_3^- exchange activity was only observed in cells expressing CFTR, strongly suggesting that the presence of CFTR is necessary for this effect.

The stimulation of apical anion exchange by CDC could be due either to a direct effect on PAT-1 activity or it could be an indirect effect caused by, for example, an increase in electrodiffusive Cl^- transport through CFTR. To directly test whether CDC could activate CFTR we performed whole cell patch clamp experiments on single guinea-pig pancreatic duct cells. Administration of 0.1 mM CDC to the bath solution had no effect on whole cell currents, whereas characteristic CFTR currents could be activated in the same cell (in 7 out of 10 cells) when it was subsequently exposed to 5 μM forskolin.

Taken together, **the stimulatory effect of 0.1 mM CDC on apical anion exchanger activity is at least partly dependent on CFTR expression, but not its Cl^- transport activity**.

Conclusions

Overall, the project provided important information on the molecular identities of acid (H^+) and base (HCO_3^-) transporters and about the regulation of anion and Na^+/H^+ exchangers in human pancreatic duct cells. We also have an improved understanding of HCO_3^- transport mechanisms in the diseased and wild-type pancreatic ductal epithelium.

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