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RESEARCH ARTICLE

The distribution and expression of CFTR restricts electrogenic anion secretion to the ileum of the brushtail possum, *Trichosurus vulpecula*

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SUMMARY

In eutherian mammals, fluid secretion is essential for intestinal function. This is driven by electrogenic Cl^- secretion, which involves a NaK2Cl cotransporter (NKCC1) in the enterocyte basolateral membrane and the cystic fibrosis transmembrane conductance regulator (CFTR) in the apical membrane. However, in the possum ileum, NKCC1 expression is low and secretagogues stimulate electrogenic HCO_3^- secretion driven by a basolateral $NaHCO_3$ cotransporter (pNBCe1). Here we investigated whether electrogenic anion secretion occurs in possum duodenum and jejunum and determined the role of CFTR in possum intestinal anion secretion. Prostaglandin E_2 (PGE_2) and forskolin stimulated a large increase in ileal short-circuit current (I_{sc}), consistent with electrogenic HCO_3^- secretion, but had little effect on the duodenal and jejunal I_{sc}. Furthermore, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) and *N*-(2-naphthalenyl)-[(3,5-dibromo-2,4-dihydroxyphenyl)methylene]glycine hydrazide (GlyH101) inhibited cloned possum CFTR in cultured cells and the PGE_2-stimulated ileal I_{sc}, implicating CFTR in ileal HCO_3^- secretion. Consistent with this, CFTR is expressed in the apical membrane of ileal crypt and lower villous cells, which also express pNBCe1 in the basolateral membrane. In contrast, duodenal and jejunal CFTR expression is low relative to the ileum. Jejunal pNBCe1 expression is also low, whereas duodenal and ileal pNBCe1 expression are comparable. All regions have low NKCC1 expression. These results indicate that cAMP-dependent electrogenic Cl^- secretion does not occur in the possum small intestine because of the absence of CFTR and NKCC1. Furthermore, CFTR functions as the apical anion conductance associated with HCO_3^- secretion and its distribution limits electrogenic HCO_3^- secretion to the ileum.

Key words: brushtail possum, *Trichosurus vulpecula*, marsupial, ileum, bicarbonate secretion, CFTR.

INTRODUCTION

In eutherian mammals, fluid secretion is essential for intestinal function as it defines the optimal composition of the luminal contents for digestion and absorption (Field, 2003; Murek et al., 2010). Fluid secretion is driven by electrogenic Cl^- secretion, which involves the transport of Cl^- across the basolateral membrane of the enterocytes by the NaK2Cl cotransporter (NKCC1) and then the exit of Cl^- across the apical membrane *via* the cystic fibrosis transmembrane conductance regulator (CFTR). This active transport of Cl^- creates a driving force for Na^+ and water and the result is the secretion of an isosmotic solution of NaCl into the lumen (Halm and Frizzell, 1990).

In addition to Cl^- secretion, HCO_3^- secretion occurs in the eutherian intestinal tract, particularly in the duodenum (Seidler et al., 2000), where HCO_3^- has an important role in the protection of the duodenal epithelium from the acidic chyme delivered from the stomach (Allen and Flemstrom, 2005). Interestingly, even in the duodenum, where the rates of HCO_3^- secretion are relatively high compared with other regions of the intestinal tract (Seidler et al., 2000), the rate of HCO_3^- secretion is markedly less than Cl^- secretion (Seidler et al., 1997). This may reflect the fact that the primary role of HCO_3^- secretion is to regulate the pH of the luminal contents rather than generate fluid flow.

Although there is clear evidence of intestinal HCO_3^- secretion in the eutherian intestine, the underlying mechanism is contentious. In the duodenum, where the mechanism of HCO_3^- secretion has been most thoroughly characterised, it is agreed that the secreted HCO_3^- is either generated in the epithelial cells through the hydration of CO_2 by carbonic anhydrase (Knutson et al., 1995; Leppilampi et al., 2005) or, more importantly (Sjöblom et al., 2009), transported into the cells by the pancreatic variant of the $NaHCO_3$ cotransporter (pNBCe1), located on the basolateral membrane (Jacob et al., 2000; Minhas et al., 1993; Praetorius et al., 2001). However, there appear to be two potential mechanisms for HCO_3^- exit across the apical membrane. The first involves the activity of a $Cl–HCO_3$ exchanger (Brown et al., 1989; Isenberg et al., 1993; Spiegel et al., 2003; Wang et al., 2002), most likely a member of the SLC26 family of anion exchangers (Jacob et al., 2002; Wang et al., 2002), and may be associated with spontaneous HCO_3^- secretion by the duodenum (Spiegel et al., 2003). In addition, acid or secretagogues stimulate electrogenic HCO_3^- secretion, which is independent of $Cl–HCO_3$ exchanger activity (Seidler et al., 1997; Spiegel et al., 2003). This suggests the presence of an apical HCO_3^- conductance through which HCO_3^- exits the cells, most likely CFTR (Akiba et al., 2005; Hogan et al., 1997), because deletion of CFTR inhibits both intestinal Cl^- and HCO_3^- secretion (Hogan et al., 1997; Seidler et al., 1997). Thus, CFTR has a central role in both electrogenic Cl^- and HCO_3^- secretion in the eutherian intestinal tract.

In the Australian common brushtail possum, *Trichosurus vulpecula*, a metatherian mammal, the mechanisms of electrolyte

transport in the intestinal tract differ from those seen in the eutherian intestine (Butt et al., 2002a; Butt et al., 2002b). This is particularly evident in the ileum where secretagogues stimulate electrogenic anion secretion, but this involves electrogenic HCO_3^- secretion, not electrogenic Cl^- secretion as seen in eutherian mammals (Bartolo et al., 2009a; Bartolo et al., 2009b). The absence of Cl^- secretion in the possum ileum is primarily due to low levels of expression of NKCC1, the major Cl^- uptake pathway of secretory epithelia in eutherian mammals (Bartolo et al., 2009a). In contrast, there are high levels of pNBCe1 expression in the basolateral membrane of the possum ileum and HCO_3^- secretion is driven by this transporter (Bartolo et al., 2009b).

The secretion of HCO_3^- by the possum ileum may be related to a specialised role of the possum ileum. The possum is a hindgut fermentor and microbial fermentation in the hindgut is dependent upon the provision of a fluid medium with sufficient buffering capacity to maintain pH within a favourable range (Rechkemmer et al., 1988). However, the hindgut of the possum has a limited ability to secrete fluid (Butt et al., 2002b). Therefore, in the absence of significant electrogenic anion secretion in the hindgut of the possum, electrogenic HCO_3^- secretion by the ileum may be a means of delivering fluid of sufficient volume and buffering capacity to the hindgut fermentation chamber.

This raises important questions relating to fluid and electrolyte secretion in the possum small intestine. Firstly, does electrogenic Cl^- secretion occur in the duodenum and jejunum of the possum, or is fluid secretion driven by HCO_3^- secretion throughout the small intestine? Secondly, does CFTR have a role in electrogenic HCO_3^- secretion in the possum intestine? This is particularly relevant because possum CFTR (pCFTR) has molecular and functional properties very similar to those of CFTR from other vertebrates and, like CFTR from other species, is primarily a protein kinase A-activated Cl^- channel (Demmers et al., 2010). Consequently, in this study we investigated the effect of cAMP-dependent secretagogues on electrogenic anion secretion in the duodenum, jejunum and ileum of the possum and related this to the expression of the critical transport proteins involved in electrogenic Cl^- and HCO_3^- secretion, NKCC1, pNBCe1 and CFTR. In addition, we used cloned pCFTR to identify effective inhibitors of pCFTR, and used these to investigate the contribution of CFTR to electrogenic anion secretion by the possum small intestine.

MATERIALS AND METHODS

Animals and tissue collection

All experimental procedures and the capture and housing of the animals were given prior approval by the AgResearch Invermay and University of Otago Animal Ethics Committees according to the Animal Welfare Act New Zealand, 1999. Adult common Australian brushtail possums, *Trichosurus vulpecula*, Kerr 1792, were used in this study. They had a live mass >2 kg and were collected and maintained as previously described (Butt et al., 2002b). Animals were killed by an intracardiac injection of barbiturate (Euthal^{TM}; Delta Veterinary Laboratories, Hornsby, NSW, Australia) administered under halothane-induced anaesthesia (Fluothane; ICI New Zealand Ltd, Lower Hutt, New Zealand), and collection of tissues for RNA and protein isolation, *in situ* hybridisation studies and Ussing chamber experiments were carried out as previously described (Bartolo et al., 2009a; Bartolo et al., 2009b).

Measurements of epithelial transport

The measurement of the active transport of ions by the ileum was carried out as described previously (Butt et al., 2002a; Butt et al., 2002b). Briefly, the underlying connective tissue and muscle layers were dissected from small pieces of small intestine, either duodenum, jejunum or ileum, and the resultant epithelial sheet was mounted in custom-made Ussing chambers (EmTech, University of Otago, Dunedin, New Zealand), bathed in HCO_3^- buffered NaCl Ringer's solution (110 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 0.5 mmol l^{-1} $MgSO_4$, 1 mmol l^{-1} $CaCl_2$, 25 mmol l^{-1} $NaHCO_3$, 10 mmol l^{-1} pyruvate/glutamine and 10 mmol l^{-1} HEPES/Tris, pH 7.4 when gassed with 95%:5% O_2:CO_2) and constantly short circuited with a computer-controlled membrane clamp (South Campus Electronics, University of Otago, Dunedin, New Zealand). Prostaglandin E_2 (PGE_2; 1 μmol l^{-1} serosal)- or forskolin (10 μmol l^{-1} mucosal and serosal)-stimulated short-circuit current (I_{sc}) was used as a measure of electrogenic HCO_3^- secretion (Bartolo et al., 2009b).

For the pharmacological characterisation of cloned pCFTR, Fischer rat thyroid (FRT) cells stably expressing pCFTR (Demmers et al., 2010) were seeded at high density (0.5×10^6 cells) on Snapwell inserts (12 mm; Corning Costar, Biolab, Auckland, New Zealand) and, after 4 to 8 days, inserts were mounted in custom-made Ussing chambers (EmTech) in the presence of a serosal to mucosal Cl^- gradient. The composition of the serosal solution was 135 mmol l^{-1} NaCl, 0.5 mmol l^{-1} $CaCl_2$, 1.2 mmol l^{-1} $MgCl_2$, 2.4 mmol l^{-1} K_2HPO_4, 0.6 mmol l^{-1} KH_2PO_4, 10 mmol l^{-1} HEPES, 10 mmol l^{-1} glucose, pH 7.4; the composition of the mucosal solution was the same, except the NaCl was replaced with 135 mmol l^{-1} Na gluconate and the $CaCl_2$ concentration was 1.2 mmol l^{-1}. The solutions were maintained at 30°C and gassed with O_2. The transepithelial potential was then clamped to zero and the basolateral membrane of the cells was permeabilised with amphotericin B (250 μg ml^{-1}, Sigma-Aldrich, St Louis, MO, USA) for 25 min. After permeabilisation with amphotericin B, the basolateral membrane was electrically eliminated so that changes in currents induced by drugs reflect the changes in conductance of the apical membrane, where pCFTR is primarily located. Therefore, the measured transepithelial current is due to Cl^- flow through pCFTR in the apical membrane and this Cl^- current (I_{Cl}) is an index of pCFTR activity (Sheppard et al., 1994). pCFTR activity was stimulated with the addition of mucosal and serosal forskolin (10 μmol l^{-1}) and 3-isobutyl-1-methylxanthine (IBMX; 100 μmol l^{-1}).

For the Ussing chamber measurements, tetrodotoxin (TTX) was purchased from Alomone Labs (Jerusalem, Israel), *N*-(2-naphthalenyl)-[(3,5-dibromo-2,4-dihydroxyphenyl)methylene]glycine hydrazide (GlyH101) was purchased from Merck Ltd (Auckland, New Zealand) whereas all other reagents were purchased from Sigma-Aldrich. Concentrated stocks of TTX were prepared in deionised water, and PGE_2 in ethanol, whereas stocks of forskolin, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), glibenclamide, GlyH101 and IBMX were made up in dimethyl sulfoxide. These drugs were then added as small aliquots of stock solutions to the appropriate side of the tissues. Control experiments demonstrated that equivalent volumes of vehicle had no effect. 4,4′-diisothiocyano-stilbene-2,2′-disulfonate (DIDS) was dissolved in Ringer's solution to give a concentration of 10 mmol l^{-1} and this was used to replace the appropriate volume of Ringer's solution in the reservoirs, to give a final concentration of 1 mmol l^{-1}.

Western blot analysis of CFTR, pNBCe1 and NKCC1

Crude membrane extracts were obtained as described previously (Bartolo et al., 2009a) and the samples were separated by SDS-polyacrylamide gel electrophoresis in 7.5% polyacrylamide gels and electroblotted onto a PVDF membrane (GE Healthcare Biosciences, Auckland, New Zealand). Membranes were blocked with 5% (w/v)

skimmed milk powder in Tris-buffered saline containing 0.1% Nonidet-P40 (TBS-NP40) for 1 h, and then incubated with specific antibodies; CFTR mouse anti-human (Ab-4) [1:100 primary, 1:3000 rabbit anti-mouse horseradish peroxidase (HRP) conjugate secondary] purchased from Lab Vision (Fremont, CA, USA); NKCC1 (N-16) goat polyclonal IgG (1:800 primary, 1:5000 donkey anti-goat IgG-HRP conjugated secondary) purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); $NaHCO_3$ cotransporter (NBC) rabbit anti-rat NBC serum (1:2000 primary, 1:6000 anti-rabbit HRP conjugate), for 2 h. The NBC antiserum was kindly donated by Dr Bernard Schmitt (Schmitt et al., 1999). Membranes were then washed with TBS-NP40 and incubated with their respective HRP-conjugated secondary antibody (GE Healthcare Biosciences) for 2 h. The specificity of the antibodies was determined by preabsorption with the control peptide for 1 h prior to incubating the membranes. Antibodies were detected using enhanced chemiluminescence (GE Healthcare Biosciences).

Quantification of CFTR transcript

Reverse transcription PCR was used to demonstrate differences in the expression of pCFTR transcript in the different regions of the possum small intestine. RNA isolation and cDNA synthesis have been described previously (Bartolo et al., 2009a; Bartolo et al., 2009b). To quantify the level of pCFTR mRNA expression between the different regions of the intestine, the expression of 18S rRNA was used as an internal control. To exclude any possibility of amplified DNA products originating from genomic DNA contamination, a subset of RNA was treated identically to the samples, but in the absence of reverse transcription. Primers for pCFTR were designed from the possum CFTR sequence (accession number AY916796) and were: forward 5′-AAGAAAGGTTTCAGACGACGCTTGG-3′ and reverse 5′-GGATCGTTCTGCTTTGTTGTCAGGA-3′. The primers for 18S rRNA were designed from the possum sequence (accession number FJ809787.1) and were: forward 5′-TGCATGTCTA-AGTACACACGGC-3′ and reverse 5′-GCCTCGAAAGA-GTCCTGTATTG-3′. These primers generated a 282 and 522 bp amplicon for CFTR and 18S rRNA, respectively. In preliminary experiments, the linear amplification range of pCFTR and 18S rRNA were determined by running PCR reactions for a varying number of cycles between 17 and 40 and quantifying the resultant product by densitometry. The final PCRs for gene expression were run for 29 and 23 cycles for pCFTR and 18S rRNA, respectively, and the products were run on a 1% agarose gel.

In situ localisation of CFTR in intestinal epithelial cells

Cellular localisation of pCFTR mRNA was determined using an *in situ* hybridization protocol as described previously (Bartolo et al., 2009a; Bartolo et al., 2009b). The probe was amplified from possum ileum cDNA using the following primers: forward 5′-CCAAAGCAGTACAGCCTCTCTT-3′ and reverse 5′-CCCAGCAGTATGCCTTAACAG-3′. The primers were designed from the pCFTR sequence available in GenBank (accession number AY916796). These primers generated a 551 bp amplicon that was cloned using the Promega pGem® T Easy vector system (Promega, Madison, WI, USA), which contains SP6 and T7 transcription sites. Sense and antisense [^{33}P] UTP (GE Healthcare, Auckland, New Zealand) labelled pCFTR probes were transcribed using an *in vitro* transcription kit (Riboprobe® *in vitro* transcription systems, Promega) according to the manufacturer's instructions. Hybridization and detection of the probes were carried out as described previously (Bartolo et al., 2009a).

Immunolocalisation of CFTR and pNBCe1

Ileal tissue samples were embedded in O.C.T. compound (Siemens Medical Solutions, Erlangen, Germany) and snap frozen in isopentane pre-cooled in liquid nitrogen. Sections (10 μm) were placed on slides coated in 2% 3-aminopropyltiethoxysilane (Sigma-Aldrich), air-dried for 10 min and stored at –80°C until required. Sections were prepared for immunohistochemistry by warming to room temperature, rinsing in PBS (137 mmol l^{-1} NaCl, 10 mmol l^{-1} phosphate, 2.7 mmol l^{-1} KCl; pH 7.4) for 5 min and fixing in 0.4% paraformaldehyde for 10 min. The sections were blocked in 1% donkey serum in PBS for 30 min, and then incubated with mouse anti-CFTR (2 μg ml^{-1} in PBS; clone M3A7; Lab Vision) and rabbit anti-rat NBC (diluted 1:300 in PBS) for 4 h. The sections were then washed in PBS and incubated with Alexa Fluor 546 donkey anti-mouse (2 μg ml^{-1} in PBS; Invitrogen Ltd, Auckland, New Zealand) and FITC-conjugated donkey anti-rabbit IgG (diluted 1.5 μg ml^{-1} in PBS; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 2 h, followed by incubation with DAPI (100 ng ml^{-1} in PBS; Sigma-Aldrich) for 20 min. The sections were then washed in PBS and mounted in Vectashield® mounting medium (Vector Laboratories, Burlingame, CA, USA). The sections were examined and photographed using a confocal scanning laser system (Zeiss 510 LSM; Carl Zeiss GmbH, Jena, Germany).

Statistics

Results of electrophysiological experiments are presented as either individual recordings or as means ± s.e.m. For measurements of ileal transport, N is the number of animals, whereas for measurements of pCFTR activity in FRT cells, N is the number of snapwells, and cells from at least three different passages were used for each experiment. Differences between means were tested, where appropriate, with either the unpaired two-tailed Student's t-test or one-way ANOVA with a Bonferroni or Dunnett's *post hoc* test as indicated in the figure legends. Differences were considered statistically significant at $P<0.05$.

RESULTS

Response of the duodenum, jejunum and ileum to secretagogues

To investigate whether the duodenum and jejunum of the possum were capable of an electrogenic secretory response similar to that of the ileum, pieces of duodenum, jejunum and ileum from the same animal were stripped of underlying connective tissue and muscle and mounted in the Ussing chamber.

As seen previously (Bartolo et al., 2009a), the ileum had a relatively high spontaneous I_{sc} (122±21 μA cm^{-2}, N=8), which was significantly reduced ($P<0.05$) by the addition of 1 μmol l^{-1} serosal TTX (ΔI_{sc} with TTX=–27±8 μA cm^{-2}). The spontaneous I_{sc} in the duodenum (38±7 μA cm^{-2}) and jejunum (30±5 μA cm^{-2}) were significantly lower ($P<0.001$ for both) than that seen in the ileum. Furthermore, TTX had no effect on the spontaneous I_{sc} in either the duodenum or jejunum (data not shown).

The subsequent addition of serosal PGE_2 (1 μmol l^{-1}) stimulated a large increase in I_{sc} in the ileum but had little effect on the I_{sc} in the duodenum or jejunum (Fig. 1). It is unlikely that the limited response to PGE_2 in the jejunum was due to damage to the tissue during preparation, as the addition of mucosal glucose (50 mmol l^{-1}) to the jejunal tissue stimulated a large increase in I_{sc} (ΔI_{sc}=70±20 μA cm^{-2}, N=8) and there was little change in transepithelial resistance (R_t) during the course of the experiment ($R_{t,initial}$=30±5 Ω cm^2, R_{t,PGE_2}=30±5 Ω cm^2). Mucosal glucose had no effect on the duodenal I_{sc}, but the R_t changed little during the time

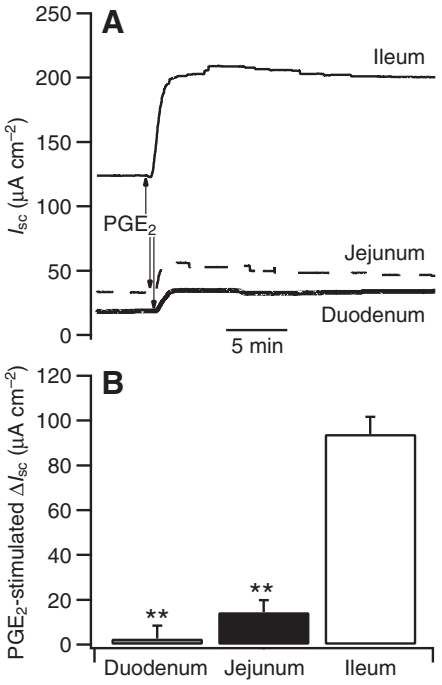

Fig. 1. Prostaglandin (PGE_2, 1 μmol l^{-1} serosal) stimulates a large increase in short-circuit current (I_{sc}) in the ileum of the possum, but has a minimal effect on the jejunum and duodenum. Ileum, duodenum and jejunum from the same animal were stripped of underlying connective tissue and muscle, mounted in Ussing chambers and pre-treated with tetrodotoxin (TTX; 1 μmol l^{-1} serosal) before the addition of PGE_2. (A) Representative experiment showing the time course of the response of the tissues from one animal. (B) Summary of the mean changes in steady-state I_{sc} (ΔI_{sc}) stimulated by PGE_2 in paired tissues from eight animals. All values are means ± s.e.m. **, significantly different (P<0.01) to response in ileum; one-way ANOVA with Dunnett's *post hoc* test.

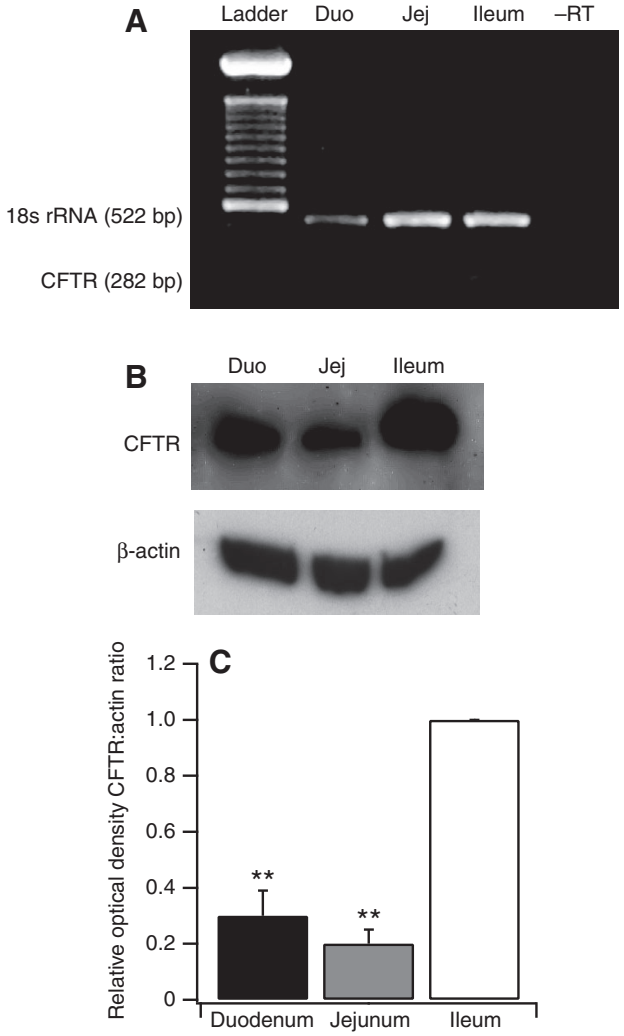

Fig. 2. The cystic fibrosis transmembrane conductance regulator (CFTR) is highly expressed in the ileum but not the duodenum or jejunum of the possum small intestine. (A) Representative gel showing the relative levels of expression of possum (p)CFTR mRNA and 18S rRNA transcript in the duodenum (duo), jejunum (jej) and ileum (ileum) of the possum. –RT, negative control (the PCR reaction contained non-reverse transcribed RNA). Representative of four animals. (B) A representative immunoblot showing the expression of pCFTR in the duodenum, jejunum and ileum. (C) Mean levels of expression of CFTR in the duodenum, jejunum and ileum illustrated as the ratio of the optical density of the pCFTR and β-actin bands measured on the immunoblots. All values are means ± s.e.m., N=4. **, significantly different (P<0.01) to value in ileum; one-way ANOVA with Dunnett's *post hoc* test.

course of the experiment ($R_{t,initial}$=67±14 Ω cm^2, R_{t,PGE_2}=64±15 Ω cm^2) suggesting that the duodenal tissue was also healthy. In a second group of five animals, forskolin (10 μmol l^{-1}, mucosal and serosal) also stimulated a large increase in I_{sc} in the ileum (ΔI_{sc}=44±11 μA cm^{-2}) but had little effect on the duodenum (ΔI_{sc}=–1.3±1.81 μA cm^{-2}) and jejunum (ΔI_{sc}=5.3±6.8 μA cm^{-2}). These data confirm our previous observations (Bartolo et al., 2009a; Bartolo et al., 2009b) that PGE_2 and forskolin stimulate large sustained increases in I_{sc} in the possum ileum and demonstrate that neither the duodenum nor jejunum of the possum are capable of electrogenic anion secretion when stimulated with these compounds.

Expression of CFTR, NKCC1 and pNBCe1 in the possum small intestine

In eutherian mammals, CFTR is intimately involved in intestinal secretion and deletion of CFTR in knockout mice results in the inhibition of both Cl^- and HCO_3^- secretion (Hogan et al., 1997; Seidler et al., 1997). Therefore, we investigated whether the expression of the pCFTR in the different regions of the possum small intestine corresponded with the magnitude of the I_{sc} response to secretagogues. Tissue was collected from the duodenum, jejunum and ileum and the relative levels of expression of CFTR transcript and mature CFTR protein in the different regions were determined using semi-quantitative PCR and western blot analysis, respectively.

There were high levels of CFTR transcript in the ileum of the possum, but much lower levels of transcript in both the duodenum and the jejunum (Fig. 2A). Immunoblots of membrane proteins extracted from epithelial cells in the duodenum, jejunum and ileum detected a strong band of ~165 kDa in the ileum (Fig. 2B). As we have shown previously (Demmers et al., 2010), this band corresponds to the size of glycosylated CFTR in both humans and rodents (Cohn et al., 1992; Denning et al., 1992; Zheng et al., 2004). Consistent with the low levels of CFTR transcript in the duodenum and jejunum, there were much lower levels of expression of CFTR in the immunoblots from the duodenum and the jejunum compared with the ileum

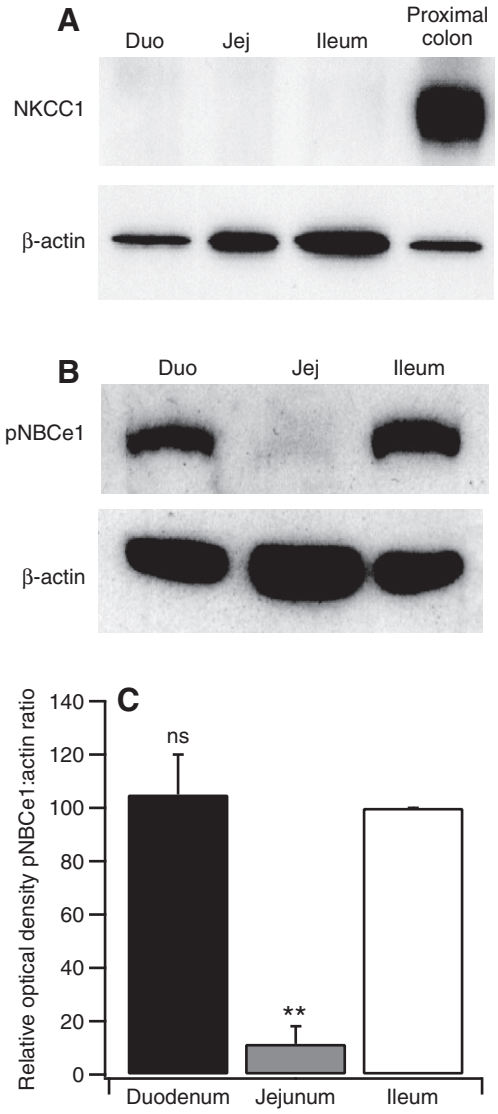

Fig. 3. Regional expression of the NaK2Cl cotransporter (NKCC1) and the pancreatic variant of the $NaHCO_3$ cotransporter (pNBCe1) in the small intestinal epithelium of the possum. (A) Immunoblot showing that NKCC1 is expressed at very low levels in all regions of the small intestine but is highly expressed in the proximal colon. Result is representative of four measurements. (B) A representative immunoblot showing that pNBCe1 is expressed at very low levels in the jejunum, but there were much higher levels of expression of pNBCe1 in the duodenum and ileum. (C) Mean levels of expression of pNBCe1 in the duodenum, jejunum and ileum illustrated as the ratio of the optical density of the pNBCe1 and β-actin bands measured on the immunoblots. All values are means ± s.e.m., N=4. ns, not significant; **, significantly different (P<0.01) to value in ileum (one-way ANOVA with Dunnett's *post hoc* test).

(Fig. 2B,C). No band was detected in samples taken from skeletal muscle or in the absence of the primary antibody (data not shown).

It is noteworthy that accompanying the low levels of expression of CFTR in the jejunum were very low levels of expression of the basolateral transport proteins associated with Cl^- and HCO_3^- secretion, NKCC1 and pNBCe1 (Fig. 3). In contrast, although the duodenum also had very low levels of expression of NKCC1, pNBCe1 was expressed at a level comparable to that seen in the ileum (Fig. 3).

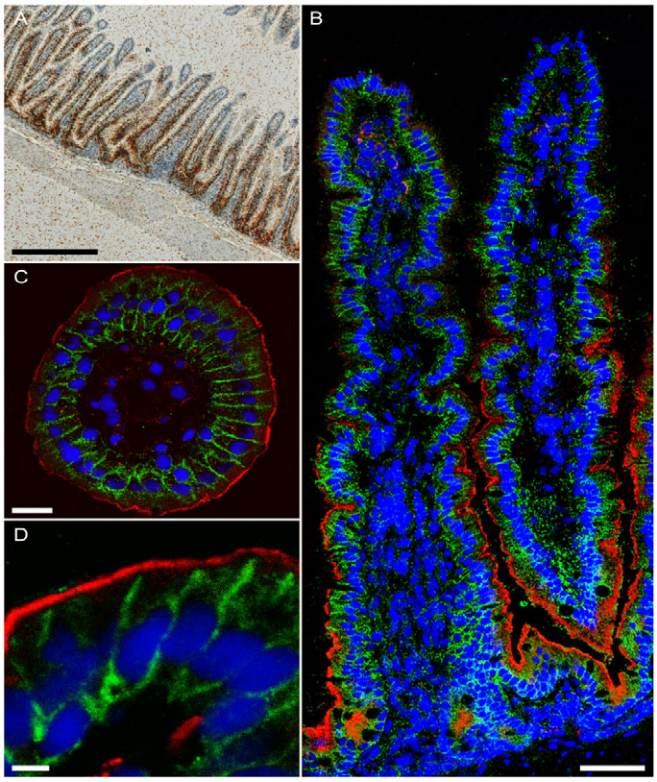

Fig. 4. Localisation of the CFTR using *in situ* hybridisation (A) and immunolocalisation of CFTR (red) and the pancreatic variant of the $NaHCO_3$ cotransporter (pNBCe1; green) in the possum ileum (B–D). (A) *In situ* localisation of CFTR mRNA in the possum ileum showing high levels of transcript in the crypts and at the base of the villous region, with CFTR mRNA levels decreasing towards the tip of the villi. (B) Immunolocalisation of CFTR and pNBCe1 in the possum ileal epithelium, showing high levels of CFTR (apical membrane) and pNBCe1 (basolateral membrane) immunoreactivity in the crypts and at the base of the villus. (C) Cross-section of an ileal villus, showing CFTR and pNBCe1 immunoreactivity at or near the apical and basolateral membrane, respectively. (D) High-power image of possum ileal epithelial cells showing that CFTR immunoreactivity is localised at the apical membrane whereas pNBCe1 is localised at the basolateral membrane. Scale bars, 500 μm (A), 50 μm (B), 20 μm (C) and 5 μm (D).

Distribution of CFTR mRNA and immunoreactivity in the possum ileum

In the possum ileum, pNBCe1, which drives the PGE_2-stimulated increase in I_{sc}, is expressed in the basolateral membrane of epithelial cells located in the crypts and the basal region of the villi (Bartolo et al., 2009b). Measurement of the distribution of CFTR in the possum ileum with *in situ* hybridisation and immunohistochemistry demonstrated a similar expression pattern for CFTR. Transcript for CFTR was most highly expressed in the cells in the crypts and the base of the villi, with decreasing amounts of transcript in the upper regions of the villi (Fig. 4A). In general there was little evidence of transcript of CFTR in cells at the tip of the villi. The distribution of CFTR immunoreactivity in the crypt/villous axis of the possum ileum indicated that it was present in the crypt cells and basal and mid regions of the villi, but absent from the tip of the villi (Fig. 4B). Significantly, CFTR and pNBCe1 immunoreactivity co-localised to the same cells within ileal epithelium, but CFTR immunoreactivity was restricted to the apical membrane of the villous cells with low cytoplasmic levels

beneath the membrane (Fig. 4C,D). In contrast, pNBCe1 was restricted to the basolateral membranes, particularly the lateral membranes of the epithelial cells (Fig. 4C,D).

Collectively, these data indicate that high levels of expression of CFTR in the ileum are associated with a significant PGE_2- and forskolin- stimulated I_{sc}. Furthermore, in the ileal epithelium, CFTR co-localises with pNBCe1, which drives electrogenic anion secretion (Bartolo et al., 2009b), and CFTR is located in the apical pole of the same cells, consistent with a role in HCO_3^- secretion.

Pharmacology of pCFTR and ileal HCO_3^- secretion

To determine whether the PGE_2-stimulated increase in I_{sc} in the ileum is dependent upon CFTR, we defined the pharmacological profile of cloned pCFTR (Demmers et al., 2010) and compared this with the pharmacological profile of the PGE_2-stimulated secretory response of the intact possum ileum. In both experiments, four inhibitors, which have been used to characterise the role of CFTR in eutherian mammals, were employed: NPPB, a non-specific inhibitor of anion conductances in epithelia (Schultz et al., 1999); DIDS, an inhibitor of Ca^{2+}-activated Cl^- channels, but not CFTR, when added to the external surface of the channel (Schultz et al., 1999); glibenclamide, which inhibits eutherian CFTR at relatively high doses (Sheppard and Welsh, 1993); and GlyH101, a recently developed highly specific inhibitor of eutherian CFTR (Muanprasat et al., 2004).

To characterise the pharmacological profile of cloned pCFTR, confluent monolayers of FRT cells stably expressing pCFTR were mounted in Ussing chambers and pCFTR activity was quantified as the cAMP-activated I_{Cl} measured across these monolayers following exposure to a Cl^- gradient and permeabilisation of the basolateral membrane with amphotericin (Sheppard et al., 1994). Under these conditions, the addition of forskolin ($10\,\mu mol\,l^{-1}$) plus IBMX ($100\,\mu mol\,l^{-1}$) resulted in the stimulation of I_{Cl} in confluent monolayers of cells transfected with pCFTR, whereas there was no increase in I_{Cl} in monolayers grown from cells transfected with the empty vector (Fig. 5A,B). Furthermore, this I_{Cl} was dependent upon the presence of a Cl^- gradient (Fig. 5C). Similar results were obtained if the monolayers were treated with 8-(4-chlorophenylthio)adenosine-3′,5′-cyclic monophospate (CPT)-cAMP (data not shown). This cAMP-stimulated I_{Cl} in the FRT cells was potently inhibited by mucosal addition of the specific CFTR inhibitor GlyH101 (IC_{50}=3.8±0.36 $\mu mol\,l^{-1}$) (Fig. 5D,E) and the non-specific anion channel inhibitor NPPB (IC_{50}=3.7±0.0.28 $\mu mol\,l^{-1}$) (Fig. 5E). In contrast, glibenclamide was a relatively ineffective inhibitor of pCFTR, $500\,\mu mol\,l^{-1}$ glibenclamide inhibiting ~50% of the I_{Cl} (Fig. 5E), and DIDS had little effect on the I_{Cl}, even at a concentration of $1\,mmol\,l^{-1}$ (data not shown).

Having established a clear pharmacological profile for cloned pCFTR, we then determined the effect of mucosal addition of these inhibitors on the PGE_2-stimulated I_{sc}, which in the possum ileum provides an index of electrogenic HCO_3^- secretion (Bartolo et al., 2009b). In all cases, four pieces of ileum from the same animal were mounted in Ussing chambers and pre-treated with $1\,\mu mol\,l^{-1}$ serosal TTX to inhibit secretion stimulated by nervous activity and reduce the variability in the spontaneous I_{sc} (Bartolo et al., 2009a). Two of the tissues were then stimulated with PGE_2 ($1\,\mu mol\,l^{-1}$, serosal) and, once the response to PGE_2 had reached a steady state, one of the inhibitors was added to the mucosal surface of one of the stimulated tissues and one of the unstimulated tissues in the continued presence of TTX and PGE_2. Vehicle was added to the other tissues and these tissues served as time-matched controls to allow for variations in both the spontaneous and PGE_2-stimulated I_{sc}.

There was very little variation in I_{sc} of the time-matched control tissues. On average, the spontaneous I_{sc} in these tissues decreased by 4.5±1.1 $\mu A\,cm^{-2}$ (N=25) during the course of the experiment. Similarly, in those tissues stimulated with PGE_2, but with no inhibitor added, the steady-state I_{sc} after the addition of PGE_2 only varied by 3.3±1.7 (N=25) during the course of the measurements. Therefore, a comparison of the effects of the inhibitors on the stimulated and unstimulated tissues allowed calculation of the effect of inhibitors on the PGE_2-stimulated response. As shown previously (Bartolo et al., 2009a), the mucosal addition of NPPB ($100\,\mu mol\,l^{-1}$) markedly inhibited the I_{sc} following stimulation with PGE_2 (Fig. 6A). In this set of experiments there was also evidence of inhibition of the spontaneous I_{sc} at this dose, but the effect of NPPB following stimulation with PGE_2 (ΔI_{sc}=–138±16 $\mu A\,cm^{-2}$, N=8) was significantly greater (P<0.001) than the effect of NPPB on the spontaneous I_{sc} (I_{sc}=–38±8 $\mu A\,cm^{-2}$), indicating a significant effect of NPPB on PGE_2-stimulated I_{sc}. Similarly, following stimulation with PGE_2, GlyH101 ($20\,\mu mol\,l^{-1}$) significantly (P<0.01) reduced the I_{sc} by 49±6 $\mu A\,cm^{-2}$ (Fig. 6D) whereas it had little effect on the I_{sc} in the unstimulated tissues (ΔI_{sc}=–5±2 $\mu A\,cm^{-2}$, N=5). In contrast, high concentrations of mucosal DIDS ($1\,mmol\,l^{-1}$) had a comparable effect on both the I_{sc} of PGE_2-stimulated tissues (–32±11 $\mu A\,cm^{-2}$, N=5) and the spontaneous I_{sc} (ΔI_{sc}=–18±12 $\mu A\,cm^{-2}$; Fig. 6B), as did mucosal glibenclamide ($250\,\mu mol\,l^{-1}$) (ΔI_{sc}-stimulated tissues=–19± 4 $\mu A\,cm^{-2}$; ΔI_{sc}-unstimulated tissues=–19±3 $\mu A\,cm^{-2}$, N=6; Fig. 6C).

In summary, $100\,\mu mol\,l^{-1}$ NPPB inhibited 80±9% and $20\,\mu mol\,l^{-1}$ GlyH101 inhibited 52±7% of the PGE_2-stimulated I_{sc}, whereas DIDS and glibenclamide had little effect on this current (Fig. 6E). The effects of GlyH101 and NPPB on the PGE_2-stimulated I_{sc} were dose dependent, although concentrations of 100 and $50\,\mu mol\,l^{-1}$ for NPPB and GlyH101, respectively, did not completely inhibit the PGE_2-stimulated I_{sc} (Fig. 6F). Consequently, only approximate IC_{50} values can be calculated from these partial dose response curves (GlyH101=22 $\mu mol\,l^{-1}$ and NPPB=29 $\mu mol\,l^{-1}$). These data suggest that in the possum ileum, CFTR is the apical conductance involved in electrogenic HCO_3^- secretion stimulated by cAMP-dependent secretagogues, such as PGE_2.

DISCUSSION

In eutherian mammals, electrogenic Cl^- secretion drives fluid secretion in the duodenum, jejunum and ileum (Field, 2003; Murek et al., 2010). This is dependent upon the basolateral NaK2Cl cotransporter, NKCC1, and the apical Cl^- channel, CFTR. NKCC1 drives the secretion of Cl^- by transporting Cl^- across the basolateral membrane and accumulating it in the secretory cells above its electrochemical equilibrium. Consequently, activation of CFTR provides an apical pathway for the exit of Cl^- from the cells and results in the active secretion of Cl^- into the lumen of the intestine. This provides a driving force for the passive movement of Na^+ and water and the net effect is the secretion of an isosmotic solution of NaCl (Field, 2003; Murek et al., 2010). In the ileum of the possum, a metatherian mammal, NKCC1 is expressed at relatively low levels compared with the eutherian intestine and cAMP and Ca^{2+}-dependent secretagogues do not stimulate electrogenic Cl^- secretion (Bartolo et al., 2009a). These secretagogues do, however, stimulate electrogenic HCO_3^- secretion in the ileum, which is driven by the pancreatic variant of the $NaHCO_3$ cotransporter, pNBCe1 (Bartolo et al., 2009b). Here we provide evidence that CFTR is essential for electrogenic HCO_3^- secretion in the possum ileum. Furthermore, because of the distribution and expression of CFTR in the possum small intestine, electrogenic anion secretion stimulated by cAMP-

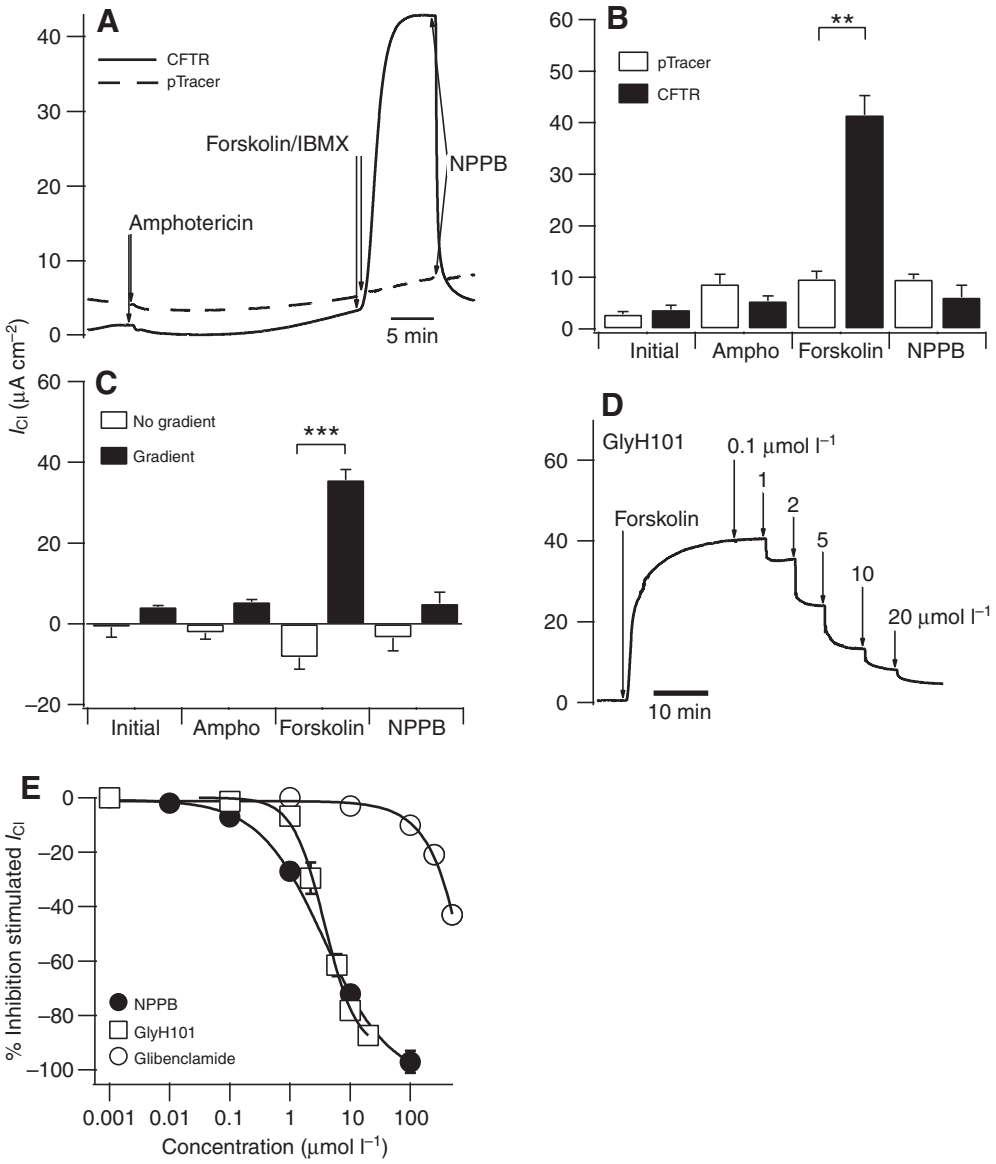

Fig. 5. Pharmacological characterisation of cloned pCFTR expressed in Fischer rat thyroid (FRT) cells. FRT cells stably expressing either pCFTR or the transfection vector (pTracer) alone were grown to confluency and then the monolayers were mounted in Ussing chambers and, in the presence of a transepithelial Cl^- gradient, the basolateral membrane was permeabilised with amphotericin (250 μg ml^{-1}). (A) Representative experiment showing that if the cells only expressed the vector, elevation of intracellular cAMP with mucosal and serosal forskolin (10 μmol l^{-1}) plus 3-isobutyl-1-methylxanthine (IBMX; 100 μmol l^{-1}) failed to stimulate an increase in Cl^- current (I_{Cl}). In contrast, in cells expressing pCFTR forskolin and IBMX stimulated a large increase in I_{Cl}, which was inhibited by 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (B) Summary of six experiments comparing the response of cells expressing the vector alone to forskolin and IBMX with that of cells stably expressing pCFTR. Ampho, steady-state I_{Cl} following the addition of amphotericin; Forskolin, steady-state I_{Cl} following the addition of forskolin and IBMX; Initial, steady-state I_{Cl} before the addition of amphotericin; NPPB, steady-state I_{Cl} following the addition of 100 μmol l^{-1} NPPB to the mucosal solution. (C) Summary of five experiments demonstrating that in the absence of a transepithelial Cl^- gradient forskolin and IBMX do not stimulate an increase in I_{Cl} across FRT monolayers stably expressing pCFTR. Labels on the abscissa are as in B. For B and C, asterisks indicate significant differences (**, P<0.01; ***, P<0.001) to control monolayers; unpaired Student's t-test. (D) Representative experiment showing the effect of increasing doses of mucosal N-(2-naphthalenyl)-[(3,5-dibromo-2,4-dihydroxyphenyl)methylene])glycine hydrazide (GlyH101) on the I_{Cl} stimulated by forskolin and IBMX in FRT cells stably expressing pCFTR. (E) Mean dose response curves for the inhibitory effect of NPPB, GlyH101 and glibenclamide on the I_{Cl} stimulated by forskolin and IBMX in FRT cells stably expressing pCFTR. The inhibitory effect of drugs expressed as percentage (%) inhibition of the forskolin and IBMX stimulated ICl by addition of NPPB, GlyH101 or glibenclamide. All values are means ± s.e.m., N=6. Curves were fit with the Hill equation. IC_{50}=3.7 μmol l^{-1} for NPPB and 3.6 μmol l^{-1} for GlyH101.

dependent secretagogues is restricted to the ileum with little evidence of either electrogenic Cl^- or HCO_3^- secretion in the duodenum or jejunum, where the expression of CFTR is significantly less than in the ileum.

In the possum small intestine, although there is evidence of expression of CFTR in the duodenum, jejunum and ileum, CFTR is expressed at much higher levels in the ileum compared with the other regions of the small intestine. This is markedly different from the expression of CFTR in the eutherian intestine, where the highest levels of expression occur in the duodenum with decreasing levels of expression in the jejunum and ileum (Ameen et al., 2000b; Strong et al., 1994; Trezise and Buchwald, 1991). Within the possum ileum,

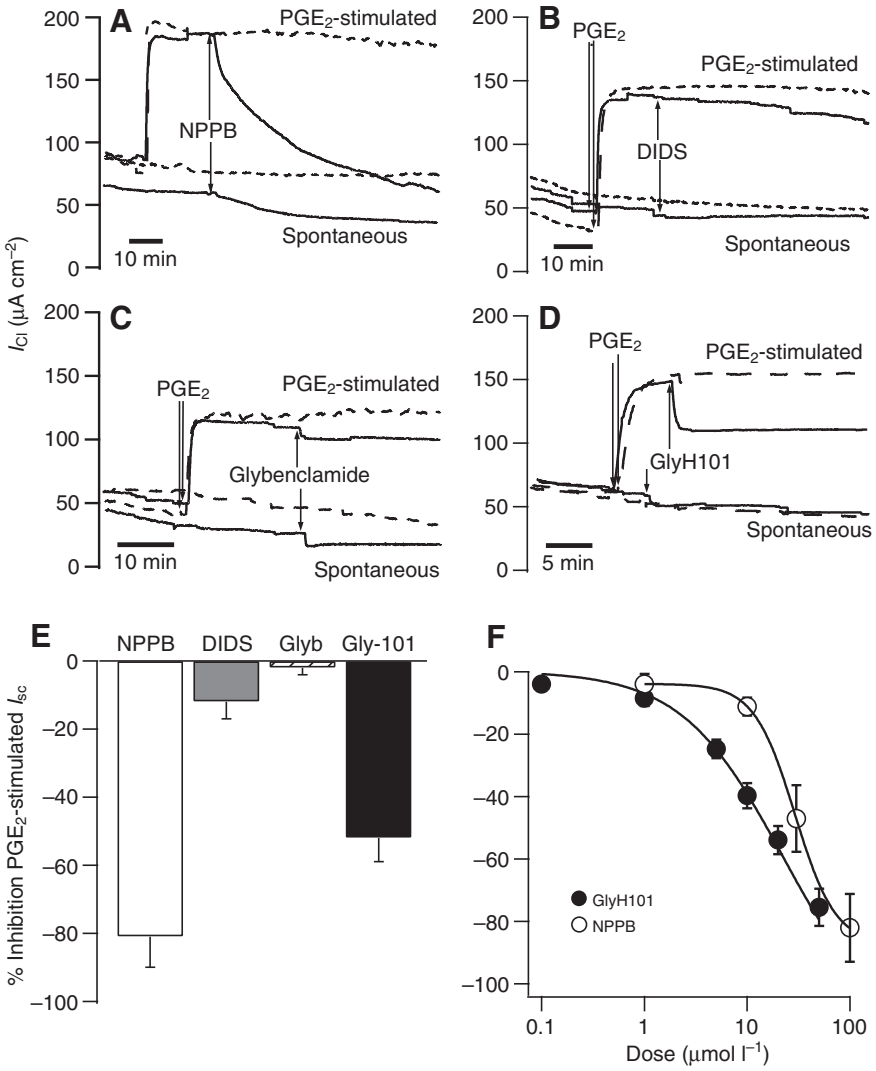

Fig. 6. The effect of mucosal addition of anion channel inhibitors on the PGE_2-stimulated I_{sc} in the possum ileum. In all experiments, four tissues from the same animal were mounted in Ussing chambers, pre-treated with serosal TTX ($1\,\mu mol\,l^{-1}$) and then two of the tissues were stimulated with PGE_2 ($1\,\mu mol\,l^{-1}$) in the continued presence of TTX. When the response to PGE_2 had reached steady state, one of the stimulated and one of the unstimulated tissues were treated with one of the anion blockers. The other two tissues served as time-match controls. The effect of (A) $100\,\mu mol\,l^{-1}$ mucosal NPPB, (B) $1\,mmol\,l^{-1}$ mucosal 4,4′-diisothiocyano-stilbene-2,2′-disulfonate (DIDS), (C) $250\,\mu mol\,l^{-1}$ mucosal glibenclamide and (D) $20\,\mu mol\,l^{-1}$ mucosal GlyH101 on the ileal I_{sc}. (E) Percentage (%) inhibition of the PGE_2-stimulated current by mucosal addition of NPPB, DIDS, glibenclamide (Glyb) and GlyH101. Values are means ± s.e.m., $N \geq 5$. The % inhibition of the PGE_2-stimulated I_{sc} was calculated as $[(\Delta I_{sc,PGE2X} - \Delta I_{sc,SponX})/\Delta I_{sc,PGE2}] \times 100$, where $\Delta I_{sc,PGE2X}$ is the change in I_{sc} induced by X (where X is either NPPB, DIDS, glibenclamide or GlyH101) in tissues stimulated with PGE_2, $\Delta I_{sc,SponX}$ is the change in spontaneous I_{sc} induced by X and $\Delta I_{sc,PGE2}$ is the change in I_{sc} induced by PGE_2. (F) Mean (±s.e.m., $N \geq 5$) cumulative dose response curve for the inhibition of the PGE_2-stimulated I_{sc} in the possum ileum by mucosal NPPB and GlyH101. Curves were fit with the Hill equation. IC_{50}=29 $\mu mol\,l^{-1}$ for NPPB and 22 $\mu mol\,l^{-1}$ for GlyH101.

CFTR is expressed in the crypts and base of the villi, with decreasing levels of expression along the villi and little or no expression in the cells at the tips of the villi. This is very similar to the expression pattern for CFTR in the crypt/villous axis of the eutherian small intestine (Ameen et al., 2000b; Strong et al., 1994; Trezise and Buchwald, 1991), although in the possum ileum we did not see any evidence of the CFTR high expresser (CHE) cells that have been reported in rats and humans (Ameen et al., 1995). However, these CHE cells are restricted to the proximal intestine of rats and humans and are absent in other eutherian mammals (Ameen et al., 2000a). In those cells of the possum ileum that express CFTR, it is localised to the apical membrane, consistent with a role in electrogenic anion secretion.

The $NaHCO_3$ cotransporter, which drives electrogenic anion secretion in the possum ileum (Bartolo et al., 2009b), is expressed in the same cells as CFTR within the ileal epithelium, but located in the basolateral membrane. The presence of CFTR and pNBCe1 in the same cells suggests that, in the possum ileum, electrogenic HCO_3^- secretion involves the transport of HCO_3^- across the basolateral membrane by the pNBCe1 and exit of HCO_3^- across the apical membrane *via* CFTR. This proposal is supported by the observation that inhibitors of cloned pCFTR also inhibit electrogenic anion secretion by the possum ileum. When expressed in the FRT cell line, a model epithelium (Sheppard et al., 1994), cloned pCFTR has a distinct inhibitor profile. The non-specific anion channel blocker, NPPB (Cabantchik and Greger, 1992), and the specific CFTR inhibitor, GlyH101 (Muanprasat et al., 2007), potently inhibit pCFTR. In contrast, glibenclamide, which until recently was the most effective CFTR inhibitor available (Hwang and Sheppard, 1999), is relatively ineffective whereas DIDS, primarily a blocker of Ca^{2+}-activated Cl^- channels (Hartzell et al., 2009) has no effect, even at very high concentrations. Except for the efficacy of NPPB, the pharmacological profile for pCFTR is comparable to that for CFTR from other species. DIDS is known not to inhibit CFTR from the external surface (Schultz et al., 1999) and, using the same method used in the present study, similar IC_{50} values for human CFTR were obtained for GlyH101 (Muanprasat et al., 2004) and glibenclamide (Ma et al., 2002). The IC_{50} values reported for the inhibition of CFTR by NPPB are quite variable, perhaps reflecting the voltage dependence of this block (Hwang and Sheppard, 1999). In general, however, they are of the order of 100–500 $\mu mol\,l^{-1}$ (Fryklund et al., 1993; Keeling et al., 1991) considerably higher than the IC_{50} of 3.7 $\mu mol\,l^{-1}$ obtained for pCFTR.

Consistent with the pharmacological profile obtained with cloned pCFTR, when these compounds were applied to the possum ileum, NPPB and GlyH101 inhibited the PGE_2-stimulated I_{sc}, whereas glibenclamide and DIDS had no effect. The IC_{50} values of both GlyH101 and NPPB were tenfold higher in the intact tissue when compared with their effect on cloned pCFTR expressed in FRT cells. However, similar discrepancies between the effect of these compounds in expression systems and native intestinal tissues have been reported for the eutherian intestine and are thought to result from diffusional barriers that limit the access of the inhibitors to the channel (Diener and Rummel, 1989; Greger et al., 1991). Given the location of CFTR at the base of the villi and in the crypts in the possum ileum, similar diffusional barriers are likely to occur in the possum.

A cellular model of CFTR-dependent electrogenic HCO_3^- secretion in the possum ileum is shown in Fig. 7. In addition to CFTR and pNBCe1, basolateral Na^+,K^+–ATPase and basolateral K^+ channels are also shown in the model. The Na^+,K^+–ATPase provides the driving force for the Na^+-dependent accumulation of HCO_3^- across the basolateral membrane, which is evident from the inhibitory affect of ouabain on the ileal secretory response (Bartolo et al., 2009a). Although pNBCe1 is likely to be electrogenic (Pushkin and Kurtz, 2006; Romero et al., 2004), the basolateral K^+ channels are necessary to maintain both charge and mass balance and the driving force for HCO_3^- exit across the apical membrane (Steward et al., 2005).

A potential limitation of the model is that pCFTR is primarily a Cl^- channel and it has a relatively low HCO_3^- permeability (P_{HCO3}:P_{Cl}≈0.25) (Demmers et al., 2010). A similar model to explain HCO_3^- secretion by the pancreatic duct of non-rodent eutherian mammals has been proposed (Park et al., 2010; Steward et al., 2005). CFTR from eutherian mammals also has a relatively low P_{HCO3} (Gray et al., 1993; Gray et al., 1990; Linsdell et al., 1997; O'Reilly et al., 2000; Poulsen et al., 1994). However, in the pancreatic duct cells it has been suggested that HCO_3^- secretion occurs *via* CFTR because there is no mechanism on the basolateral membrane to accumulate Cl^-, whereas there is a mechanism for HCO_3^- accumulation (Fernández-Salazar et al., 2004). As a result, following stimulation of CFTR, the intracellular Cl^- activity ($[Cl^-]i$) rapidly falls and approaches its equilibrium value, thus there is no driving force for Cl^- exit across the apical membrane *via* CFTR (Ishiguro et al., 2002a). However, through the activity of the basolateral $NaHCO_3$ cotransporter and, to a lesser extent, hydration of CO_2, the intracellular HCO_3^- activity ($[HCO_3^-]i$) is maintained above its equilibrium value and so HCO_3^- is secreted *via* CFTR (Ishiguro et al., 1996; Ishiguro et al., 2002b). In addition, it has recently been demonstrated that there is a change in selectivity of CFTR associated with stimulation of HCO_3^-secretion, with P_{HCO3}:P_{Cl} increasing from 0.3–0.5 to 1.5 (Park et al., 2010). This is also dependent upon the fall in $[Cl^-]i$ that accompanies the stimulation of secretion and subsequent activation of Cl^--dependent kinases (Park et al., 2010). Similar Cl^--dependent mechanisms may be operating in the possum ileum. NKCC1, which is the main mechanism for Cl^- accumulation in secretory epithelia (Gamba, 2005), is expressed at very low levels in the possum ileal epithelium (Bartolo et al., 2009a). Thus activation of CFTR is likely to result in a fall in $[Cl^-]i$ whereas pNBCe1, which is expressed at high levels in the ileal secretory cells (Bartolo et al., 2009b), will maintain $[HCO_3^-]i$. This, however, will need to be confirmed through measurements of $[Cl^-]i$, as intracellular Cl^- accumulation can also occur in intestinal epithelia through Cl^-–HCO_3^- exchange (Gawenis et al., 2010; Walker et al., 2002).

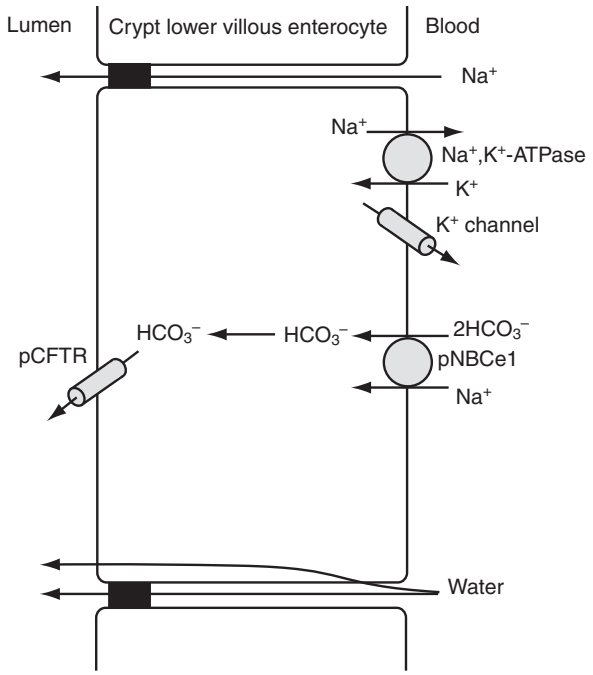

Fig. 7. Proposed cellular model of electrogenic anion secretion in the epithelial cells of the crypt and lower villous region of the possum ileum.

Although there was clear evidence of electrogenic HCO_3^- secretion in the possum ileum, there was little evidence that significant electrogenic HCO_3^- or Cl^- secretion occurred in the duodenum and jejunum of the possum. The spontaneous ileal I_{sc} was significantly higher than that of the duodenum and jejunum and, as seen previously (Bartolo et al., 2009a; Bartolo et al., 2009b), serosal TTX inhibited the spontaneous I_{sc} in the ileum, whereas it had no effect in the duodenum and jejunum. In the eutherian small intestine, the inhibition of the spontaneous I_{sc} by TTX is associated with the inhibition of intestinal anion secretion stimulated by submucosal nervous activity (Grubb, 1995; Grubb and Gabriel, 1997). In addition, PGE_2, which stimulated a large sustained increase in I_{sc} by the possum ileum (Bartolo et al., 2009a; Bartolo et al., 2009b), had little effect on the I_{sc} of either the duodenum or jejunum. It is possible that the limited effect of PGE_2 in the duodenum and jejunum is due to the absence of the appropriate PGE_2 receptors in these two tissues. However, forskolin, which elevates intracellular cAMP through direct activation of adenylate cyclase (Seamon et al., 1981), also did not stimulate an appreciable increase in I_{sc} in the duodenum and jejunum.

Given the demonstrated role of CFTR in electrogenic HCO_3^- secretion in the possum ileum, the much lower levels of expression of CFTR in the duodenum and jejunum provide a reasonable explanation for the absence of appreciable electrogenic Cl^- or HCO_3^- secretion stimulated by cAMP-dependent secretagogues in these tissues. However, an important function of HCO_3^- secretion in the duodenum is protection of the duodenal epithelium from the acidic chyme delivered from the stomach into the small intestine (Allen and Flemstrom, 2005). In eutherian mammals, duodenal secretion consists of a mixture of CFTR-dependent electrogenic Cl^- and HCO_3^- secretion and HCO_3^- secretion in association with Cl^- absorption (Clarke and Harline, 1998; Seidler et al., 1997; Singh et al., 2008). The main Cl^-–HCO_3^- exchangers in the apical membrane

of the eutherian duodenum are SLC26A3 and SLC26A6 (Jacob et al., 2002; Singh et al., 2008; Tuo et al., 2006; Walker et al., 2009). However, SLC26A6 contributes little to HCO_3^- secretion (Wang et al., 2005) whereas SLC26A3 is responsible for ~60% of the spontaneous HCO_3^- secretion and ~50% of the cAMP-stimulated secretion, CFTR and a small paracellular leak accounting for the remainder (Walker et al., 2009). It is generally accepted that SLC26A6 is electrogenic (Jiang et al., 2002; Ko et al., 2002; Xie et al., 2002) with an apparent stoichiometry of $1Cl^-$:$2HCO_3^-$ (Shcheynikov et al., 2006), and early evidence suggested that SLC26A3 was also electrogenic, but with a stoichiometry of $2Cl^-$:$1HCO_3^-$, opposite that of SLC26A6 (Ko et al., 2002; Shcheynikov et al., 2006). However, more recent evidence indicates that SLC26A3 is electroneutral (Alper et al., 2010; Lamprecht et al., 2005; Melvin et al., 1999). Both SLC26A3 and SLC26A6 are expressed in the possum intestine (Gill et al., 2009), but the very low basal I_{sc} in the possum duodenum suggests that if basal HCO_3^- secretion does occur, the overall process is electroneutral. Similarly, the minimal increase in I_{sc} stimulated by PGE_2 or forskolin suggests that any HCO_3^- secretion stimulated by these secretagogues is also electroneutral. Both processes may involve SLC26A3 or, as has been suggested for eutherian epithelia (Shcheynikov et al., 2006), simultaneous activities of equal magnitude of SLC26A3 and SLC26A6. Because of their opposite stoichiometries, this would result in electroneutral transport.

In the absence of appreciable CFTR expression in the possum duodenum, HCO_3^- secretion dependent on Cl^-–HCO_3^- exchange must provide sufficient protection against the gastric acid. If this involves the accumulation of HCO_3^- across the basolateral membrane by pNBCe1 it would provide an explanation for the expression of pNBCe1 in the duodenum at levels comparable to that seen in the ileum. It is notable that in the possum ileum, CFTR expression is limited to the crypts and the base of the villi, whereas pNBCe1 expression extends further towards the tip of the villi. In eutherian mammals, the SLC26 transporters associated with HCO_3^- secretion are expressed primarily in the villi (Simpson et al., 2007; Wang et al., 2002). Furthermore, HCO_3^- secretion through Cl^-–HCO_3^- exchange also occurs in the ileum and proximal colon of eutherian mammals (Knickelbein et al., 1985; Mugharbil et al., 1990; Rajendran and Binder, 1993) and is an important mechanism of HCO_3^- secretion in other vertebrates, particularly marine teleosts (Grosell, 2006). Therefore, it is possible that ileal HCO_3^- secretion involves both CFTR-dependent and SLC26-dependent secretion.

The limited expression of CFTR in the duodenum and jejunum not only accounts for the absence of appreciable levels of electrogenic HCO_3^- secretion in the duodenum and jejunum, but also the lack of cAMP-stimulated electrogenic Cl^- secretion in these regions. However, the expression of NKCC1 is also very low in the duodenum and jejunum. This would limit electrogenic Cl^- secretion in these regions of the intestine, even if CFTR were present in the apical membrane, as this transporter generally drives electrogenic Cl^- secretion by accumulating Cl^- in the epithelial cells (Gamba, 2005). Indeed, the expression patterns of NKCC1 and CFTR suggest that electrogenic Cl^- secretion does not occur in any region of the possum small intestine. This is surprising. As noted above, in eutherian mammals it is generally accepted that intestinal fluid secretion has a number of significant roles in intestinal function (Barrett and Keely, 2000), and invariably intestinal fluid secretion is driven primarily by electrogenic Cl^- secretion (Murek et al., 2010). Support for this comes from the effect of cystic fibrosis in humans or in animal models. In both instances, electrogenic Cl^- secretion is reduced or absent in the intestine (Grubb, 1997; Oloughlin et al., 1991; Seidler et al., 1997) and intestinal function is compromised. In cystic fibrosis patients this is evident as a high incidence of meconium ileus at birth (Taylor and Hardcastle, 2006) and distal intestinal obstruction in older patients (Dray et al., 2004). In mouse models of cystic fibrosis, intestinal function is compromised to such an extent that death results, usually just after weaning (Grubb and Gabriel, 1997). Given the importance of fluid secretion in the small intestine, it is possible that in the possum small intestine electrogenic Cl^- secretion involves other transport mechanisms and is modulated by other secretagogues. In the duodenum of NKCC1 knockout mice, the parallel activity of a basolateral $NaHCO_3$ cotransporter and Cl^-–HCO_3^- exchanger drives Cl^- secretion (Walker et al., 2002). Also, in the duodenum and jejunum of the possum, a Cl^- channel other than CFTR, such as the Ca^{2+}-activated Cl^- channel TMEM16A (Caputo et al., 2008), could provide a route for apical Cl^- exit from the epithelial cell, although it should be noted that a range of Ca^{2+}-dependent secretagogues stimulated HCO_3^- secretion but not Cl^- secretion in the possum ileum (Bartolo et al., 2009a). Alternatively, in the possum, gastric, pancreatic and biliary secretions combined with selective absorption of solutes may be sufficient to define the composition of the luminal contents for efficient digestion and absorption of ingested food. In relation to this, it is notable that the effect of reduced secretion in the animal models of cystic fibrosis is concentrated in the terminal portion of the ileum (Delaney et al., 1996; Snouwaert et al., 1992; Zeiher et al., 1995) and the proximal colon (Snouwaert et al., 1992). This suggests that, in the absence of intestinal secretion, fluid delivered from the stomach into the upper regions of the small intestine is sufficient to hydrate the intestinal mucus and provide an aqueous environment for digestion and absorption, whereas in the terminal region of the small intestine, intestinal secretion has a more significant role.

In conclusion, CFTR plays a significant role in defining intestinal secretion by the possum intestine. In the ileum, CFTR is expressed at high levels in the apical membrane of the crypt cells and the cells at the base of the villi and provides an apical anion conductance for electrogenic HCO_3^- secretion. In contrast, low levels of expression of CFTR in the duodenum and jejunum limit cAMP-stimulated electrogenic Cl^- and HCO_3^- secretion in these regions of the possum small intestine, and suggest that in the possum either intestinal fluid secretion is not essential for efficient digestion and absorption of nutrients in the duodenum and jejunum or alternative mechanisms of fluid secretion exist. This differs markedly to the situation in eutherian mammals, where fluid secretion, driven primarily by electrogenic Cl^- secretion, occurs throughout the small intestine and has an essential role in intestinal function.

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