Abolition of Ca^{2+}-mediated intestinal anion secretion and increased stool dehydration in mice lacking the intermediate conductance Ca^{2+}-dependent K^+ channel Kcnn4

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Intestinal fluid secretion is driven by apical membrane, cystic fibrosis transmembrane conductance regulator (CFTR)-mediated efflux of Cl^- that is concentrated in cells by basolateral $Na^+-K^+-2Cl^-$ cotransporters (NKCC1). An absolute requirement for Cl^- efflux is the parallel activation of K^+ channels which maintain a membrane potential that sustains apical anion secretion. Both cAMP and Ca^{2+} are intracellular signals for intestinal Cl^- secretion. The K^+ channel involved in cAMP-dependent secretion has been identified as the KCNQ1–KCNE3 complex, but the identity of the K^+ channel driving Ca^{2+}-activated Cl^- secretion is controversial. We have now used a *Kcnn4* null mouse to show that the intermediate conductance IK1 K^+ channel is necessary and sufficient to support Ca^{2+}-dependent Cl^- secretion in large and small intestine. Ussing chambers were used to monitor transepithelial potential, resistance and equivalent short-circuit current in colon and jejunum from control and *Kcnn4* null mice. Na^+, K^+ and water content of stools was also measured. Distal colon and small intestinal epithelia from *Kcnn4* null mice had normal cAMP-dependent Cl^- secretory responses. In contrast, they completely lacked Cl^- secretion in response to Ca^{2+}-mobilizing agonists. Ca^{2+}-activated electrogenic K^+ secretion was increased in colon epithelium of mice deficient in the IK1 channel. Na^+ and water content of stools was diminished in IK1-null animals. The use of *Kcnn4* null mice has allowed us to demonstrate that IK1 K^+ channels are solely responsible for driving intestinal Ca^{2+}-activated Cl^- secretion. The absence of this channel leads to a marked reduction in water content in the stools, probably as a consequence of decreased electrolyte and water secretion.

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Fluid secretion in the small and large intestine is a highly regulated process driven by electrogenic, active transport of Cl^- ions (throughout this paper, we refer to a current consistent with anion secretion as Cl^- secretion. We cannot discard the idea that a portion of this current is carried by HCO_3^- (see Discussion section)) across the epithelium (Barrett & Keely, 2000; Kunzelmann & Mall, 2002). The importance of this regulation is highlighted by the consequences of its disruption in pathophysiological states such as secretory diarrhoea and cystic fibrosis. Secretion is dependent upon the activation of apical CFTR Cl^- channels. Such activation allows passive efflux of Cl^- into the lumen of the intestine from those cells where it is found accumulated above electrochemical equilibrium. Intracellular accumulation is generated by uphill Cl^- entry at the basolateral membrane via the $Na^+-K^+-2Cl^-$ cotransporter NKCC1. The ion gradients which drive Cl^- influx through NKCC1 are maintained by the Na^+-K^+ pump, also present at the basolateral membrane. Basolateral membrane K^+ channels are central in the anion secretion process, as they maintain a membrane potential favourable to continuous Cl^- efflux and serve to recycle K^+ taken up by NKCC1 and the Na^+-K^+ pump. Secretion of Cl^- in intestinal epithelium is stimulated by various secretagogues, which differ in the intracellular mediator involved in their signalling. The primary intracellular mediators of intestinal Cl^- secretion are cAMP (or cGMP)

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and intracellular Ca^{2+}. In mammalian colon, K^+ secretion occurs in addition to Cl^- secretion, making an important contribution to K^+ homeostasis (Kunzelmann & Mall, 2002).

Ca^{2+}-dependent anion secretion in distal colon and in small intestinal epithelia requires the simultaneous activity of apical CFTR Cl^- channels, activated by cAMP, and basolateral K^+ channels activated by Ca^{2+} (Mall *et al.* 1998). Life-threatening rotaviral-induced secretory diarrhoea is known to be due to alterations in Ca^{2+} homeostasis (Ramig, 2004). On the other hand, if the magnitude of Ca^{2+}-dependent Cl^- secretion is sufficiently high to have an impact on the hydration of intestinal contents, it might determine the severity of the intestinal disease in cystic fibrosis (Bronsveld *et al.* 2001). The identification of the K^+ channel involved in Ca^{2+}-dependent intestinal Cl^- secretion could provide a useful pharmacological target to modulate this type of secretion.

Three classes of Ca^{2+}-activated K^+ channels have been distinguished from their single channel conductance: the small-, intermediate- and large-conductance channels SK, IK1 and BK, respectively. SK channels are encoded by three genes, *Kcnn1–3*; IK1 and BK channels are the products of the *Kcnn4* and *Slo* (*Kcnma1*) genes, respectively (Vergara *et al.* 1998). Ca^{2+}-activated K^+ channels of all three types have been identified in small intestine and colon (Hay-Schmidt *et al.* 2003; Joiner *et al.* 2003; Chen *et al.* 2004) and could participate in Ca^{2+}-dependent anion secretion. There is evidence that IK1, encoded by the *Kcnn4* gene, is the K^+ channel involved in Ca^{2+}-dependent intestinal Cl^- secretion (Warth *et al.* 1999), but this has been strongly challenged in recent work (Halm *et al.* 2006). These investigations used mainly pharmacological tools to identify the conductance in question, thus alternative approaches are required to address this discrepancy.

In this report we tested the hypothesis that the intermediate conductance IK1 (Kcnn4) channel is important in Ca^{2+}-dependent Cl^- secretion in the small and large intestine, by investigating the effect of inactivating the *Kcnn4* gene in the mouse. Our results strongly suggest that IK1 is necessary and sufficient for Ca^{2+}-dependent Cl^- secretion. Besides this, the genetic ablation of IK1 induces changes in the handling of Na^+ and water leading to a marked increase in stool dehydration. Some of these results have been published previously in abstract form (Flores *et al.* 2005).

Methods

Animals

Male mice (in C57Bl/6J background) aged 2–5 months were used. They were bred at CECS mouse facility in Valdivia. The *Kcnn4* null animal generation and their genotyping have been previously described (Begenisich *et al.* 2004).

Tissue isolation and Ussing chamber experiments

Animals were killed by cervical dislocation, a procedure performed according to international regulations for animal care and with the approval of the local bioethics committee. A segment of distal colon or jejunum was excised, rinsed with warm 0.9% NaCl, and cut open lengthwise through the mesenteric border. For colon, a partially stripped mucosal sheet was obtained by scraping the mucosa surface with a glass microscope slide (Catalán *et al.* 2004). The sheet obtained was mounted on a tissue-holding slider (aperture, 0.1 cm^2) and put as a dividing membrane in a modified Ussing chamber (Physiologic Instruments Inc., San Diego, CA, USA). The transepithelial potential difference referred to the serosal compartment was measured continuously using an EVC4000 amplifier (World Precision Instruments, Sarasota, FL, USA). The current was clamped at zero and at 20 s intervals 1 s pulses to 10–20 μA were given. The voltage pulses were generated with pCLAMP 6 software (Axon Instruments, Union City, CA, USA) through a Labmaster interface, which also served to acquire the voltage. The difference in current and voltage was used to calculate the tissue resistance and equivalent short-circuit currents (I_{sc}s) according to Ohm's law (Warth *et al.* 1999). A correction for the resistance of the fluid measured in the absence of tissue (60–64 Ω) was applied. The ΔI_{sc} values were calculated as follows: amiloride-sensitive as I_{sc} before minus I_{sc} after drug addition; cAMP-induced as I_{sc} after minus I_{sc} before addition of forskolin/IBMX; chromanol 293B as I_{sc} before minus I_{sc} after addition of the drug in the continued presence of forskolin/IBMX, minus cAMP-induced; carbachol-induced as I_{sc} after minus I_{sc} before addition of the agonist in the continued presence of forskolin/IBMX and chromanol 293B. The bath solution was continuously gassed with CO_2 5% and O_2 95% and contained the following (mM): 120 NaCl, 25 $NaHCO_3$, 3.3 KH_2PO_4, 0.8 K_2HPO_4, 1.2 $MgCl_2$, 1.2 $CaCl_2$ and 10 D-glucose. When using jejunum, D-glucose was replaced by D-mannitol in the apical bath solution, to avoid Na^+-coupled glucose currents. Addition of drugs was by injection of small volumes from concentrated stocks in dimethylsulphoxide (DMSO). Addition of solvent only was without effect. Wash out of drugs was done by two washes of the appropriate compartment by complete fresh solution replacement. Most reagents were obtained from Sigma Chemical Company (USA). Mepyramine and chromanol 293B were purchased from Tocris Bioscience (USA).

Immunohistochemistry

Colonic tissue was removed and washed with warm saline before being fixed for 24 h at 4°C, and then for 24 h at room temperature, in PBS containing

2% picric acid and 2% paraformaldehyde. Immunohistochemistry was performed as previously described (Peña-Münzenmayer *et al.* 2005). Briefly, paraffin sections (4 μm thick) were incubated with anti-mouse BK antibody (APC-021, Alomone, Israel) diluted at 1 : 50, 1 : 100 or 1 : 200 in PBS with 0.5% BSA, pH 7.4 at 22°C overnight. Bound antibodies were detected with the aid of the biotin–streptavidin–peroxidase technique. After peroxidase was developed, with diaminobenzidine and hydrogen peroxide, sections were counterstained with Harris haematoxylin.

RT-PCR reactions

Cells were isolated as previously described (Catalán *et al.* 2004). Total RNA from surface cells and crypts were isolated using Trizol reagent (Invitrogen) and then RT-PCR was performed. Three micrograms of total RNA were reverse transcribed with the SuperScript II system (Invitrogen), using the oligo (dT) primer and random hexamer primers. PCR amplification primers for *KCNN1*, *KCNN2*, *KCNN3*, *KCNN4*, *KCNMA1*, *KCNMB1* and *KCNMB4* are given in Supplementary Table 1. The reaction mixture contained aliquots of cDNA, 0.2 μM of each primer, 2.5 units Taq DNA polymerase (Promega), 2 mM dNTPs, and 1.5 mM $MgCl_2$ in a total volume of 25 μl. Conditions were: initial denaturation at 95°C for 2 min, 30 cycles at 95°C for 30 s, annealing at 58°C for 30 s and extension at 74°C for 30 s, and final extension at 74°C lasting 5 min. cDNA samples from tissues with known expression of each of the transcripts were used as positive controls.

Water and electrolytes in stools

Freshly defecated faeces were collected, weighed immediately (WW) and then dried for 48 h, weighed again (DW), resuspended on 1 ml of deionized water and heated to 65°C for 30–60 min. Suspension were centrifuged at 12,000 g for 5 min and Na^+ and K^+ assays were performed on the supernatant using a Jenway PFP7 flame photometer (Essex, UK) under manufacturer's instructions. Calculation of water content was done as (WW − DW)/WW.

Data handling

Results are generally given as mean ± S.E.M. and the significance of differences tested by *t* test.

Results

Cl^- secretion induced by the muscarinic agonist carbachol in large and small intestine shows an absolute dependence upon K^+ channel IK1

Muscarinic activation leads to Ca^{2+}-dependent Cl^- secretion in colonic epithelium from various species (Strabel & Diener, 1995; Mall *et al.* 1998; Warth *et al.* 1999; Carew & Thorn, 2000; Halm *et al.* 2006). Here we show the response to carbachol in the colon from our control animals. The upper panel of Fig. 1 shows a recording of transepithelial potential (V_{te}). A lumen-negative V_{te} corresponds to anion secretion or

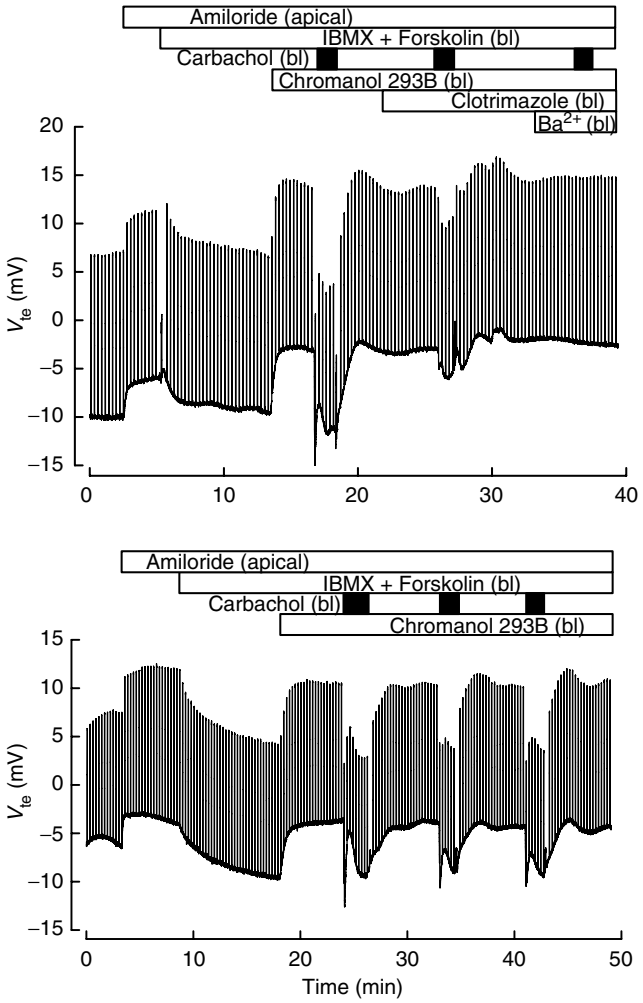

Figure 1. Effect of secretagogues on wild-type mouse colon mucosa
The traces show continuous recordings of transepithelial potential (V_{te}) as function of time obtained in Ussing chamber experiments of mouse distal colon. The voltage deflections are caused by current pulse injection. Addition of 100 μM isobutylmethylxanthine (IBMX) plus 1 μM forskolin in both experiments, induces a negative change in V_{te} that was fully reversed by 10 μM chromanol 293B. Addition of 100 μM carbachol (black boxes) yielded fast negative changes in V_{te} that were maintained during repeated application (lower panel). Carbachol effect was almost completely blocked by the Kcnn4 inhibitor clotrimazole (CTZ, 3 μM) and totally blocked by further addition of 5 mM serosal Ba^{2+} (upper panel). In both experiments apical 10 μM amiloride was used to inhibit ENaC-mediated sodium currents. Apical and bl indicate additions to the mucosal and serosal compartment, respectively. Tissue resistances were, respectively, 86 and 83 Ω cm^2 at the beginning and end of the experiment shown in the upper panel. The respective numbers for the lower panel experiment were 78 and 75 Ω cm^2.

cation absorption. Apical amiloride (10 μM) was added to block Na^+ current through the epithelial sodium channels (ENaC). Increasing intracellular cAMP by serosal addition of forskolin (1 μM) and isobutylmethylxanthine (IBMX, 100 μM) induced anion secretion, as revealed by the negative deflection in V_{te}. This secretion was abolished by serosal chromanol 293B (10 μM), a blocker of KCNQ1–KCNE3 K^+ channels. Although chromanol 293B has been reported to inhibit recombinant CFTR expressed in *Xenopus* oocytes (Bachmann *et al.* 2001) and a mucosal effect of 293B, albeit with a threefold lower affinity, has been reported for rabbit colon mucosa (Lohrmann *et al.* 1995), in isolated rat colonic crypts 293B blocks K^+ conductance but has no effect on cAMP-evoked depolarization induced by the opening of apical (CFTR) Cl^- channels (Diener *et al.* 1996). In our hands mucosal chromanol 293B had no effect on cAMP-induced anion secretion in mouse colon (not shown), suggesting a lack of effect upon CFTR in our experimental protocol. Addition of serosal carbachol in the presence of 293B induced a robust anion secretion response. The effect of carbachol was reduced > 70% by the IK1 K^+ channel inhibitor clotrimazole, and abolished in the combined presence of Ba^{2+} and clotrimazole in the serosal side. The partial effect of clotrimazole could be due to the rather low concentration used here to avoid undesired non-specific effects. Clotrimazole had no effect on cAMP-induced anion secretion (not shown). Separate control experiments, an example of which is shown in the lower panel of Fig. 1, demonstrated that the effect of successive additions of carbachol was comparable over time without any evidence for rundown. Carbachol did not always evoke anion secretion without pre-treatment with IBMX and forskolin (not shown). In tissues treated with indomethacin, to prevent spontaneous increase in cAMP via prostaglandin E_2 release (Carew & Thorn, 2000), there was no Cl^- secretory response at all (see Fig. 6, further described below). This suggests a lack of Ca^{2+}-dependent apical chloride channel (CaCC) activity under the conditions of the experiment.

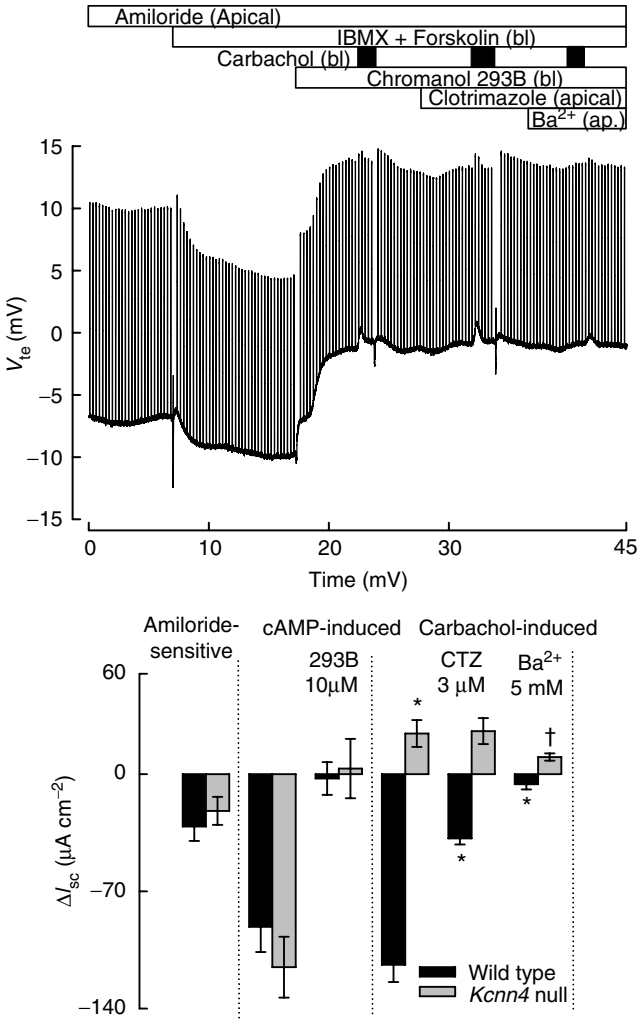

Figure 2. Effect of secretagogues on colon mucosa from *Kcnn4* null mice

All details for the addition of agonists and blockers are as in Fig. 1. When colon strips of *Kcnn4* null mice were challenged with 100 μM serosal carbachol there were positive instead of negative changes in V_{te} that were fully blocked by mucosal Ba^{2+} but not by clotrimazole. Tissue resistance at the beginning and end of the experiment were 89 and 76 Ω cm^2. The lower panel summarizes differences in equivalent short-circuit currents (ΔI_{sc}) obtained under different conditions with wild-type and *Kcnn4* null mice colonic tissue. A $P < 0.0005$ level of significance for the difference with the effect of carbachol on WT tissue is indicated by *. † indicates $P < 0.05$ compared with carbachol effect on *Kcnn4* null. Data are means ± S.E.M. of 5 and 4 separate experiments for WT and and KO, respectively.

The upper panel in Fig. 2 shows a similar experiment to that in the upper panel of Fig. 1, but done on tissue from a *Kcnn4* null mouse. After apical addition of amiloride to block ENaC, increasing cAMP induced the hyperpolarization expected during activation of cAMP-dependent Cl^- secretion. As in wild-type (WT) colon, the effect was abolished by 293B. In the presence of 293B, addition of carbachol to the serosal side of the epithelium did not evoke the hyperpolarizing response observed in the WT epithelium. A rather small depolarizing response was induced by addition of the agonist. The IK1 channel inhibitor clotrimazole had no effect, but 5 mM apical Ba^{2+} (or 10 mM tetraethylammonium (TEA), not shown) abolished the modest depolarizing effect of carbachol in *Kcnn4* null mice.

The average change in equivalent short-circuit current (ΔI_{sc}) across colon epithelium under the different treatments is summarized in the lower panel of Fig. 2. Both the Na^+ absorption through ENaC channels and the 293B-inhibitable cAMP-dependent Cl^- secretory currents were similar in colon from WT and IK1 knockout (KO) mice. The large anion secretory response seen in

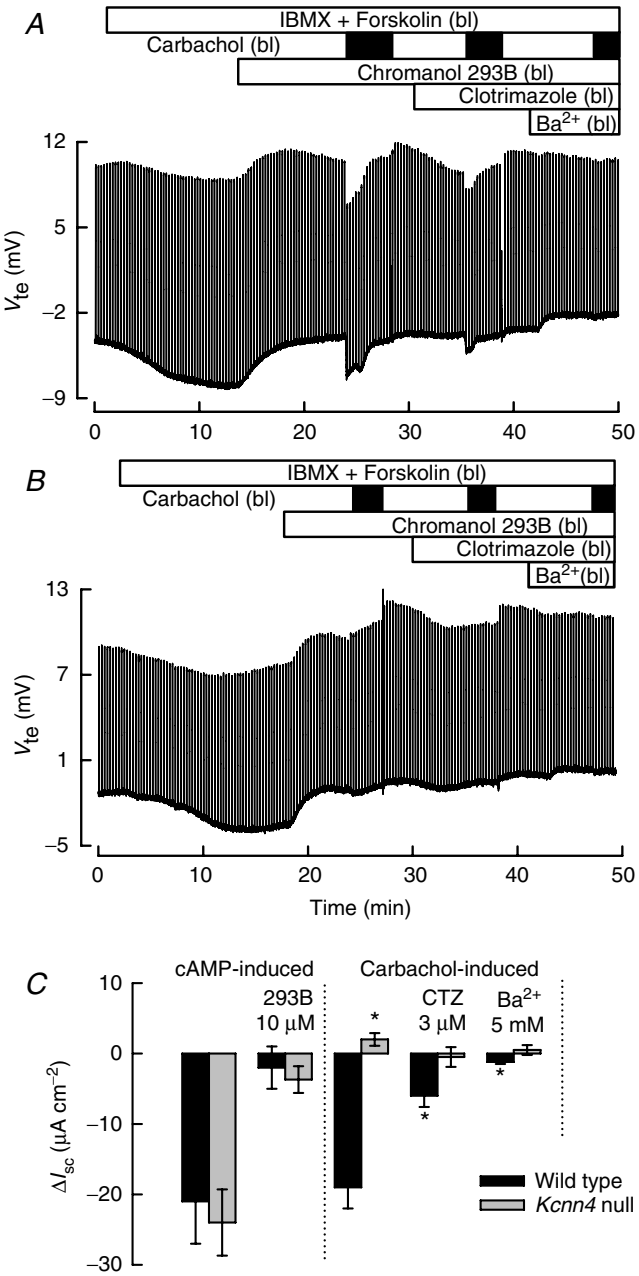

Figure 3. Effect of secretagogues on jejunum epithelia from WT and *Kcnn4* null mice
All details for the addition of agonists and blockers are as in Fig. 1. When jejunum of WT mice (*A*) was challenged with 100 μM serosal carbachol, there were negative changes in V_{te} that were partially blocked by 3 μM clotrimazole and fully blocked by additional 5 mM Ba^{2+}. *B* shows results for a jejunum from *Kcnn4* null mouse, where carbachol addition did not produce the negative changes related with anionic secretion. *C*, summary of ΔI_{sc} values for a series of experiments performed on jejunum epithelium from wild-type and *Kcnn4* null mice. Data are means ± S.E.M. of 5 individual experiments for each animal. A $P < 0.0005$ level of significance for the difference with the effect of carbachol on WT tissue is indicated by *. None of the effects of carbachol on Kcnn4 KO tissue was significantly different from zero.

WT colon contrasted with the small, but significant, cation secretory current in the *Kcnn4* null epithelium. Clotrimazole significantly inhibited carbachol-dependent anion secretion of WT epithelium but had no effect on the apparent cation secretion evoked in the colon from KO animals.

A further series of experiments was conducted to ascertain whether similar Ca^{2+}-dependent anion secretion could be evoked in the small intestine. In Fig. 3*A* and *B* experiments with jejunal epithelium are illustrated. These follow a similar protocol as for colonic epithelium. Treatment with IBMX and forskolin evoked a hyperpolarizing response in jejunal epithelia from both control (*A*) and *Kcnn4* null (*B*) animals. This response was abolished by the 293B K^+ channel blocker in both types of tissue. The response to serosal carbachol addition in control jejunum was a transient hyperpolarization, which was greatly diminished by clotrimazole and abolished in the combined presence of serosal clotrimazole and Ba^{2+}. Carbachol was without effect in jejunum from *Kcnn4* null mouse. Figure 3*C* summarizes the calculated ΔI_{sc} values for the different treatments in jejunal tissue from WT and KO animals.

Na^+-coupled D-glucose- and L-phenylalanine-stimulated currents are not affected in the jejunum of IK1 KO mice

Na^+-coupled absorption of sugars and amino acids in the small intestine is electrogenic and requires an active basolateral K^+ conductance to avoid driving force collapse (Schultz, 1981). We tested therefore whether IK1 might contribute to this phenomenon by comparing the electrical

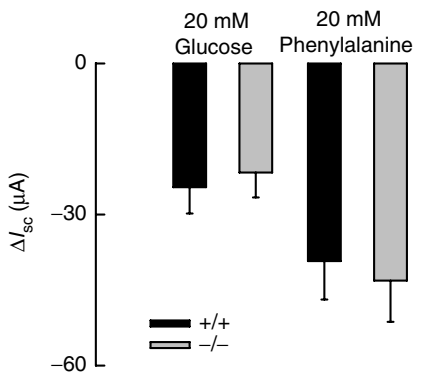

Figure 4. Sodium-coupled sugar and amino acid transport on jejunum
Jejunum strips from WT and IK1 KO mice, respectively, were challenged with 20 mM D-glucose or with 20 mM L–phenylalanine. Tissue was previously incubated with 2 μM indomethacin to avoid cAMP-induced currents. ΔI_{sc} values obtained with the indicated metabolite are shown. There were no differences between tissues. Data are means ± S.E.M. of 4 individual experiments each for WT and IK1 KO animals.

effects of transported metabolites between WT and IK1 KO small intestine. Glucose and phenylalanine were increased from 0 to 20 mM in the apical side of jejunum strips placed in Ussing chambers and V_{te} monitored. Both metabolites induced negative changes on V_{te} consistent with Na^+-coupled absorption. Calculation of the average change in equivalent short-circuit current in response to L-phenylalanine or D-glucose showed no significant differences between WT and KO tissues (Fig. 4). These results suggest that IK1 is not a key factor in the Na^+-coupled absorption of sugars and amino acids in the small intestine.

Histamine-evoked Cl^- and K^+ secretion in colonic epithelium

Histamine increases intracellular Ca^{2+} in intestinal crypts (Lindqvist *et al.* 2002) and induces Cl^- secretion in rabbit (McCabe & Smith, 1984), human (Bronsveld *et al.* 2001) and rat (Schultheiss *et al.* 2006) large intestine. As expected, after activation of secretion by increasing cAMP and blockade of basolateral KCNQ1–KCNE3 with chromanol 293B, WT colon exhibited a robust, transient Cl^- secretion (Fig. 5*A*), but only a cation secretory response in colon from IK1 KO animals (Fig. 5*B*). It is interesting to note that the Cl^- secretory effect of histamine

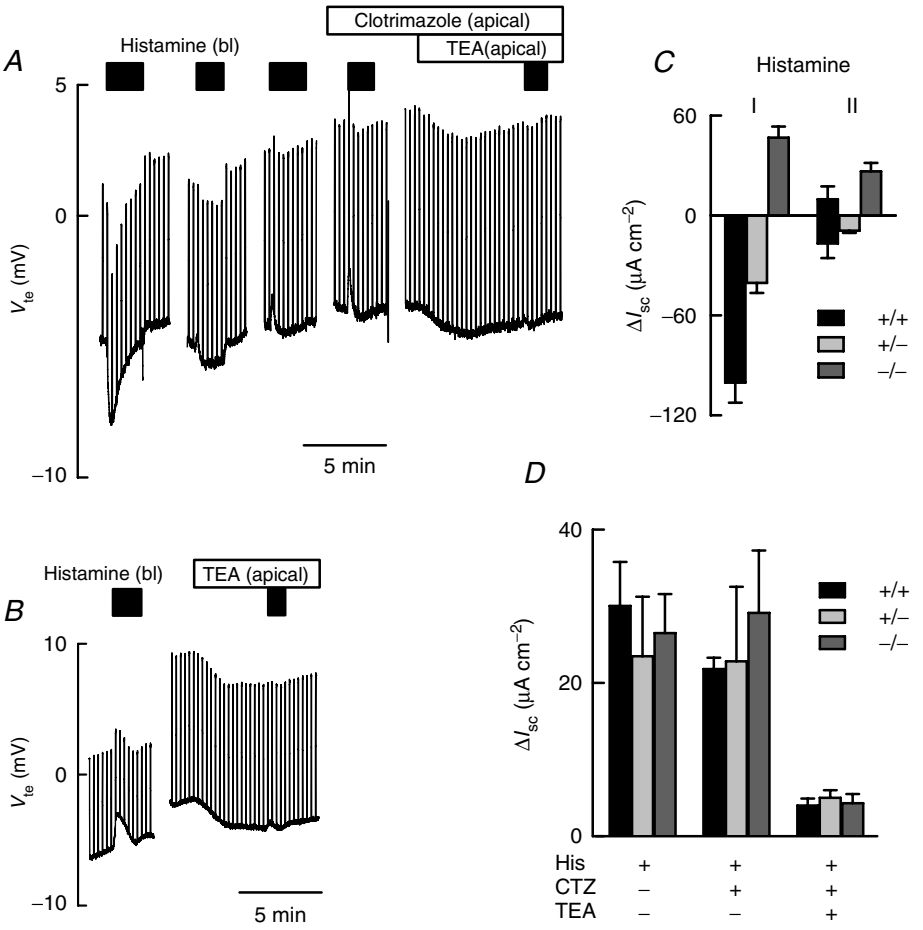

Figure 5. Histamine effect on colonic ion secretion
Tissues were initially treated with 100 μM IBMX plus 1 μM forskolin in all experiments, followed by serosal addition of 10 μM chromanol 293B as in Fig. 1. Serosal addition of histamine (150 μM) induced a transient Cl^- current in the wild-type tissue. Further pulses of histamine on the same colonic preparation did not evoke a negative change in V_{te}. At a third pulse of histamine, a 10 mM TEA-sensitive K^+ secretion is unmasked (*A*). Serosal histamine addition always evoked positive V_{te} change in the *Kcnn4* null colon, which was also abolished by mucosal TEA (*B*). In *C*, a summary of evoked ΔI_{sc} values in response to the first 2 histamine challenges on wild-type (+/+), heterozygous (+/–) and homozygous *Kncc4* null (–/–) mice. Values are means ± S.E.M., $n = 9$ (+/+), $n = 4$ (+/–) and $n = 7$ (–/–). *D* shows ΔI_{sc} for histamine-induced K^+ secretion in tissues of the three different genotypes. The responses were measured successively from the third histamine application onwards, when the signal became stable. Values are means ± S.E.M., $n = 6$ (+/+), $n = 3$ (+/–) and $n = 4$ (–/–).

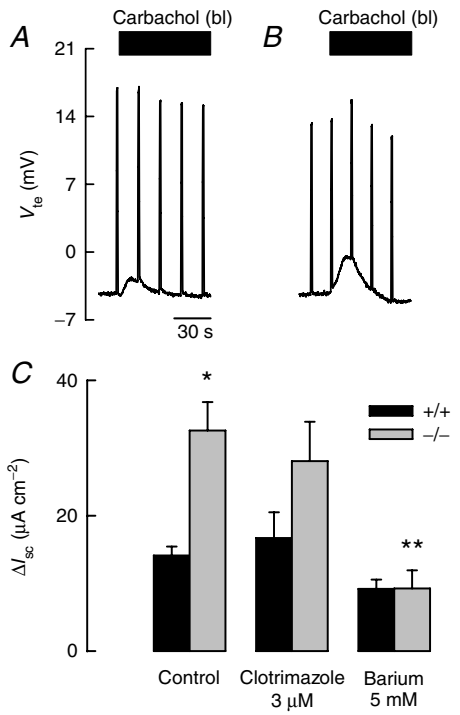

Figure 6. Carbachol-induced K^+ secretion on mice colon
After pre-incubation with indometacin (2 μM), colonic strips were challenged with carbachol. The inhibitory effect of indometacin on the cAMP-induced anionic secretion unmasked K^+ secretion that was enhanced in the IK1 KO tissue (*B*) compared with that of the WT animal (*A*). The K^+ secretion was not inhibited by clotrimazole but partially blocked by apical Ba^{2+} in both animals. *C* shows ΔI_{sc} calculated for each condition. Values are means ± S.E.M., $n = 4$ (+/+) and $n = 6$ (−/−). *Significantly different from +/+ control; **significantly different from −/− control (*t* test).

promptly desensitized, having disappeared at a second challenge, and becoming a cation secretory current from a third addition onwards. This effect is summarized by plotting the average responses to the first and second histamine challenges in Fig. 5*C*, which also reports results from experiments done with tissues from heterozygous animals (+/−). Notice that a second addition of histamine evoked either a small cation secretory response or a small anion secretion in WT colon. These two types of response are shown separately in Fig. 5*C* (black columns in histamine addition II). The idea that the cation secretory current corresponds to transient K^+ secretion is supported by its blockade by apical TEA, but not clotrimazole (Fig. 5*A*). A similar result was obtained with the KO epithelia, where apical TEA largely eliminated the K^+ secretory current (Fig. 5*B*). A summary for the results of the third to the fifth histamine application is shown in Fig. 5*D*.

IK1 is not involved in K^+ secretion in colonic epithelium stimulated by carbachol

Experiments in colon from *Kcnn4* null mice suggested the presence of cation secretion (Fig. 2). To test whether this might correspond to Ca^{2+}-dependent K^+ secretion, experiments were carried out with tissues previously incubated with indomethacin (2 μM), to avoid basal release of prostaglandin and consequent cAMP increase. Carbachol evoked only cation secretory current in colon from WT (Fig. 6*A*) and KO (Fig. 6*B*) animals under these conditions. In both types of tissue the effect could be partially inhibited (Fig. 6*C*) by mucosal Ba^{2+} but not clotrimazole (added to both the mucosal and the serosal side), consistent with K^+ secretion. These results suggest that Ca^{2+}-dependent K^+ secretion in the colon does not involve the IK1 channel. Moreover, K^+ secretion appeared to be increased in the KO tissue, further supporting the idea that IK1 is not involved in this process. RT-PCR on isolated epithelial cells from the colon of both animals showed expression of various Ca^{2+}-activated K^+ channels. We confirmed the presence in both WT and IK-1 KO tissues of message for KCNN1 (SK1), KCNN3 (SK3) and KCNMA1 (BK, Slo, Maxi-K) and its β-subunits KCNMB1 and KCNMB4 (Fig. 7). KCNN4 was found in WT tissues only, as expected. KCNN2 transcript was not detected in either type of tissue (not shown).

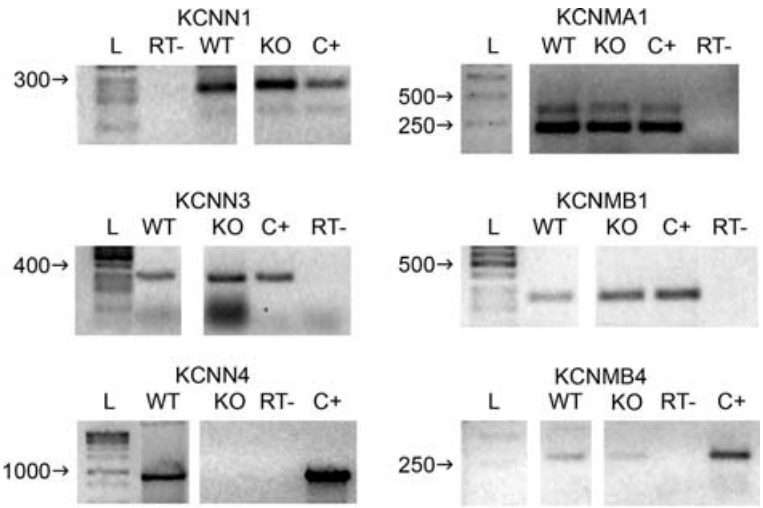

Figure 7. Ca^{2+}-activated K^+ channel expression in colon tissue from WT and *Kcnn4* null (KO) mice
Agarose gel electrophoresis of RT-PCR products is shown for the indicated transcripts. Predicted product sizes were KCNN1 (241 bp), KCNN3 (275 bp), KCNN4 (957 bp), KCNMA1 (392 and 218 bp), KCNMB1 (246 bp) and KCNMB4 (285 bp). RT–, negative control omitting reverse transcriptase. C+ is a positive control from parotid gland cDNA, except for KCNN1 and KCNN3 which were done on brain cDNA. Arrows point to different number of base pairs as indicated on 100 or 1000 bp ladders.

Immunohistochemical studies demonstrated that BK immunoreactivity was present in colonic epithelial tissue of both WT (Fig. 8*A*) and IK1 KO animals (Fig. 8*B*). BK-positive cells were encountered deeper into the crypts of KO colon (Fig. 8*D*) than in WT tissue (Fig. 8*C*), where the reaction was restricted to surface cells. This observation could explain the augmented K^+ secretion in KO tissue when stimulated by carbachol. With the purpose of obtaining a stronger signal, anti-BK dilution was decreased to 1 : 50 for WT (Fig. 8*C*), but kept at 1 : 200 for the tissues from KO animals (Fig. 8*D*). A similar staining intensity was obtained despite the use of different antibody dilutions,

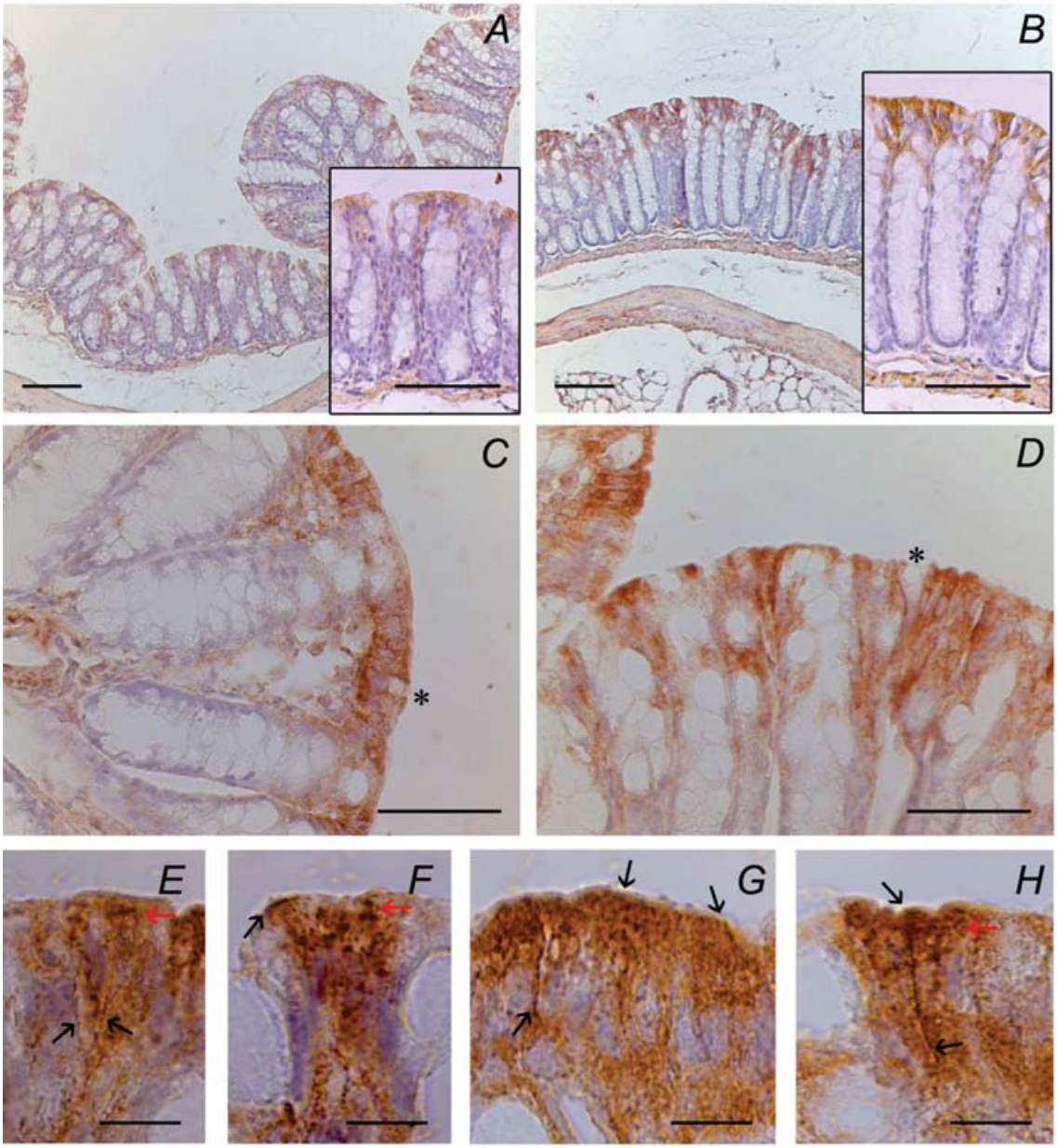

Figure 8. Immunohistochemical localization of BK channels within mice colonic epithelium
A and *B*, BK immunoreactivity using 1 : 200 antibody dilution in WT (*A*) and IK1 KO (*B*) colon; insets show details of representative areas of each sample. *C* and *D* are higher magnification of WT and IK1 KO colon using 1 : 50 and 1 : 200 dilution, respectively. *E–H*, surface colonic epithelium displaying basolateral and apical immunoreactivity (black arrows); red arrows point out granular immunostaining concentrated in the upper portion of colonocytes. Asterisks mark absence of staining in goblet cells. Biotin–streptavidin–peroxidase-DAB technique. The images are representative of experiments with 3 different mice for each the WT and IK1 KO groups. Scale bars: *A* and *B*, 100 μm; *C* and *D*, 50 μm; *E–H*, 10 μm.

Table 1. Water and cation content of stools and serum electrolytes

		Wild-type	Kcnn4 null
Serum electrolytes	Na^+ (mEq l^{-1})	127 ± 12 (3)	132 ± 6 (3)
	K^+ (mEq l^{-1})	4.2 ± 0.4 (3)	4 ± 0.2 (3)
Faeces electrolytes	Na^+ (mEq (g dry wt)$^{-1}$)	122 ± 14 (7)	73 ± 7 (6)*
	K^+ (mEq (g dry wt)$^{-1}$)	87 ± 10 (7)	58 ± 8 (6)*
	Na^+ (mEq l^{-1})	90 ± 10 (7)	84 ± 6 (5)
	K^+ (mEq l^{-1} l)	58 ± 6 (7)	67 ± 7 (6)
	Water content of stools (%)	60 ± 3 (7)	46 ± 2 (6)*

Na^+ and K^+ concentrations in serum and faecal water in WT and IK1 KO mice. Na^+ and K^+ are also given as contents in dried stools. Data are means ± S.E.M. of the number of animals indicated in brackets. * indicates significant ($P < 0.05$) difference from WT figures.

which might suggest a higher expression level in tissues from KO animals. We failed to confirm this, however, in Western blots of protein extracts from total epithelium (not shown). Notice that the reaction was not restricted to any membrane domain, being present at apical (Fig. 8*F*, *G* and *H*) and basolateral (Fig. 8*E*, *G* and *H*) locations, in addition to strong cytoplasm staining.

Kcnn4 null mice present decreased Na^+ and water contents in the faeces

Animals were given free access to food and water. Analyses of serum demonstrated that Na^+ and K^+ concentrations did not differ between WT and IK1 KO animals (Table 1). In contrast, Na^+ and K^+ content was diminished in stools obtained from IK1 KO animals compared with WT. This phenomenon was paralleled by a marked decrease in water content in the faeces of IK1 KO animals, which meant that there was no significant change in cation concentration in stools water (Table 1).

Discussion

Cl^- and fluid secretion in the intestinal epithelium depends crucially upon the activation of a basolateral membrane K^+ conductance which both maintains a hyperpolarized membrane potential favourable to Cl^- exit despite activation of CFTR, and recycles K^+ taken up during Cl^- accumulation. The K^+ channel that supports cAMP-activated Cl^- secretion is a complex formed by KCNQ1 and KCNE3 (Schroeder *et al.* 2000). Recent work with a KO mouse lacking KCNQ1 has confirmed this view (Vallon *et al.* 2005). Similarly, work with rat and rabbit colon suggests that, of the different Ca^{2+}-activated K^+ channels present in this epithelium, IK1 is a strong candidate to underlie the basolateral K^+ conductance which supports Ca^{2+}-dependent Cl^- secretion (Warth *et al.* 1999). In addition there is immunocytochemical evidence for the presence of IK1 in the basolateral membrane of intestinal crypt epithelium (Furness *et al.* 2003; Joiner *et al.* 2003; Halm *et al.* 2006). There is, however, no agreement about the function of this channel as recent results obtained using guinea-pig colon suggest that the activity of basolateral IK1 is not necessary to support Ca^{2+}-dependent Cl^- secretion. These experiments relied mainly upon the use of pharmacological agents to dissect the different conductance pathways. It is clear, however, that these compounds can have non-specific effects that will obscure the interpretation of results. This makes it necessary to employ alternative approaches to help in understanding these complex epithelial transport mechanisms. We have now used an IK1 KO mouse to explore the role of this channel in intestinal Cl^- secretion. Our data demonstrate that Ca^{2+}-dependent Cl^- secretion in mouse colon and small intestine is absolutely dependent upon the presence of the IK1 intermediate conductance, Ca^{2+}-dependent K^+ channel encoded by the *Kcnn4* gene. Data presented here confirm and extend our results, communicated previously in abstract form, demonstrating lack of carbachol-induced anion secretion in IK1 KO mice (Flores *et al.* 2005). A very recent report also reaches a similar conclusion (Matos *et al.* 2007). The authors demonstrate the absence of carbachol-induced anion secretion in the colon of a different *Kcnn4* null mouse, confirming our result in a different genetic background (129SV/C57Bl6 F2 generation and 129SV inbred). It is interesting to note that, by comparison with the mice used here (C57Bl/6J inbred) the response to carbachol is monophasic, lacking the fast component we describe here and that is also seen in rat and rabbit colon mucosa (Warth *et al.* 1999) and in mouse (strain not given) tissue (Carew & Thorn, 2000).

We attribute the anion secretory response observed in the present work to Cl^- electrogenic flow. We cannot discard the idea, however, that part of this negative current in response to secretagogues is carried by HCO_3^-. Experiments with mouse colon have revealed that around 70% of total secretory current in the presence of HCO_3^- can be inhibited by blockade of basolateral NKCC1. In the absence of HCO_3^-, NKCC1 blockade achieved nearly

full inhibition (Cuthbert *et al.* 1999). This could be interpreted to imply the presence of a HCO_3^- component to the anion secretory current. However, as HCO_3^-—Cl^- exchange across the basolateral membrane has been shown to contribute to Cl^- accumulation across this membrane (Grubb *et al.* 2000), an indirect effect of HCO_3^- removal on Cl^- current could be taking place. Given the fact that CFTR has a low HCO_3^- permeability under physiological extracellular Cl^- concentration (Shcheynikov *et al.* 2004), we believe that under our experimental conditions most anion secretory current will correspond to Cl^- secretion.

The IK1-deficient mouse presents anomalies in osmotic responses in its T-lymphocytes and red blood cells, as expected from the proposed role of IK1 in these cells. Rather surprisingly, however, *Kcnn4* KO mice have no apparent defect in Ca^{2+}-dependent parotid gland fluid secretion nor cell volume regulation (Begenisich *et al.* 2004). Instead, the large conductance BK K^+ channel, as demonstrated in a study using BK KO mice, is critical for the regulatory volume decrease of acinar cells and plays an important role in the sodium uptake and potassium secretion process in the ducts of these fluid-secreting salivary glands (Romanenko *et al.* 2006). In another report, KO animals for IK1 presented increased blood pressure due to impaired vascular dilatation induced by the endothelial-derived hyperpolarizing factor (Si *et al.* 2006). Our present examination of colonic and small intestinal epithelium shows normal Cl^- secretory responses to increasing intracellular cAMP in the IK1 KO mice. These responses are probably supported by the KCNQ1–KCNE3 K^+ channel (Vallon *et al.* 2005), as they were blocked by chromanol 293B, an inhibitor of this type of channel. The Cl^- secretory response elicited by either the Ca^{2+} agonist carbachol or histamine in WT colon and small intestine was completely absent from the IK1-deficient animals, suggesting that this K^+ channel is essential for Ca^{2+}-dependent Cl^- secretion. Despite the fact that several other Ca^{2+}-activated K^+ channels are known to be present in intestinal epithelium, none of them is capable of substituting for the basolateral IK1 channel. This might point to a failure to activate these channels at the intracellular Ca^{2+} concentrations reached or to their localization in different membrane domains or cell populations (Joiner *et al.* 2003). Indeed, the large conductance BK channel was found on the apical membrane where it supports K^+ secretion (Sausbier *et al.* 2006). Our localization experiments found it to be on apical and basolateral locations of only surface cells. We hypothesize that in this cellular location BK cannot substitute for IK1 as the basolateral Ca^{2+}-activated K^+ channel needed for Ca^{2+}-dependent Cl^- secretion, which probably occurs in the crypts.

Ca^{2+}-dependent intestinal Cl^- secretion requires the simultaneous activation of CFTR by cAMP (Clarke *et al.* 1994; Strabel & Diener, 1995; Mall *et al.* 1998) and, as shown here, of IK1 Ca^{2+}-dependent K^+ channels. No CaCC channels appear to be expressed in adult mouse intestine to allow a CFTR-independent Cl^- secretion. This appears at variance with what has been observed recently in rat distal colon, where fast transient muscarinic activation of an apical Cl^- conductance takes place (Schultheiss *et al.* 2003, 2005). Nevertheless, in the IK1-null mouse intestine, or when CFTR activation is avoided by treating WT tissue with indomethacin, no Cl^- secretion can be elicited by carbachol or histamine. What could be the physiological role of Ca^{2+}-activated IK1 channels in the intestinal epithelium? They could provide an additional way to increase Cl^- secretion which is rate-limited by the basolateral K^+ conductance (Warth *et al.* 1999). The observation that the heterozygous IK1 colon has only about 40% of the histamine (Fig. 5*C*) or carbachol (not shown) Cl^- secretory response of the WT, supports the view that the number or activity of basolateral K^+ channels is rate-limiting to apical Cl^- exit. IK1 channels apparently support CFTR-mediated secretion in the absence of cAMP-activated basolateral K^+ conductance, providing an explanation for the lack of intestinal phenotype in KCNQ1 KO mice (Lee *et al.* 2000; Casimiro *et al.* 2001). This contrasts with the generalized intestinal obstruction observed in CFTR KO mice which severely limits their survival (Snouwaert *et al.* 1992; Ratcliff *et al.* 1993). The cAMP-induced Cl^- secretion in jejunum from KCNQ1 KO mice is only reduced by about 50% (Vallon *et al.* 2005), suggesting that additional basolateral K^+ channels must be present.

The physiological relevance of functional IK1 channels is underlined by the finding that its genetic ablation causes increased stool dehydration accompanied by decreased faecal Na^+ and K^+ content although not in concentration in faecal water. This might be caused by an increased absorption or a decreased secretion of water and electrolytes. Neither small intestinal Na^+-coupled glucose and amino acid absorption nor ENaC-mediated colonic absorptive Na^+ current are affected in the IK1 KO animals. Given the role of IK1 in Ca^{2+}-dependent Cl^- secretion in small and large intestine demonstrated here, we favour the hypothesis of a decreased secretion of water and electrolytes in *Kcnn4* null animals. This would also be consistent with the decrease in faecal water content occurring without change in electrolyte concentration, suggestive of a deficit in an isotonic fluid secretion. The site of secretion for electrolytes and water has long been hypothesized to be in the crypt epithelium (Welsh *et al.* 1982). There is also evidence, however, that hypertonic water absorption occurs in distal colon crypts which dehydrate faeces from around 80% water content in the distal small intestine and proximal colon, to 60% water content in distal colon (Naftalin *et al.* 1999;.Thiagarajah *et al.* 2001). If the enhanced dehydration of faeces observed in the IK1 KO

mouse is due to decreased secretion in the small intestine, this would provide drier initial luminal contents for the colon. On the other hand, it could occur in the distal colon, a tissue whose secretory capacity is affected by inactivation of *Kcnn4*, and which is also believed to be the sole segment capable of effectively dehydrating intestinal contents (Naftalin *et al.* 1999). In this case, we would conclude that distal colon crypts both absorb and secrete, thus providing fine control for faeces dehydration in distal colon.

In the IK1 KO colon, or when CFTR activation is avoided by treating the tissue with indomethacin, only cation secretion can be elicited by carbachol or histamine. This corresponds to Ca^{2+}-activated electrogenic K^+ secretion as it can be inhibited by K^+ channel blockers added at the mucosal side of the epithelium (Binder & Sandle, 1994; Warth & Barhanin, 2003). Ca^{2+}-dependent K^+ secretion elicited by ATP in the colon depends absolutely upon the activity of apical membrane large conductance BK channels (Sausbier *et al.* 2006), and thus it was not expected to be affected in the IK1 KO mice as observed here. Surprisingly, it appears that IK1 ablation leads to an increased electrogenic K^+ secretion in colon which correlates with a wider distribution of BK channels in the epithelium. A negative regulation of BK channels by IK1 has been demonstrated both in parotid glands and in a heterologous expression system (Thompson & Begenisich, 2006) and a direct interaction between the channels was proposed to take place to mediate this effect. In addition to its expression in crypt cells, IK1 has been reported at a similar location as BK in surface colonic epithelium (Joiner *et al.* 2003; Furness *et al.* 2003; Halm *et al.* 2006). An interaction between the channels at this location might possibly account for an increased BK activity in the absence of IK1. The mechanism for the effect of IK1 ablation on BK expression in colon remains to be elucidated.

Histamine can also promote Ca^{2+}-dependent Cl^- secretion in WT colon. Unlike what is seen with carbachol, the histamine effect on Cl^- secretion rapidly desensitizes so that further challenges with the agonist lead to K^+ secretion only. The fact that desensitization of the Cl^-, but not the K^+ secretory response to histamine, takes place suggests that these two processes occur in different cell populations within the epithelium. Cl^- secretion occurs mainly in crypt cells (Welsh *et al.* 1982; Kunzelmann & Mall, 2002). Ca^{2+}-dependent K^+ secretion has an absolute dependence upon large conductance BK channels, which mediate K^+ exit across apical epithelial membranes (Sausbier *et al.* 2006). Our present immunocytochemical localization study gives a mainly surface epithelial location for BK in mouse colon, supporting the idea that Ca^{2+}-dependent K^+ and Cl^- secretion do indeed occur in different cell populations. This contrasts with a mainly crypt location also reported for mouse colon (Sausbier *et al.* 2006). This discrepancy might be due to the different genetic background of those animals (hybrid 129SV/C57Bl6, F2 generation) compared with ours (C57Bl/6J inbred). Other work shows BK channels localized to the apical membrane of surface cells of rabbit colon (Hay-Schmidt *et al.* 2003) and to the cytosol of surface and upper crypt cells in human colon (Mathialahan *et al.* 2005). Functional evidence indicates a surface, rather than crypt, cell location for aldosterone-regulated K^+ secretion in rat distal colon (Grotjohann *et al.* 1998), whilst single channel recordings have given an apical membrane, surface cell location in rat (Butterfield *et al.* 1997) and human (Sandle *et al.* 2007) colon. Given the exceptionally high single channel conductance of BK, and the presence of β-subunits that very strongly modify its Ca^{2+} and voltage dependence (Orio *et al.* 2002), the presence of low numbers of BK channels in surface cells might suffice to account for electrogenic K^+ secretion in colon. An alternative to the proposal that K^+ and Cl^- secretion are harboured in different cell populations would be that they are responsive to separate histamine receptors with differing desensitization properties. There is indeed evidence that histamine-induced K^+ and Cl^- secretion are controlled by different receptors in rat colon (Schultheiss *et al.* 2006). However, the specific H1 receptor antagonist mepyramine (Hill, 1990; Stack *et al.* 1995) abolished both K^+ and Cl^- secretion response in mouse colon (Supplementary Fig. 1). This would suggest that both these responses to histamine in mouse colon are mediated by H1 receptors. Differences in desensitization properties of histamine-dependent K^+ and Cl^- secretion could be due to differences in signalling compartmentalization associated with different membrane domains or a different cellular distribution of K^+ and Cl^- secretory functions.

In conclusion, inactivation of the *Kcnn4* gene demonstrates that IK1 channels are responsible for the Ca^{2+}-activated K^+ conductance involved in Ca^{2+}-dependent Cl^- secretion of mouse intestinal epithelium, a process that appears to contribute to the hydration of faeces. Electrogenic K^+ colonic secretion activated by the Ca^{2+}-mobilizing agonists carbachol and histamine, is present in tissue from *Kcnn4* KO mice. This observation suggests that IK1 is not part of this latter process. The key finding that IK1 is the only Ca^{2+}-activated K^+ channel driving Cl^- secretion, suggests a unique role for IK1 channels in pathological states where Ca^{2+}-dependent Cl^- secretion is enhanced. This might arise in conditions such as rotaviral-induced diarrhoea where Ca^{2+} homeostasis in intestinal epithelium is known to be affected (Ramig, 2004). IK1 could serve as a useful target for new drugs for the treatment and/or prevention of virus-induced diarrhoea.

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Supplemental material

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