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Secretagogues stimulate electrogenic HCO_3^- secretion in the ileum of the brushtail possum, *Trichosurus vulpecula*: evidence for the role of a Na^+/HCO_3^- cotransporter

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SUMMARY

Fluid secretion is essential for intestinal function and, in eutherian mammals, is driven by electrogenic Cl^- transport, which is dependent upon a bumetanide-sensitive, basolateral $Na^+/K^+/2Cl^-$ cotransporter, NKCC1. However, ileal secretion in the brushtail possum, a marsupial, involves a fundamentally different process, since NKCC1 expression is low in this tissue and the secretagogue-induced short circuit current (I_{sc}) is insensitive to bumetanide. In view of these differences we have investigated the basis of the secretory response of the possum ileum. In the Ussing chamber the secretory I_{sc} is independent of Cl^- but dependent upon Na^+ and serosal HCO_3^-/CO_2, suggesting that secretagogues stimulate electrogenic HCO_3^- secretion. In agreement with this, serosal DIDS (4,4′-diisothiocyano-stilbene-2,2′-disulfonate; 1 mmol l^{-1}) inhibited the secretory response. However, acetazolamide (1 mmol l^{-1}) and serosal amiloride (1 mmol l^{-1}) had little effect, indicating that HCO_3^- secretion is driven by HCO_3^- transport from the serosal solution into the cell, rather than hydration of CO_2 by carbonic anhydrase. Consistent with this the pancreatic variant of the electrogenic Na^+/HCO_3^- cotransporter (pNBC) is highly expressed in the ileal epithelium and is located in the basolateral membrane of the epithelial cells, predominantly in the mid region of the villi, with lower levels of expression in the crypts and no expression in the villous tips. We conclude that the secretory response of the possum ileum involves electrogenic HCO_3^- secretion driven by a basolateral pNBC and that the ileal HCO_3^- secretion is associated with a specialised function of the possum ileum, most probably related to hindgut fermentation.

Key words: brushtail possum, *Trichosurus vulpecula*, marsupial, ileum, bicarbonate secretion, $NaHCO_3$ cotransport.

INTRODUCTION

Fluid secretion in the small intestine maintains the composition of the luminal contents in order to optimise digestion and the absorption of nutrients, and is driven by the net movement of salt into the lumen, thus creating an osmotic gradient for the flow of water (Halm and Frizzell, 1990). In the duodenum and ileum of eutherian mammals, this transport mechanism is primarily driven by epithelial Cl^- secretion, but HCO_3^- transport also occurs and functions to maintain pH and the composition of the luminal fluid (Minhas et al., 1993; Seidler et al., 1997; Spiegel et al., 2003). The HCO_3^- secretion consists of two components. The first results from the absorption of Cl^-, and involves the exchange of luminal Cl^- for intracellular HCO_3^- across the apical membrane (Spiegel et al., 2003). This anion exchange is thought to be mediated by the SLC26 family of transporters, although their role in intestinal secretion is somewhat controversial (for a review, see Dorwart et al., 2007). The second component of intestinal HCO_3^- secretion in eutherian mammals is associated with electrogenic Cl^- secretion, which drives intestinal fluid secretion in these mammals *via* the cystic fibrosis transmembrane conductance regulator (CFTR) channel (Poulsen et al., 1994). Secretagogues that stimulate Cl^- secretion *via* CFTR, also stimulate electrogenic HCO_3^- secretion, but the HCO_3^- component of secretion is generally significantly less than that for Cl^- (Minhas et al., 1993; Seidler et al., 1997; Spiegel et al., 2003). Although it has recently been shown that duodenal HCO_3^- secretion can also occur independently of CFTR (Sellers et al., 2008), it is notable that in humans suffering from cystic fibrosis and in CFTR knockout mice, both Cl^- and HCO_3^- secretion are severely compromised (Oloughlin et al., 1991; Grubb, 1997).

The first step in electrogenic Cl^- secretion is the entry of Cl^- into the cell across the basolateral membrane of the epithelial cells, through the activity of the $Na^+/K^+/2Cl^-$ cotransporter (NKCC1) (Halm and Frizzell, 1990). The activity of this transporter accumulates Cl^- in the cell above its electrochemical equilibrium and, as a result, activation of the apical Cl^- channel, CFTR, results in the secretion of Cl^- across the apical membrane (Welsh et al., 1983).

The ileum of the Australian common brushtail possum, *Trichosurus vulpecula*, which is a metatherian mammal, expresses low levels of the NKCC1 and neither cAMP- nor Ca^{2+}-dependent secretagogues stimulate electrogenic Cl^- secretion (Bartolo et al., 2009). This differs markedly from the situation in the ileum of eutherian mammals, were a high level of NKCC1 expression in the crypt cells is associated with the secretion of Cl^-, which drives intestinal fluid secretion (Field, 2003). Despite the low levels of expression of NKCC1 in the possum, secretagogues do stimulate a secretory response. This response is independent of Cl^- and is not inhibited by bumetanide (Bartolo et al., 2009), a known inhibitor of NKCC1 (Gamba, 2005). However, it is dependent upon HCO_3^-, which suggests it may involve electrogenic HCO_3^- secretion (Bartolo et al., 2009).

The HCO_3^- that is secreted by epithelia can be derived either from the hydration of CO_2 by carbonic anhydrase within the cell or the transport of HCO_3^- across the basolateral membrane. The electrogenic Na^+/HCO_3^- cotransporter, NBCe1, is a member of the bicarbonate transporter family and is involved in sodium-dependent bicarbonate

transport across the basolateral membrane of both absorptive and secretory epithelia (Gross and Kurtz, 2002). NBCe1 has two major variants that are spliced from the *SLC4A4* gene using different promoter sites (Abuladze et al., 2000); NBCe1-A was first cloned from the kidney of salamander (Romero et al., 1997) and human (Burnham et al., 1997), while NBCe1-B was cloned from human pancreas (Abuladze et al., 1998). Human NBCe1-A and -B are identical over the 994 amino acids at the C-terminus, but have unique N-terminal sequences and tissue expression varies (Abuladze et al., 2000). The two variants are generally referred to as kNBC and pNBC, which denotes the tissue from which they were first isolated, kidney and pancreas, respectively, and they are localised on the basolateral membrane of epithelial cells (Marino et al., 1999; Schmitt et al., 1999). The direction of bicarbonate transport is determined by the stoichiometry of the different transporters, which is thought to be tissue dependent, and the gradients for Na^+ and HCO_3^- across the basolateral membrane (Gross et al., 2001). In general, pNBC is recognised as a secretory variant, whereas kNBC as an absorptive variant.

We have previously shown that the electrogenic secretory response of the possum ileum does not involve Cl^- secretion, and proposed that it involves HCO_3^- secretion (Bartolo et al., 2009). In the current study we have tested this proposal. We have used the Ussing technique to identify the source of HCO_3^- secreted by the possum ileum, discriminating between the transport of HCO_3^- across the basolateral membrane and hydration of CO_2 by carbonic anhydrase, and provided a preliminary identification of the transporter involved by characterising the pharmacology of the secretory response. Also we have cloned and sequenced possum p- and kNBC and determined their distribution within the ileal epithelium in order to identify the specific variant involved in the secretory response of this tissue in the common Australian brushtail possum, a metatherian mammal.

MATERIALS AND METHODS

Animals and tissue collection and preparation

Adult Australian common brushtail possums, *Trichosurus vulpecula* Kerr 1792, were used in this study. They had a live weight greater than 2 kg, and were trapped and housed as previously described (Butt et al., 2002b). All experimental procedures were performed with the approval of the AgResearch Invermay and Otago University Animal Ethics Committees according to the Animal Welfare Act, 1999. Male and female animals were used in this study to exclude any sex bias. All tissues used in the current study were prepared as previously described (Bartolo et al., 2009).

Measurements of epithelial transport

The secretory response was quantified as the change in short circuit current (I_{sc}) in response to prostaglandin E_2 (PGE_2), forskolin or carbachol (CCH). To reduce variability in the responses, all tissues were pre-treated with 1 μmol l^{-1} serosal tetrodotoxin (TTX) to inhibit neurally mediated spontaneous secretion (Bartolo et al., 2009). I_{sc} and transepithelial resistance (R_t) were determined as previously described (Butt et al., 2002b; Butt et al., 2002a). Briefly, after removal of the ileum from the animal and flushing of the luminal contents, the underlying muscle and connective tissue were removed by blunt dissection and the epithelium mounted as a flat sheet in the Ussing chamber and constantly short-circuited. The Ringer's solutions employed in the Ussing chamber experiments are summarised in Table 1. TTX was purchased from Alomone Labs. (Jerusalem, Israel), while all other reagents were purchased from Sigma Aldrich (St Louis, MO, USA). Concentrated stocks of TTX and CCH were prepared in deionised water, forskolin in dimethyl sulphoxide (DMSO), PGE_2 in ethanol and 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA) in methanol. 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. Acetazolamide was dissolved in a $NaHCO_3$ solution and this solution was used to prepare a Ringer's solution containing 1 mmol l^{-1} acetazolamide. DIDS was prepared as a 10 mmol l^{-1} stock in Ringer's solution (hereafter referred to as Ringer's) and this was used to replace the appropriate volume of Ringer's in the reservoirs, to give a final concentration of 1 mmol l^{-1}. Amiloride was added as a small aliquot of dry powder directly to the serosal chamber to give a final concentration of 1 mmol l^{-1}.

Cloning and expression of pancreatic and kidney NBC isoforms

RNA isolation and cDNA synthesis were as described previously (Bartolo et al., 2009). RACE PCR was carried out using a GeneRacer RACE kit (3′ RACE; Invitrogen, Auckland, New Zealand) or a BD SMART RACE kit (5′ RACE; Clontech Laboratories, Mountain View, CA, USA) according to the manufacturers protocol. Since NBC variants can be involved in either absorption or secretion of HCO_3^-, it was important to determine if both p- and kBNC were expressed in the possum ileum.

Primers (NBC-For 1 and NBC-Rev 1, see Table 2 for a summary of primer sequences) were designed from highly conserved regions of the pNBC from human (acc. no. AF011390.1), rat

Table 1. Composition of the Ringer's solutions (in mmol l^{-1}) employed in the Ussing chamber studies

Solution	1 $NaCl/HCO_3^-$	2 Cl^- free	3 HCO_3^- free	4 Na^+ free
NaCl	110	0	110	0
Sodium gluconate	0	110	25	0
N-methyl-D-glucamine Cl	0	0	0	110
KCl	5	0	5	5
Potassium gluconate	0	5	0	0
$MgSO_4$	0.5	0.5	0.5	0.5
$CaCl_2$	1	0	1	1
Calcium gluconate	0	1	0	0
$NaHCO_3$	25	25	0	0
Choline bicarbonate	0	0	0	25
Hepes/Tris	10	10	10	10
Pyruvate/glutamine	4	4	4	4
Gas	95%O_2: 5%CO_2	95%O_2: 5%CO_2	100%O_2	95%O_2: 5%CO_2
pH	7.4	7.4	7.4	7.4

Table 2. PCR primer sequences for amplification of possum NBCs

Primer name	Primer sequence (5′–3′)
Primers for cloning and expression	
NBC-For 1	ATCAGTGATTTTGCCATTAT
NBC-Rev 1	ATGTGAGCAATGGAGATGAC
NBC-For 2	GCCTTGCTGGTCACCATCCTGATT
NBC-Rev 2	TGGGCTACTTGCACTGGAGA
pNBC-For 1	CAARCTGGAGGAGCGACGGAAG
kNBC-For 1	ATCCCACCWRTSTTTAACC
kNBC-Rev 1	CCGAGGTAAAAATACTACGGTTAAAGA
pNBC-For 2	ATGGAGGATGAAGCCGTCCTG
kNBC-For 2	ACCAAATTGAAGCTGGGATCT
Primers for *in situ* probe	
Forward	TGGAGGATGAAGCTGTCCTG
Reverse	TGAGTGGTTTGAGGATGCTAC

(AF107265.1), mouse (NM_018760.2) and rabbit (AF149418) that are common to the respective kNBC sequence of each species. Using ileal cDNA as a template, these primers amplified a 377 bp cDNA that was then used to design possum-specific primers for RACE PCR. The NBC-For 2 primer was used in 3′ RACE PCR using ileal cDNA made with the GeneRacer RACE kit (Invitrogen, Auckland, New Zealand). Attempts to amplify the 5′ end of NBC from the possum ileum using 5′ RACE were unsuccessful. However, we designed a forward primer, pNBC-For 1, on a highly conserved region in the 5′ UTR of human and mouse pNBC sequences. This primer was then used with NBC-Rev 2 in PCR to amplify a 711 bp amplicon of pNBC.

Since it was probable that kNBC was expressed at low levels in intestinal tissues (Seidler et al., 2000; Praetorius et al., 2001), we amplified and sequenced kNBC using possum kidney cDNA. A forward primer (kNBC-For 1) was designed within the coding region of possum, *Monodelphis domestica* kNBC from sequence data obtained from the Ensembl genome database (www.ensembl.org/Monodelphis_domestica). This primer was used with the NBC-Rev 2 primer in PCR, and produced an amplicon of the expected size (498 bp) that was used to design a primer for use in 5′ RACE PCR. The 5′ end of possum kNBC was amplified using the kNBC-Rev 1 and the BD SMART RACE kit (Clontech Laboratories), according to the manufacturer's instructions.

All RACE PCRs were carried out using a touchdown PCR program, as per the BD SMART RACE kit instructions (Clontech Laboratories). All other PCR reactions were carried out using the following cycling parameters: initial denaturation at 94°C for 60 s, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 60 s. A final extension was carried out at 72°C for 10 min. The resultant products were sequenced and submitted to the NCBI GenBank. Alignments of possum NBC sequences were carried out to determine identity with human sequences using ClustalW2 (http://www.ebi.ac.uk/tools/clustalw/).

To determine which variants of NBC where expressed in the possum ileum, forward primers that targeted the unique 5′ ends of p- and kNBC were used, whereas the reverse primer targeted a region of sequence common to both possum p- and kNBC. The forward primers were pNBC-For 2 and kNBC-For 2 and the reverse primer was NBC-Rev 2 (Table 2).

Western blot analysis of NBC

Crude membrane extracts were obtained as described previously (Bartolo et al., 2009) and the samples separated by SDS-polyacrylamide gel electrophoresis in 7.5% polyacrylamide gels and electroblotted onto a PVDF membrane (GE Healthcare Biosciences, Auckland, NZ). 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 a 1:2000 dilution of rabbit anti-rat NBC serum for 2 h. The NBC antiserum was kindly donated by Dr Bernard Schmitt, Department of Physiology, Otago University, New Zealand (Schmitt et al., 1999). Membranes were then washed with TBS-NP40 and incubated with 1:6000 donkey anti-rabbit horseradish-peroxidase-conjugated secondary antibody (GE Healthcare Biosciences, Auckland, NZ) for 2 h. The specificity of the NBC antibody was determined by preabsorbtion with the control peptide for 1 h prior to incubating the membranes. Antibodies were detected using enhanced chemiluminescence (GE Healthcare Biosciences, Auckland, NZ).

Localisation of pNBC mRNA by in situ hybridisation

Cellular localisation of pNBC mRNA in the ileum was determined using an *in situ* hybridisation (ISH) protocol as described previously (Bartolo et al., 2009). The probe used for ISH experiments was amplified from possum ileum cDNA using a primer pair (see Table 2) that generated a 252 bp cDNA that was cloned using the Promega pGem® T Easy vector system (Promega Corporation, Madison, WI, USA). [^{33}P]dUTP (NZ Scientific, Auckland, New Zealand)-labelled sense and antisense probes were transcribed using an *in vitro* transcription kit (Riboprobe® *in vitro* transcription systems, Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Hybridisation and detection of the probe were carried out as described previously (Bartolo et al., 2009).

Immunolocalisation of NBC

Ileal tissue samples were embedded in OCT compound (Siemens Medical Solutions, Erlangen, Germany) and snap frozen in isopentane that was pre-cooled in liquid nitrogen. Sections (10 μm) were placed on slides coated with 2% 3-aminopropyltiethoxysilane (Sigma Aldrich, St Louis, MO, USA), 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 (in mmol l^{-1}; 137 NaCl, 8.1 Na_2HPO_4 and 1.9 KH_2PO_4, 2.7 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 rabbit anti-rat NBC (diluted 1:400 in PBS) for 2 h. The sections were then washed in PBS and incubated with FITC-conjugated donkey anti-rabbit IgG (diluted 1:200 in PBS; Jackson ImmunoResearch Laboratories, PA, USA) for 2 h, followed by incubation with DAPI (Sigma Aldrich; 100 ng ml^{-1} in PBS) for 20 min. The sections were then washed in PBS and mounted in Vectashield® mounting medium (Vector Laboratories, Burlingame, CA, USA). The specificity of staining was determined by preabsorbing the primary antibody with the control peptide for 1 h before adding the antibody to the sections. 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 the mean ± standard error (s.e.m.); *N*, number of animals. 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 to be statistically significant where $P<0.05$.

RESULTS

Ion dependence of secretory response

Previously we have demonstrated that in the possum ileum, secretagogues stimulate an increase in I_{sc} that is dependent upon HCO_3^-, but independent of Cl^-, using Ussing chamber experiments (Bartolo et al., 2009). The total independence of Cl^- eliminates the possibility that the increase in I_{sc} induced by secretagogues in the possum ileum involves Cl^- secretion driven by a HCO_3^--dependent process. Rather it suggests that the response involves HCO_3^- secretion. To investigate this further, we considered the ion dependence of this process in more detail. Firstly, we confirmed that the secretory response was independent of Cl^-, as shown previously (Bartolo et al., 2009).

Symmetrical replacement of Cl^- with the impermeant anion gluconate in the Ringer's solution (solution 2; Table 1) had little effect on the response to PGE_2 or CCH (Fig. 1), which stimulate I_{sc} *via* increases in intracellular cAMP and Ca^{2+}, respectively. PGE_2 resulted in a sustained increase in I_{sc} of comparable magnitude in tissues bathed in both NaCl and sodium gluconate Ringer's. Similarly, CCH stimulated a transient increase in I_{sc} of comparable magnitude (Fig. 1) and duration (control, 8.3±0.6 min; Cl^--free Ringer's 7.6±0.7 min) in tissues bathed in the two Ringer's solutions. By contrast, the responses to PGE_2 and CCH were highly dependent upon HCO_3^-/CO_2, since the replacement of HCO_3^-/CO_2 (solution 3; Table 1) in both the mucosal and serosal bathing solutions markedly reduced the secretagogue-stimulated increases in I_{sc} (Fig. 2). However, this effect was entirely dependent on the replacement of HCO_3^-/CO_2 on the serosal side of the tissue. The removal of HCO_3^-/CO_2 from the mucosal solution alone had little effect on the response to either PGE_2 or CCH, whereas removal of serosal HCO_3^-/CO_2 inhibited the responses to a comparable extent as removal of both mucosal and serosal HCO_3^-/CO_2. The secretory responses were also profoundly dependent upon Na^+, since the replacement of Na^+ in the mucosal and serosal bathing solutions with *N*-methyl-D-glucamine (nMDG; solution 4; Table 1) markedly inhibited both the cAMP-stimulated and the Ca^{2+}-stimulated increase in I_{sc} (Fig. 3). Collectively, these data indicate that secretagogues stimulate a Cl^--independent, but Na^+- and HCO_3^--dependent increase in I_{sc} in the ileum of the possum, consistent with electrogenic HCO_3^- secretion.

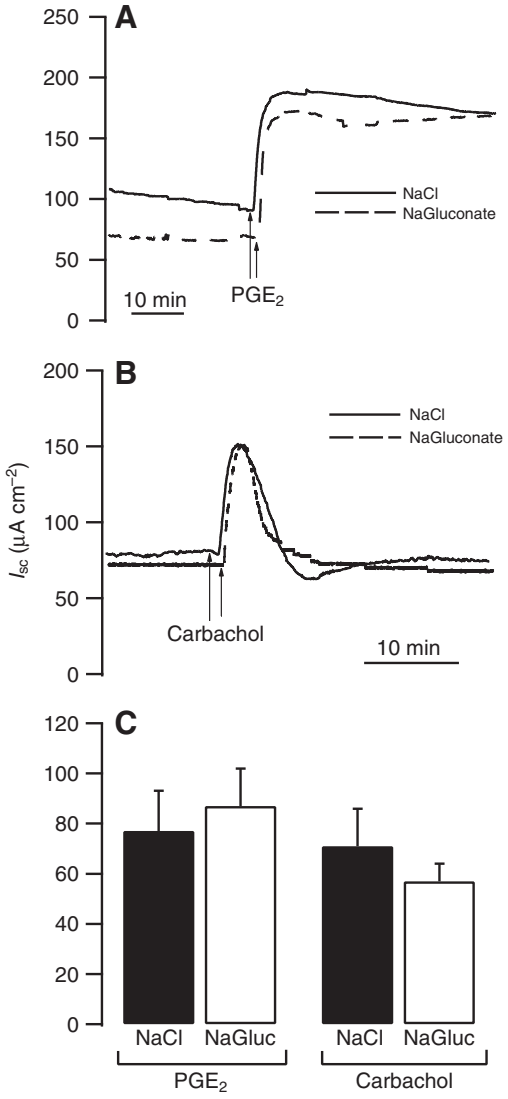

Fig. 1. Symmetrical (mucosal and serosal) replacement of Cl^- with the impermeant anion gluconate (Gluc, solution 2, Table 1) does not affect the short circuit current (I_{sc}) stimulated by PGE_2 (1 μmol l^{-1}, serosal) or carbachol (100 μmol l^{-1}, serosal). (A,B) Representative experiments showing the effect of Cl^- replacement with sodium gluconate on the response to PGE_2 (A) or carbachol (B). (C) Summary of the effects of Cl^- replacement with gluconate on the response to PGE_2 and carbachol. All values are mean ± s.e.m., *N*=8 for each.

The pharmacology of the HCO_3^- secretory response

The HCO_3^- secreted by epithelia is either transported into the cell across the basolateral membrane by Na^+/HCO_3^- cotransporters (NBC) or generated within the cell through the hydration of CO_2 by carbonic anhydrase (Steward et al., 2005). To determine the contribution of these two processes to the secretion of HCO_3^- in the possum ileum, we first investigated the effect of acetazolamide, which inhibits the hydration of CO_2 by carbonic anhydrase (Wu et al., 1998), on the I_{sc} stimulated by PGE_2 and CCH. Paired tissues from each animal were mounted in the Ussing chamber and one was incubated with 1 mmol l^{-1} mucosal and serosal acetazolamide for 60 min to inhibit carbonic anhydrase activity. The tissues were then stimulated with either PGE_2 or CCH. In the presence of acetazolamide, PGE_2 stimulated a sustained increase in I_{sc} of comparable magnitude to that seen in the control tissues (ΔI_{sc} control=81±15 μA cm^{-2}, acetazolamide=76±11 μA cm^{-2}; *N*=8). Similarly, the change in I_{sc} stimulated by CCH following pre-treatment with acetazolamide was of similar magnitude (ΔI_{sc}=55±9 μA cm^{-2}) as in the absence of acetazolamide (ΔI_{sc}=61±10 μA cm^{-2}) and both responses were of comparable duration (duration control, 10±1.1 min; duration plus acetazolamide, 10±0.7 min, *N*=7). Thus acetazolamide, at a dose in excess of that shown to inhibit carbonic anhydrase-dependent HCO_3^- secretion in the eutherian pancreas (Cheng et al., 1998), had no effect on the secretory current in the possum ileum, suggesting that the hydration of CO_2 contributes little to HCO_3^- secretion in the ileum. This was supported by the observation that the addition of serosal amiloride also had little effect on the PGE_2-stimulated response. The hydration of CO_2 by carbonic anhydrase generates both HCO_3^- and H^+ ions and the extrusion of the H^+ ions across the basolateral membrane by the Na^+/H^+ exchanger NHE1, maintains the driving force for HCO_3^- secretion (Novak and Greger, 1988). However, 1 mmol l^{-1} serosal amiloride, which inhibits NHE1 activity (Harris and Fliegel, 1999), had little effect on the PGE_2-stimulated increase in I_{sc} (Fig. 4). Similar results were obtained following stimulation with forskolin and with the use of EIPA (100 μmol l^{-1}, serosal; data not shown),

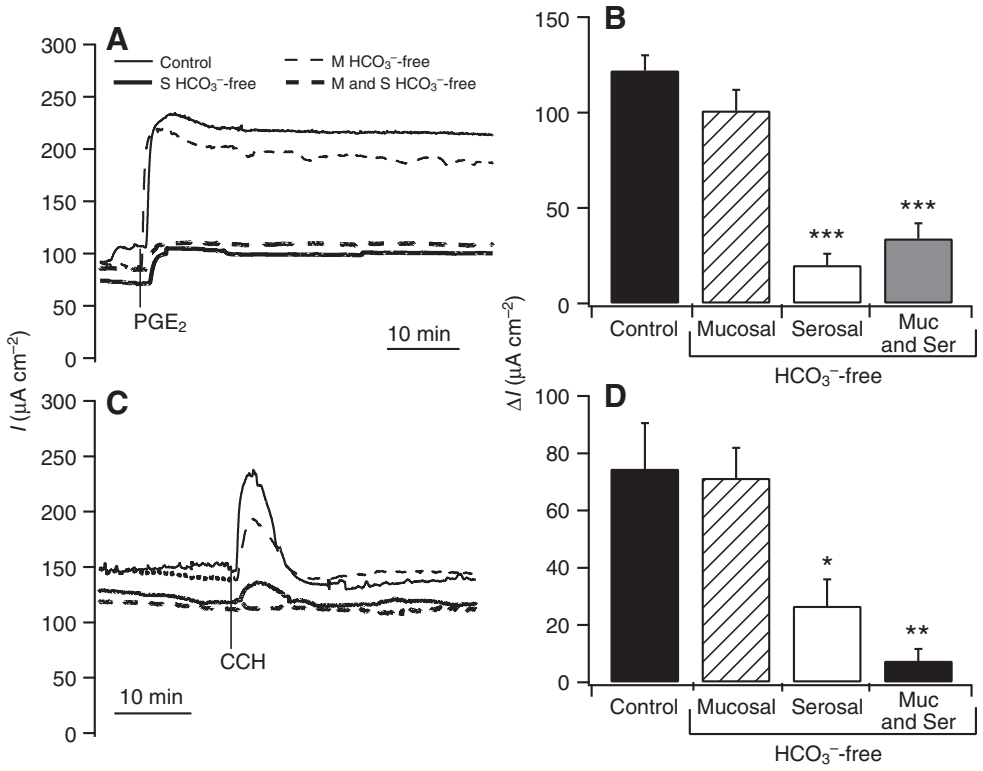

Fig. 2. The effect of replacement of HCO_3^- in the mucosal (M), serosal (S) or both mucosal and serosal (M and S) solutions on the response of the ileum to either PGE_2 or carbachol. In each case four tissues from the same animal were used. One tissue (control) was bathed in NaCl/HCO_3^- Ringer's (solutions 1, Table 1) on both sides, and one (M and S HCO_3^--free) in HCO_3^--free NaCl Ringer's (solution 3, Table 1) on both sides. The remaining two tissues were bathed in HCO_3^--free NaCl Ringer's on the mucosal (M HCO_3^--free) or serosal (S HCO_3^--free) on one side and NaCl/HCO_3^- Ringer's on the other side. All tissues were then stimulated with either PGE_2 (1 μmol l^{-1}, serosal) or carbachol (CCH, 100 μmol l^{-1}, serosal). (A) A representative experiment showing the different responses to PGE_2. (B) The mean change in transepithelial current (ΔI) in eight identical experiments, showing that the PGE_2-stimulated response was dependent upon serosal HCO_3^-. (C) A representative experiment showing the different responses to carbachol (CCH). (D) A summary of eight experiments showing that the mean ΔI stimulated by CCH was also dependent upon serosal HCO_3^-. All values are mean ± s.e.m. *A significant difference from the response in NaCl Ringer's; one-way ANOVA with a Dunnett's *post-hoc* test. *P<0.05, **P<0.01, ***P<0.001.

an amiloride analogue specific for Na^+/H^+ exchangers (Noel and Pouyssegur, 1995).

The absence of an effect of acetazolamide and amiloride imply that the secretory response is dependent upon a basolateral Na^+/HCO_3^- cotransporter. To support this conclusion we investigated the effect of serosal DIDS, which inhibits HCO_3^--dependent transporters, such as the NBCs (Cullifordet al., 2003). Following stimulation of the I_{sc} with PGE_2, serosal DIDS (1 mmol l^{-1}) completely inhibited the PGE_2-stimulated I_{sc} increase, whereas it had little effect on the spontaneous I_{sc} (Fig. 5A,B). Similarly, pre-treatment of paired tissues from the same animal with 1 mmol l^{-1} serosal DIDS eliminated the I_{sc} response to CCH (Fig. 5C,D). Similar effects of DIDS were seen when the tissues were stimulated with either PGE_2 or CCH in Cl^--free Ringer's (data not shown).

Identification of possum NBC variants and detection of NBC protein in the ileum

Collectively the physiological data indicate that HCO_3^- secretion in the possum ileum is dependent upon a NBC located in the basolateral membrane of the secretory cells. Therefore, we investigated whether the possum ileum expresses a NBC, in particular pancreatic (p)NBC which is associated with HCO_3^- secretion by eutherian epithelia (Praetorius et al., 2001; Bachmann et al., 2003; Steward et al., 2005). Initially we cloned and sequenced possum pNBC from ileal tissue and (k)NBC from kidney and submitted the sequences to the NCBI data bank, under the accession numbers EU159119.2 and EU192930 for pNBC and kNBC, respectively.

Possum pNBC was 1070 amino acids long, whereas kNBC was 1028 amino acids long. The C-terminal 985 amino acids of possum p- and kNBC share very high sequence identity, as there are only two amino acids that are different, both of which are the result of single nucleotide substitutions. However, the N-terminal 85 amino acids of possum pNBC and the N-terminal 43 amino acids of possum kNBC have very low sequence identity, which is a characteristic of the two variants in other species (Abuladze et al., 2000). The deduced amino acid sequence of possum pNBC shares 95.0% sequence identity with human pNBC (accession number AAC39840), whereas possum kNBC shares 93.2% sequence identity to human kNBC (accession number AAC51645). The N-terminus of possum pNBC has a high sequence identity to the N-terminus of human pNBC (94.1%; Fig. 6A), but the N-terminus of possum kNBC was only 56.1% similar to the N-terminus of human kNBC (Fig. 6B).

In order to determine whether both NBC variants are expressed in the possum ileum we used specific primers that target the N-terminal region of possum p- and kNBC in PCR. A 712 bp partial cDNA of possum pNBC was clearly amplified from ileal cDNA with the pNBC-specific primers (Fig. 6C). A 721 bp partial cDNA

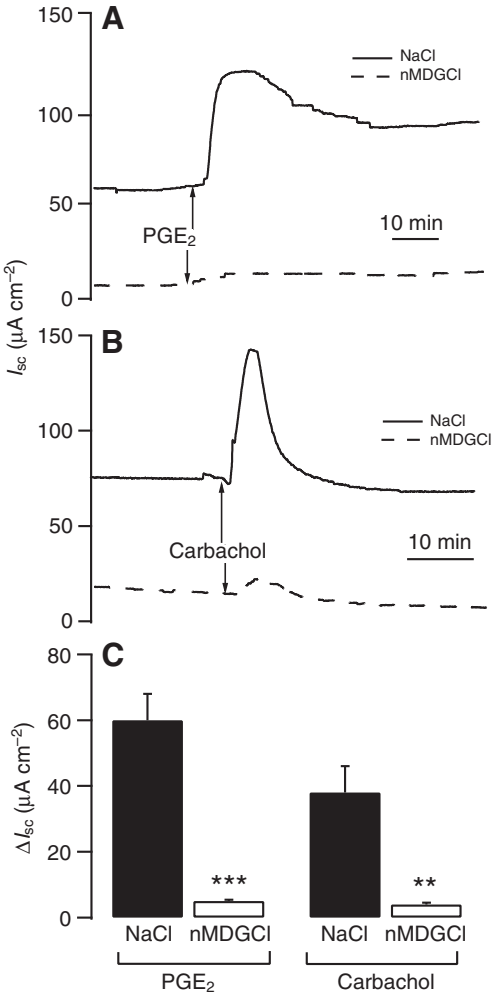

Fig. 3. Symmetrical (mucosal and serosal) replacement of Na^+ with the impermeant cation *N*-methyl-D-glucamine ($nMDG^+$, Solution 4, Table 1) inhibits the increase in short circuit current (I_{sc}) stimulated by PGE_2 (1 mmol l^{-1}, serosal) or carbachol (100 μmol l^{-1}, serosal) in the ileum. Representative experiments showing the effect of Na^+ replacement on the response to PGE_2 (A) or carbachol (B). (C) Summary of the effects of Na^+ replacement on the change in I_{sc} (ΔI_{sc}) stimulated by PGE_2 or carbachol. All values are mean ± s.e.m., *N*=7 for each. *A significant difference from the response in NaCl Ringer's solution. $^{**}P<0.01$; $^{***}P<0.0001$, Student's *t* test.

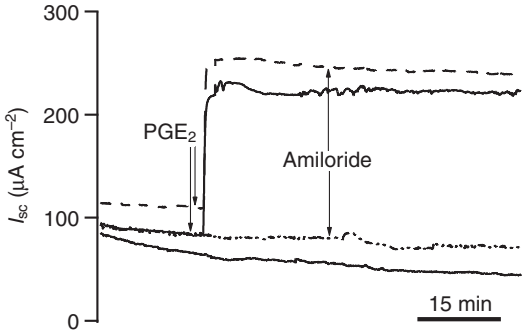

Fig. 4. Serosal amiloride had no effect on the PGE_2-stimulated short circuit current (I_{sc}) in the possum ileum. Four tissues from the same animal were mounted in Ussing chambers and pre-treated with TTX (1 μmol l^{-1}, serosal). Then two of the tissues were stimulated with PGE_2 (1 μmol l^{-1}, serosal), while the other two served as unstimulated controls. When the response to PGE_2 had reached a steady state, amiloride (1 mmol l^{-1}, serosal) was added to one of the stimulated and one of the unstimulated tissues. Traces are representative of eight experiments.

was also amplified from the ileum using kNBC-specific primers, but the kNBC amplicon was very faint (Fig. 6C). The use of the same primer pair on kidney cDNA resulted in the amplification of a greater abundance of cDNA, which was confirmed as kNBC by sequencing (data not shown). Using ileal RNA as a template for PCR confirmed that we were not amplifying genomic DNA with the kNBC primers (Fig. 6C). These data suggest that both pNBC and kNBC are expressed in the possum ileum, although it would appear that pNBC is the main variant that is expressed.

Consistent with the PCR results we were able to detect NBC protein in the ileal epithelium of the possum. The anti-NBC antibody we used was raised in rabbit against a C-terminal region common to both rat p- and kNBC (Schmitt et al., 1999), thus it did not discriminate between the two variants. This antibody detected possum NBC protein of approximately 130 kDa in size from ileal protein extracts (Fig. 6D). Possum NBC was not detected when the primary antibody was pre-absorbed with the control peptide.

Localisation of pNBC mRNA and NBC immunoreactivity in the possum ileum

In the intestine of eutherian mammals the transport proteins associated with absorption and secretion are predominately expressed in the villous and crypt cells, respectively (Field, 2003). To determine whether there was a discrete localisation of the NBC variants within the crypt–villous axis of the possum ileum we used probes specific for the N-terminal regions of pNBC and kNBC in *in situ* hybridisation studies. These experiments demonstrated a high level of pNBC mRNA expression in the crypts and base of the villous epithelium, with decreasing levels of expression along the length of the villi (Fig. 7A). With the use of a probe specific for kNBC we were unable to demonstrate the presence of appreciable levels of kNBC transcript in the possum ileum, although the probe clearly demonstrated high levels of kNBC transcript in the kidney (data not shown). These results support the conclusion that pNBC is the main variant expressed within the ileal epithelium.

The distribution of NBC 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 it was absent from the tip of the villi (Fig. 7B,C). NBC immunoreactivity was the most intense in the mid region of the villi in the basolateral membrane of ileal epithelial cells (Fig. 7D). The discrepancy in the distribution of pNBC mRNA and NBC immunoreactivity in the crypt–villous axis of the possum ileum is probably because of the constant migration of intestinal epithelial cells from the crypts to the tip of the villi, where they are shed (Barker et al., 2008).

DISCUSSION

Electrogenic Cl^- secretion has been shown to drive fluid secretion in elasmobranches, birds and eutherian mammals and is the underlying mechanism driving fluid secretion in the small intestine of eutherian mammals (Sullivan and Field, 1991). This process is critically dependent upon the $Na^+/K^+/2Cl^-$ cotransporter, NKCC1, which is expressed in high levels in secretory epithelia (Russell, 2000). NKCC1 is located in the basolateral membrane of secretory epithelial cells and is the primary transporter that accumulates Cl^- in the cells above equilibrium, thus driving the Cl^- secretory process (Welsh, 1983; Greger et al., 1986). In the possum ileum, cAMP- and Ca^{2+}-dependent secretagogues stimulate electrogenic secretory

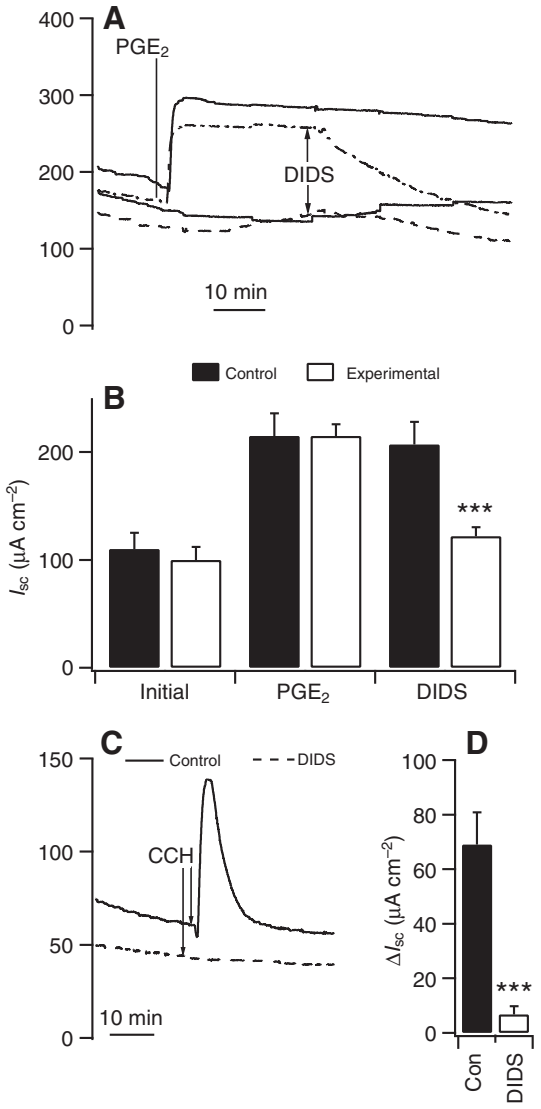

Fig. 5. Serosal 4,4′-diisothiocyano-stilbene-2,2′-disulfonate (DIDS) inhibited the short circuit current (I_{sc}) stimulated by either PGE_2 or carbachol (CCH). (A) A representative experiment showing that DIDS (1 mmol l^{-1}) did not inhibit the spontaneous I_{sc}, but inhibited the PGE_2-stimulated (1 μmol l^{-1}, serosal) I_{sc}. Four tissues from the same animal were mounted in Ussing chambers and pre-treated with serosal TTX (1 μmol l^{-1}). Two of the tissues were then stimulated with PGE_2, and when the response had reached a steady state one stimulated and one unstimulated tissue were treated with DIDS. (B) A summary of the effect of PGE_2 and DIDS on the I_{sc} in the possum ileum. All values are mean ± s.e.m., N=8. *A significant difference from control tissues; one-way ANOVA with a Bonferroni *post-hoc* test, ***P<0.001. (C) A representative experiment showing the effect of pre-treatment with serosal DIDS (1 mmol l^{-1}) on the response to CCH (100 μmol l^{-1} serosal). One of two tissues from the same animal was pre-treated with serosal DIDS for 30 min and then both tissues were stimulated with CCH. (D) A summary of the change in I_{sc} (ΔI_{sc}) stimulated by CCH in the presence and absence of serosal DIDS. Values are mean ± s.e.m., N=8. *A significant difference from control tissues, Student's t-test, ***P<0.001.

responses that are qualitatively similar to the Cl^- secretory responses seen in eutherian mammals. PGE_2 and, to a lesser extent, forskolin stimulate a sustained increase in I_{sc}, whereas the Ca^{2+}-dependent secretagogue CCH stimulates a transient increase in I_{sc}. However, these responses do not involve electrogenic Cl^- secretion as they are independent of Cl^- and are insensitive to bumetanide. Here we present evidence indicating that the electrogenic secretory response of the possum ileum involves electrogenic HCO_3^- secretion driven by a basolateral Na^+/HCO_3^- cotransporter.

In the ileum of the possum the levels of expression of NKCC1 are relatively low compared with secretory epithelia in eutherian mammals (Bartolo et al., 2009). Consequently, the secretory responses seen in the possum ileum are independent of NKCC1 activity. Recently, it was demonstrated that in NKCC1 knockout mice and in colonic surface epithelial cells that do not express NKCC1, Cl^- secretion was driven by HCO_3^--dependent accumulation of Cl^- *via* a basolateral Cl^-/HCO_3^- exchanger (Jacob et al., 2001; Walker et al., 2002). Although the levels of expression of NKCC1 in the possum ileum are very low (Bartolo et al., 2009) it is unlikely that the secretory response involves Cl^- secretion driven by a HCO_3^--dependent process since the complete replacement of Cl^- in the bathing solution with an impermeant anion had little effect on secretion. By contrast, the removal of HCO_3^-/CO_2 from the bathing solution profoundly inhibited the secretory responses. This dependence on serosal HCO_3^-/CO_2, combined with its total independence of Cl^-, is consistent with the notion that, in the possum ileum, secretagogues stimulate electrogenic HCO_3^- secretion.

In HCO_3^--secreting epithelia of eutherians, HCO_3^- can be either generated by hydration of CO_2, through the action of carbonic anhydrase (Jacob et al., 2000) or transported into the epithelial cells from the serosal solution (Ishiguro et al., 1996; Bachmann et al., 2003). The CO_2 that is hydrated by carbonic anhydrase is derived from either metabolic CO_2 or the diffusion of CO_2 into the cells from the bathing solution. However, in the possum ileum, acetazolamide, which inhibits carbonic anhydrase activity (Wu et al., 1998), had no effect on secretion, indicating that the hydration of CO_2 does not contribute to the secretory response. Furthermore, serosal amiloride had no effect on the secretory responses. The hydration of CO_2 generates both HCO_3^- and H^+ ions. As a result, the activity of an amiloride-sensitive Na^+/H^+ exchanger on the basolateral membrane of epithelial cells plays an integral role in the secretion of HCO_3^- generated by carbonic anhydrase, since the extrusion of H^+ ions across the basolateral membrane maintains the driving force for HCO_3^- secretion across the apical membrane (Novak and Greger, 1988).

Thus, it would appear that secretion by the possum ileum is primarily dependent upon serosal HCO_3^-. This proposal is supported by the observation that the removal of HCO_3^-/CO_2 from the serosal solution alone inhibited secretion to the same extent as removal from both sides. Indeed the dependence of the stimulated I_{sc} on Na^+, serosal HCO_3^- and its inhibition by serosal DIDS, suggest that in the possum ileum secretagogues stimulate electrogenic HCO_3^- secretion, which is driven by a Na^+/HCO_3^- cotransporter on the basolateral membrane of the secretory cells. This was supported by the demonstration that NBC is expressed in the possum ileum and the NBC immunoreactivity was localised to the basolateral membrane of the ileal epithelial cells.

In eutherian epithelia the pancreatic (p) and kidney (k) variants of the electrogenic Na^+/HCO_3^- cotransporter (NBC) are associated with the transport of HCO_3^- across the basolateral membrane of epithelial cells. However, these two variants of NBC have markedly different distributions and functions. kNBC is predominately located in epithelia of the kidney (Bok et al., 2001; Roussa et al., 2004), where it is involved in the reabsorption of HCO_3^- (Boron et al., 1997). This cotransporter is located in the basolateral membrane of the proximal tubule epithelial cells and has a Na^+:HCO_3^- stoichiometry of 1:3. This stoichiometry, combined with the

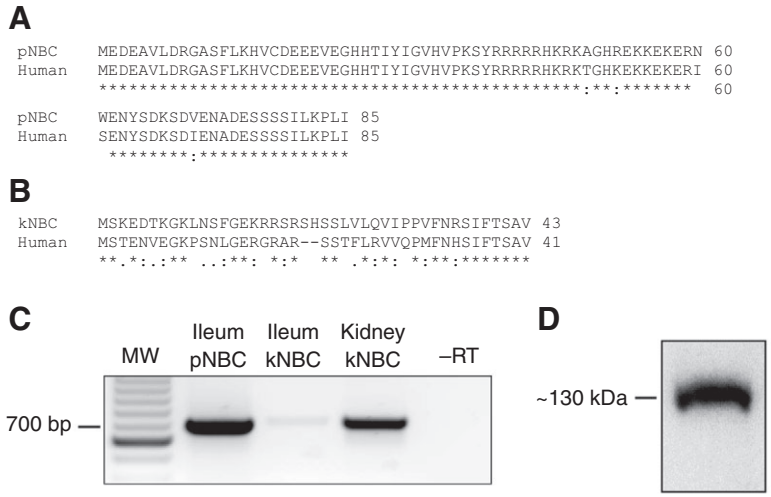

Fig. 6. Alignment of the N-terminus of possum pNBC (A) and kNBC (B) with the respective human NBC variants. Asterisks indicate identity between sequences; colons indicates conserved substitutions; dots indicate semi-conserved substitutions. (C) Using possum ileal cDNA as a template for PCR, we demonstrated that p- and kNBC were expressed in the possum ileum, and with kidney cDNA we showed that kNBC was highly expressed in the kidney; bands in the MW lane are molecular mass markers; –RT, PCR using kNBC-specific primers without reverse transcription of ileal RNA. (D) Possum pNBC protein was shown to be approximately 130 kDa in size by western blot experiments.

electrochemical gradients present in the proximal tubular cells, results in the extrusion of HCO_3^- across the basolateral membrane of these cells (Pushkin and Kurtz, 2006), and this transporter plays an important role in HCO_3^- absorption in the kidney. By contrast, pNBC has a broader distribution within the body and is expressed at high levels in tissues that secrete HCO_3^-, such as the pancreas (Thevenod et al., 1999; Roussa et al., 2004) and duodenum (Jacob et al., 2000; Seidler et al., 2000). In these tissues, pNBC has a Na^+:HCO_3^- stoichiometry of 1:2 (Romero et al., 2004; Pushkin and Kurtz, 2006) and is essential to the secretion of HCO_3^- (Praetorius et al., 2001; Bachmann et al., 2003; Steward et al., 2005), as it transports HCO_3^- into the cell across the basolateral membrane (Ishiguro et al., 1996; Praetorius et al., 2001). Both variants of NBC are expressed in the intestine of eutherians, but pNBC is the predominant variant, particularly in the duodenum (Praetorius et al., 2001) and the proximal colon (Seidler et al., 2000), which secrete HCO_3^- at relatively high rates. Results from PCR experiments suggest that both pNBC and kNBC are expressed in the possum ileum. However, using kNBC-specific PCR primers and ileal cDNA, we were only able to demonstrate a very faint band for kNBC on a DNA gel, and we were unable to demonstrate significant levels of kNBC transcript in the possum ileal epithelial cells using *in situ* hybridisation. This suggests that in the possum ileum, as in the eutherian small intestine, pNBC is the dominant variant of NBC expressed, and is responsible for HCO_3^- secretion.

Using a pNBC-specific probe, we demonstrated with *in situ* hybridisation that the distribution of pNBC mRNA expression was highest within the crypt and lower region of the villous epithelium. By contrast, NBC immunoreactivity was concentrated in the epithelial cells of mid region of the villi, with less intense levels in the crypts and no detectable NBC immunoreactivity at the tip of the villi. Although the antibody that we used did not discriminate between pNBC and kNBC, given the low levels of kNBC transcript seen in the possum ileum, it is probable that the pattern of immunoreactivity seen is representative of pNBC expression within the crypt–villous axis of the ileum. Thus, the distribution of pNBC immunoreactivity differs from that of pNBC mRNA. Similar discrepancies between mRNA and protein distribution within the crypt–villous axis have been reported for other transport proteins in the small intestine of other species (Hwang et al., 1991; Lee et al., 1994; Balen et al., 2008) and are thought to be a consequence of the development and maturation of the intestinal epithelial cells. Within the small intestine all epithelial cells are derived from stem cells in the basal region of the crypts, which are constantly dividing. The epithelial cells then migrate from the crypts and along the length of the villi to the tip where they are shed into the intestinal lumen 2–5 days latter (Barker et al., 2008). As the cells migrate towards the tip of the villi they undergo maturation and there is a change in expression pattern of transport proteins (Field, 2003). Thus, the probable explanation for the difference in the patterns of pNBC mRNA and immunoreactivity in the possum ileum, is that as the ileal epithelial cells mature they stop transcribing pNBC mRNA, but the turnover of pNBC protein is a much slower process. This is supported by the observation that no pNBC mRNA or immunoreactivity could be detected at the tips of the villi.

The high levels of expression of pNBC within the mid-region of the villous cells of the possum ileum imply that HCO_3^- secretion primarily occurs within the villi of the possum. In the small intestine of eutherian mammals it is generally felt that secretion occurs within the crypts (Field, 2003). This is certainly the case for Cl^- secretion, as the high levels of NKCC1 expression associated with Cl^- secretory epithelia are limited to the crypt cells (Bartolo et al., 2009). However, there is very little evidence of the distribution of pNBC within the ileum of eutherian mammals. Early functional assays suggested that NBC, presumably pNBC, was present in the crypts of the rabbit (Knickelbein et al., 1988; Minhas and Field, 1994) and guinea pig (Macleod et al., 1996), but this has not been confirmed with direct measurements of the distribution of pNBC. Interestingly, in mouse (Praetorius et al., 2001) and rat (Seidler et al., 2000) duodenum, which are known to secrete HCO_3^- (Isenberg et al., 1993; Hogan et al., 1997), pNBC is expressed in the villous cells but not the crypt cells. Thus, at least in these rodents electrogenic HCO_3^- secretion driven by pNBC is primarily a function of the villous cells, as it is in the possum.

Whether other secretory epithelia in the possum utilise the same HCO_3^--dependent mechanism to drive fluid secretion as the ileum is yet to be determined. In eutherian epithelia, although the dominant mechanism of fluid secretion is electrogenic Cl^- secretion, many epithelia also have a limited ability to secrete HCO_3^- in parallel with Cl^-. However, it is only in the pancreatic duct that secretion is entirely dependent upon HCO_3^-, and this is associated with a specific function of pancreatic fluid to neutralise the acid delivered into the duodenum from the stomach (Steward et al., 2005). Accordingly, the secretion of HCO_3^- by the possum ileum may be unique to the ileum and be associated with a specific function of the ileum, possibly related to the fermentation of food material in

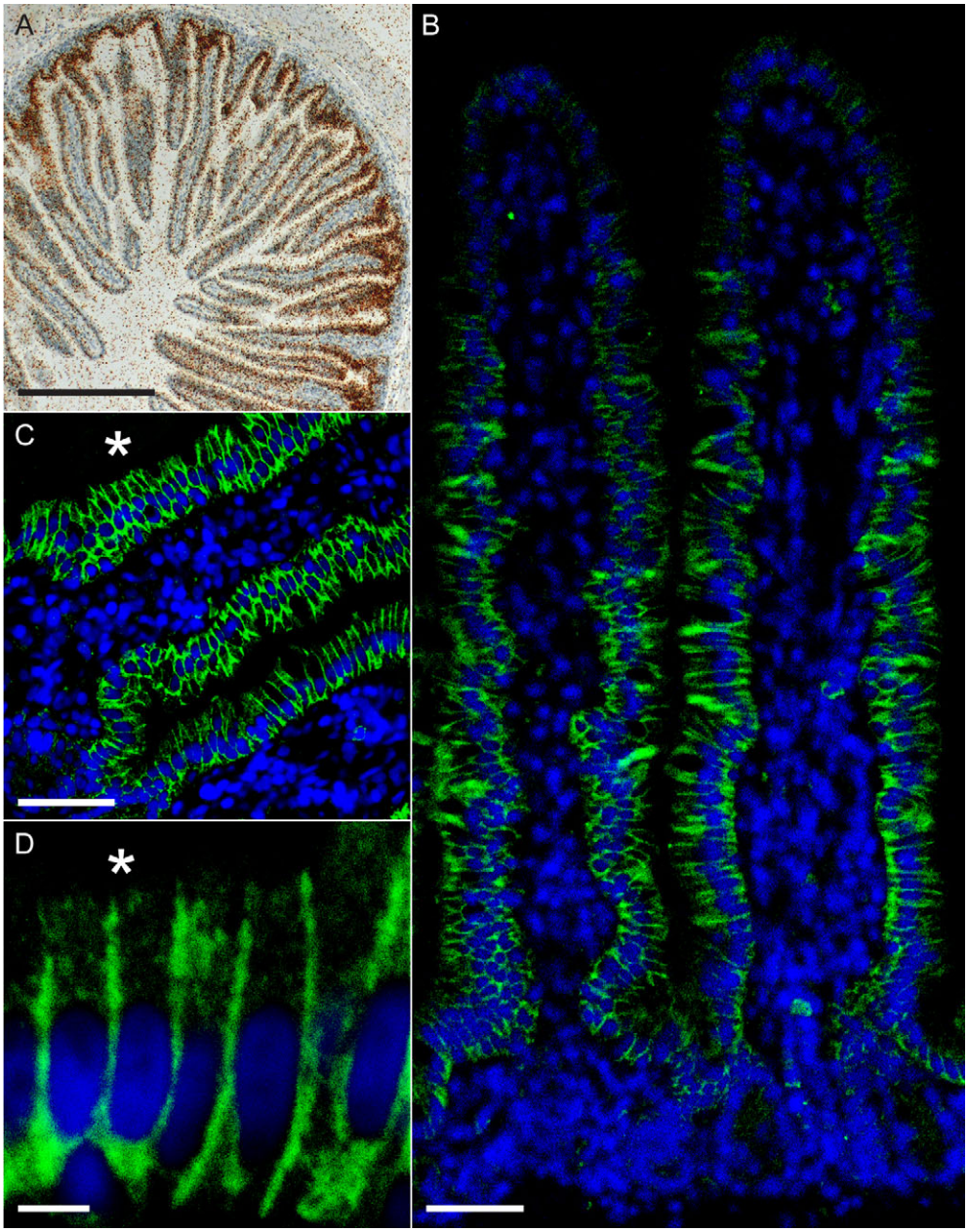

Fig. 7. Localisation of possum pNBC in the ileum using *in situ* hybridisation (A) and immunohistochemistry (B-D). (A) In the possum ileum, pNBC mRNA appeared to be primarily distributed in the crypts and at the base of villous epithelium, with a decreasing amount of pNBC mRNA along the crypt–villus axis towards the tip of the villi. (B,C) Using immunohistochemistry, we demonstrated that pNBC immunoreactivity (green) was present in the crypts, but was most intense in the mid-region of the villus epithelium (B,C), whereas very little pNBC immunoreactivity was detected at the tip of the villi (B). In the ileal epithelial cells that showed pNBC immunoreactivity, pNBC was located at the basolateral membrane (D). In C and D, an asterisk indicates the intestinal lumen. Scale bars, 500 μm (A), 50 μm (B,C) and 5 μm (D).

the caecum and proximal colon. The possum is a hindgut fermenter (Hume, 1982), and microbial fermentation in the hindgut requires a suitable environment for microbial growth and absorption of short chain fatty acids that result from the fermentation process. This includes a fluid medium and sufficient buffering capacity to maintain pH within a favourable range (Rechkemmer et al., 1988). In ruminants, HCO_3^- ions derived from salivary secretion are an important buffer in the foregut fermentation chamber (Allen, 1997). In the absence of significant electrogenic secretion in the hindgut of the possum (Butt et al., 2002b), the electrogenic HCO_3^- secretion by the ileum may be an important means of not only regulating the luminal pH, but also ensuring a sufficient volume of fluid for microbial growth. By contrast, the proximal colon of eutherian hindgut fermenters is capable of both fluid and HCO_3^- secretion (Clauss, 1986; Sullivan, 1986; Hyun et al., 1994; Kawamata et al., 2006). Therefore, it may not be necessary to deliver similar volumes of HCO_3^--rich fluid from the ileum. As a result, it may be sufficient to elevate ileal HCO_3^- concentration primarily through Cl^-/HCO_3^- exchange, which will alter the composition of the luminal fluid, but will not drive net fluid secretion. Certainly, along the length of the small intestine of eutherian mammals there is a progressive rise in luminal HCO_3^- concentration and a reciprocal fall in intestinal Cl^- concentration as a result of Cl^-/HCO_3^- exchange (Argenzio, 1991). Furthermore, in the ileum of eutherian mammals HCO_3^- is secreted at a relatively low rate compared with the duodenum and proximal colon (Seidler et al., 1997; Seidler et al., 2000). Interestingly, possums are very efficient in the digestion of fibre in the hindgut, particularly when compared with eutherian hindgut fermenters (Wellard and Hume, 1981). Therefore, the specialisation seen in ileal HCO_3^- transport may be a feature unique to marsupials or the possum.

In conclusion, secretagogues stimulate an electrogenic secretory response in the ileum of the brushtail possum, which involves electrogenic HCO_3^- secretion and is dependent upon the activity of

the pancreatic variant of the electrogenic Na^+/HCO_3^- cotransporter, pNBC. This is different from the situation in eutherian mammals in which the dominant mechanism of secretion in the ileum involves electrogenic Cl^- secretion. This difference may reflect an evolutionary divergence of secretion mechanisms between marsupials and eutherians or, alternatively, it may reflect a specialised function of the possum ileum that increases the efficiency of nutrient metabolism.

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