Basolateral K^+ channel involvement in forskolin-activated chloride secretion in human colon

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1. In this study we investigated the role of basolateral potassium transport in maintaining cAMP-activated chloride secretion in human colonic epithelium.

2. Ion transport was quantified in isolated human colonic epithelium using the short-circuit current technique. Basolateral potassium transport was studied using nystatin permeabilization. Intracellular calcium measurements were obtained from isolated human colonic crypts using fura-2 spectrofluorescence imaging.

3. In intact isolated colonic strips, forskolin and prostaglandin E_2 (PGE_2) activated an inward transmembrane current (I_{SC}) consistent with anion secretion (for forskolin $\Delta I_{SC} = 63{\cdot}8 \pm 6{\cdot}2\ \mu A\ cm^{-2}$, $n = 6$; for PGE_2 $\Delta I_{SC} = 34{\cdot}3 \pm 5{\cdot}2\ \mu A\ cm^{-2}$, $n = 6$). This current was inhibited in chloride-free Krebs solution or by inhibiting basolateral chloride uptake with bumetanide and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS).

4. The forskolin- and PGE_2-induced chloride secretion was inhibited by basolateral exposure to barium (5 mM), tetrapentylammonium (10 μM) and tetraethylammonium (10 mM).

5. The transepithelial current produced under an apical to serosal K^+ gradient in nystatin-perforated colon is generated at the basolateral membrane by K^+ transport. Forskolin failed to activate this current under conditions of high or low calcium and failed to increase the levels of intracellular calcium in isolated crypts

6. In conclusion, we propose that potassium recycling through basolateral K^+ channels is essential for cAMP-activated chloride secretion.

The key event in colonic fluid secretion is the electrogenic transport of chloride ions, with sodium and water following by passive diffusion. It has been shown in the immortalized intestinal-derived cell line T-84 and in dogfish rectal gland (Greger & Schlatter, 1984; Halm *et al.* 1988; Dharmsathaphorn *et al.* 1988) that during chloride secretion chloride ions enter the secreting cells via the Na^+–K^+–$2Cl^-$ cotransporter and leave the luminal surface through apical chloride channels. This results in an accumulation of K^+ by the secreting cell. Basolateral K^+ channels therefore play an important role in maintaining the driving force for chloride secretion.

A number of K^+ channels have been identified on the basolateral membrane of mammalian colon. In rat colon there appear to be at least four distinct K^+ channels: a < 3 pS cAMP-activated channel (Greger *et al.* 1997*a*), a 16 pS Ca^{2+}-activated channel (Burckhardt & Goegelein, 1992), a maxi-K channel (Burckhardt & Goegelein, 1992) and a non-selective cation channel (Siemer & Goegelein, 1992). In rabbit colon the predominant basolateral K^+ channel is a calcium-activated maxi-K channel (Klaerke, 1997). In T-84 cells at least two K^+ channels have been identified on the basolateral membrane; these are an inwardly rectifying Ca^{2+}-activated channel (Tachbarani *et al.* 1994) and a large conductance Ca^{2+}-independent channel (Devor & Frizzell, 1998). We have identified two pharmacologically distinct potassium conductances in the basolateral membrane of human colonic epithelial cells, ATP-regulated potassium channels (K_{ATP}) and calcium-activated potassium channels (K_{Ca}) (Maguire *et al.* 1994). Both channels are sensitive to the non-specific potassium channel blocker barium. K_{ATP} channels are activated by increasing intracellular pH and inhibited by the sulphonylurea tolbutamide. K_{Ca} channels are activated by cytosolic calcium and inhibited by quaternary ammonium cations such as tetrapentylammonium (TPeA) and tetra-ethylammonium (TEA).

Electrogenic chloride secretion in colonic cells may be activated by either of two second messenger systems. Secretagogues such as prostaglandin E_2 (PGE_2) and forskolin activate secretion through cAMP, while agonists such as histamine, bradykinin, acetylcholine and carbachol act by increasing intracellular calcium (Bridges *et al.* 1983;

Diener *et al.* 1988; Pickles & Cuthbert, 1991). In rat colon the inter-relationship between apical chloride conductance, basolateral K^+ conductance and intracellular Ca^{2+} during chloride secretion is well understood. During carbachol- and acetylcholine-activated chloride secretion a 16 pS Ca^{2+}-dependent K^+ channel is activated (Nielsen *et al.* 1998). In contrast, during cAMP-mediated chloride secretion, cAMP directly activates apical chloride secretion; in addition there is a parallel activation of small conductance basolateral K^+ channels (Greger *et al.* 1997*b*), and this is accompanied by inhibition of the Ca^{2+}-dependent channel. However, there are significant interspecies differences in colonic ion transport mechanisms (Binder & Sandle, 1994). It is therefore particularly important to describe the exact ionic transport mechanisms that occur in human colon. In intact native human colonic epithelium the relationship between chloride secretion, basolateral K^+ transport and intracellular Ca^{2+} during cAMP-activated chloride secretion has not been well described. In this study we have investigated which of the K^+ channel types are involved in cAMP-mediated chloride secretion in the human distal colon and the effects of cAMP and raised intracellular calcium levels on basolateral K^+ transport.

METHODS

Source and preparation of colonic mucosa

Non-diseased samples of human distal colon from patients undergoing resection for carcinoma were used. The use of human tissue in these experiments was approved by the Cork University Teaching Hospitals' Ethics Committee, and experiments were performed with patients' full consent and in accordance with the Declaration of Helsinki. The sample was transported to the laboratory in 0·9% saline within 30 min of surgery.

The epithelial layer was separated from underlying smooth muscle and connective tissue by blunt dissection. Sheets of this tissue (0·5 cm^2) were mounted in modified Perspex Ussing chambers (ADInstruments UK Ltd). Experiments on chloride secretion in intact cells were performed in Krebs solution of the following ionic composition (mM): NaCl, 118; $NaHCO_3$, 25; glucose, 11; KCl, 4·7; $CaCl_2$, 2·5; $MgSO_4$, 1·2; and KH_2PO_4, 1·2. The solution was equilibrated in 5% CO_2 in oxygen, pH 7·4. The bath temperature was maintained at 37 °C using a heated water jacket.

Electrophysiological techniques

The spontaneous transmembrane potential was measured using an EVC 4000 Amplifier (WPI). The potential was clamped to 0 mV by the application of a short-circuit current (I_{SC}), which is a measure of electrogenic ion transport (Koefoed-Johnsen & Ussing, 1958). Transepithelial resistance (R_t) was calculated by measuring the current response to a 5 mV pulse. The current signal was digitized using an MP100 analog–digital converter (Biopac Systems Inc., USA) and analysed using Acqknowledge version 3.0 software (Biopac Systems Inc., USA) on an Apple Macintosh Quadra 650 personal computer. All drugs and chemicals were obtained from Sigma.

Studies with K^+ channel blockers

In nystatin permeabilization experiments the basolateral membrane was bathed in a low chloride Krebs solution of the following ionic composition (mM): sodium gluconate, 100; NaCl, 20; $NaHCO_3$, 25; glucose, 11; KCl, 4·7; $CaCl_2$, 2·5; $MgSO_4$, 1·2; and KH_2PO_4, 1·2. pH was maintained at 7·4 by gassing with 95% O_2–5% CO_2. The apical membrane was bathed in potassium-rich solution of the following ionic composition (mM): potassium gluconate, 120; NaCl, 20; $MgSO_4$, 3; KH_2PO_4, 1·2; glucose, 11; EDTA, 5; and Hepes, 6. $CaCl_2$ was added to give a final free calcium concentration of 10 nM, 50 nM, 100 nM or 1 μM at 37 °C and pH 7·2; the amount of $CaCl_2$ to be added was calculated using a computer program (Chang *et al.* 1988), and pH was adjusted to 7·2 by the addition of KOH. Electrogenic Na^+ transport was abolished by treatment of the apical membrane with amiloride (100 μM). The use of symmetrical low chloride solutions was found to be necessary to avoid cell volume changes.

The apical membrane was treated with the polyene antibiotic nystatin (500 i.u. ml^{-1} in < 0·01% methanol) and the transepithelial current allowed to reach a steady state. Nystatin forms cation-permeable pores in the apical membrane, and isolates the basolateral membrane electrically (Lewis *et al.* 1977). Under the conditions of a mucosa to serosa K^+ gradient, the transepithelial current is generated by the K^+ conductance of the basolateral membrane and K^+ transport across the paracellular pathway. Transport across the paracellular pathway is insensitive to K^+ channel blockers. In our nystatin experiments the current remaining after K^+ channel inhibition accounted for < 10% of the total.

Statistical methods

All values are expressed as the mean ± standard error of the mean. Student's *t* test was used to determine statistical significance. We used half-maximal inhibitory concentration (IC_{50}) as a summary statistic to compare the concentration–inhibition characteristics of the K^+ channel blockers used in these experiments. IC_{50} was calculated by fitting concentration and response values to the following equation, which is analogous to the Michaelis–Menten equation. This was done using a commercially available computer program (SPSS for windows, SPSS Inc. UK).

$$\Delta I = \Delta I_{max}[\text{Drug}]/(IC_{50} + [\text{Drug}]), \quad (1)$$

where ΔI is the inhibition of I_{SC} or I_K at a given concentration of drug, and ΔI_{max} is the maximum inhibition of I_K or I_{SC} produced by the drug.

Intracellular calcium measurements

Human colonic epithelium is a complex tissue. It is therefore difficult to precisely measure intracellular calcium in individual cells and to extrapolate these results to the conditions that apply in Ussing chamber experiments. To make qualitative measurements of the changes in intracellular calcium levels that occur during cAMP-mediated chloride secretion we measured intracellular Ca^{2+} in intact colonic crypts using the following techniques. Intact human crypts were isolated by exposing mucosal segments, microdissected from resected segments, to a calcium chelation solution (composition (mM): NaCl, 96; KCl, 1·5; Hepes-Tris, 10; NaEDTA, 27; sorbitol, 45; and sucrose, 28) for 30 min at room temperature. A pellet of isolated crypts was formed by centrifugation at 2000 *g* for 1 min and was resuspended in Krebs solution. Freshly isolated crypts were exposed to 5 μM fura-2 AM at room temperature for 60 min. They were then rinsed twice and stored in the dark in fresh Krebs solution on ice.

Crypts were transferred to glass coverslips treated with poly-L-lysine and were mounted on an inverted epi-fluorescence microscope (Diaphot 200, Nikon). The light from a xenon lamp (Nikon) was filtered through alternating 340 and 380 nm interference filters (10 nm bandwidth, Nikon). The emitted fluorescence was passed through a 400 nm dichroic mirror, filtered at 510 nm and then

collected using an intensified CCD camera system (Darkstar, Photonic Science). Images were digitized and analysed using the Starwise Fluo system (Imstar, Paris). The system was calibrated *in vitro* and the cytosolic Ca^{2+} concentration was calculated using the equation of Grynkiewicz *et al.* (1985). We determined the changes in intracellular calcium levels in response to varying extracellular $[Ca^{2+}]$ in the presence of nystatin (500 i.u. ml^{-1} in $<0{\cdot}01\%$ methanol). The nystatin pore has a low but finite permeability to Ca^{2+}, and changes in extracellular Ca^{2+} over the range 10 nM to 100 μM produce a sustained increase in cytosolic $[Ca^{2+}]$ from 50 to 800 nM. In the presence of nystatin, the intracellular Ca^{2+} level at the following $[Ca^{2+}]_o$ was found to be as follows: at an $[Ca^{2+}]_o$ of 10 nM, 50 nM, 100 nM, 1 μM, 10 μM and 100 μM, the corresponding $[Ca^{2+}]_i$ was 47 ± 11, 110 ± 19, 225 ± 31, 295 ± 51, 385 ± 51 and 515 ± 126 nM ($n = 18$), respectively.

RESULTS

Basolateral chloride transport involved in forskolin-induced chloride secretion

Addition of forskolin to the basolateral membrane produced an immediate and sustained increase in short-circuit current (I_{SC}) consistent with anion secretion (Fig. 1). This effect was concentration dependent with maximal stimulation of current occurring at a forskolin concentration of 15 μM (basal $I_{SC} = 2{\cdot}1 \pm 0{\cdot}3$ μA cm^{-2}, +forskolin $I_{SC} = 65{\cdot}9 \pm 5{\cdot}8$ μA cm^{-2}, corresponding to a mean increase in current (ΔI_{SC}) of $63{\cdot}8 \pm 6{\cdot}2$ μA cm^{-2}, $n = 6$). The concentration–response characteristics for the forskolin response are shown in Fig. 2. This was associated with a fall in transepithelial resistance (control $R_t = 312 \pm 16$ Ω cm^2, forskolin $R_t = 276 \pm 10$ Ω cm^2, $n = 6$, $P < 0{\cdot}01$).

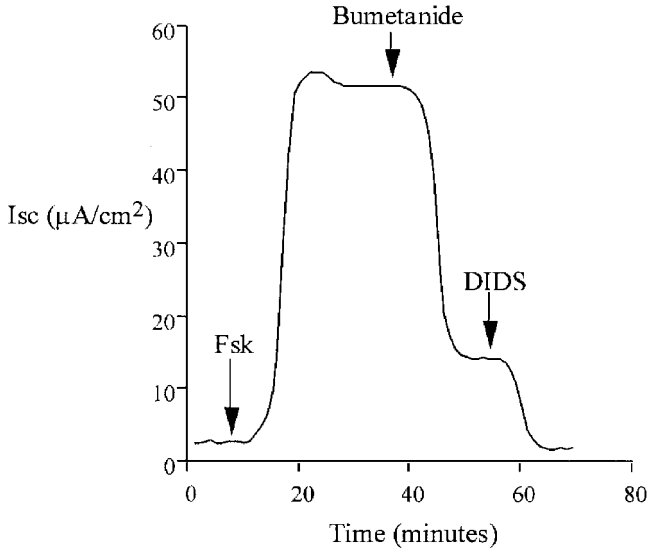

Figure 1

Addition of forskolin (Fsk) to the basolateral side of isolated colon epithelium resulted in an immediate increase in transepithelial short-circuit current, I_{SC}, consistent with anion secretion. This representative trace shows that secretion was inhibited by blockade of basolateral Na^+–K^+–$2Cl^-$ cotransport with bumetanide (100 μM) and Cl^-–HCO_3^- exchange with DIDS (100 μM). Replacement of basolateral Cl^- with gluconate also prevented this response.

Exposure of the colonic epithelium to a low chloride Krebs solution on both sides (NaCl replaced by sodium gluconate) prevented activation of current by forskolin (basal $I_{SC} = 1{\cdot}7 \pm 0{\cdot}8$ μA cm^{-2}, +forskolin $I_{SC} = 3{\cdot}2 \pm 0{\cdot}9$ μA cm^{-2}, $\Delta I_{SC} = 1{\cdot}4 \pm 0{\cdot}4$ μA cm^{-2}, $n = 6$). These results indicate that the forskolin-activated current is carried by chloride ions. Treatment of the basolateral membrane with bumetanide (100 μM), a known inhibitor of Na^+–K^+–$2Cl^-$ cotransport, produced an immediate inhibition of I_{SC} (Fig. 1) (forskolin $I_{SC} = 62{\cdot}2 \pm 4{\cdot}9$ μA cm^{-2}, forskolin + bumetanide $I_{SC} = 20{\cdot}4 \pm 3{\cdot}8$ μA cm^{-2}, bumetanide-inhibited current (ΔI_{SC}) = $41{\cdot}8 \pm 3{\cdot}2$ μA cm^{-2}, $n = 6$). The remaining current was sensitive to the stilbene derivative 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), a known inhibitor of Cl^-–HCO_3^- exchange (forskolin + bumetanide $I_{SC} = 20{\cdot}2 \pm 3{\cdot}6$ μA cm^{-2}, forskolin + bumetanide + DIDS $I_{SC} = 1{\cdot}3 \pm 0{\cdot}6$ μA cm^{-2}, DIDS-sensitive current (ΔI_{SC}) = $18{\cdot}9 \pm 2{\cdot}8$ μA cm^{-2}, $n = 6$). In our experiments we found a small and variable amount of amiloride-sensitive current under basal conditions (basal $I_{SC} = 4{\cdot}9 \pm 2{\cdot}2$ μA cm^{-2}, +amiloride $I_{SC} = 2{\cdot}7 \pm 0{\cdot}4$ μA cm^{-2}, $\Delta I_{SC} = 2{\cdot}2 \pm 0{\cdot}8$ μA cm^{-2}, $n = 6$). We did not detect any change in amiloride-sensitive current following forskolin treatment (forskolin $I_{SC} = 64{\cdot}9 \pm 4{\cdot}1$ μA cm^{-2}, +amiloride $I_{SC} = 62{\cdot}4 \pm 5{\cdot}4$ μA cm^{-2}, $\Delta I_{SC} = 2{\cdot}5 \pm 1{\cdot}3$ μA cm^{-2}, $n = 6$, $P = 0{\cdot}39$). Because of the small amount of amiloride-sensitive current it is not possible to base any definite conclusions on these amiloride data.

DIDS partially inhibits Cl^- channels. To confirm the role of HCO_3^- exchange we performed similar experiments in the nominal absence of CO_2/HCO_3^-. When experiments were performed in the nominal absence of CO_2/HCO_3^- (Hepes-buffered Krebs solution, pH 7·4, gassed with 100% oxygen), the current response to forskolin was blunted (basal $I_{SC} = 1{\cdot}9 \pm 0{\cdot}6$ μA cm^{-2}, +forskolin in Hepes buffer $I_{SC} = 44{\cdot}1 \pm 4{\cdot}2$ μA cm^{-2}, $\Delta I_{SC} = 42{\cdot}2 \pm 3{\cdot}9$ μA cm^{-2}, $n = 6$, significantly different from response in CO_2/HCO_3^- buffer,

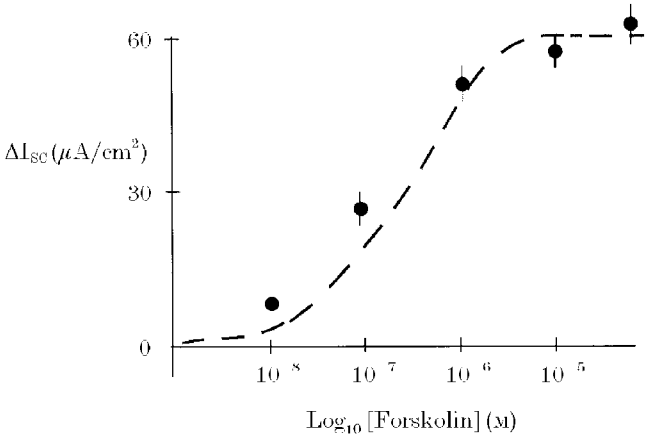

Figure 2. Concentration–response relationship for the effect of forskolin on I_{SC}

Maximal response to forskolin was obtained at a concentration of 10 μM. Dashed lines show the best-fit curve for an equation analagous to the Michaelis–Menten equation.

$P < 0{\cdot}001$). Under Hepes buffer conditions, the forskolin-activated current was almost completely inhibited by bumetanide (forskolin + Hepes $I_{SC} = 41{\cdot}6 \pm 3{\cdot}7\ \mu A\ cm^{-2}$, forskolin + Hepes + bumetanide $I_{SC} = 2{\cdot}5 \pm 0{\cdot}6\ \mu A\ cm^{-2}$, bumetanide-inhibited current (ΔI_{SC}) $= 39{\cdot}1 \pm 2{\cdot}5\ \mu A\ cm^{-2}$, $n = 6$) and DIDS had little inhibitory influence (forskolin + bumetanide $I_{SC} = 2{\cdot}6 \pm 0{\cdot}4\ \mu A\ cm^{-2}$, forskolin + bumetanide + DIDS $I_{SC} = 1{\cdot}4 \pm 0{\cdot}3\ \mu A\ cm^{-2}$, $\Delta I_{SC} = 1{\cdot}2 \pm 0{\cdot}2\ \mu A\ cm^{-2}$, $n = 6$). These results indicate that the cAMP-mediated chloride secretion is dependent on both Na^+–K^+–$2Cl^-$ cotransport and Cl^-–HCO_3^- exchange. They imply that approximately two-thirds of chloride secretion in human colon is mediated by Na^+–K^+–$2Cl^-$ cotransport.

Basolateral potassium transport involved in forskolin-induced chloride secretion

The effects of K^+ channel blockers on the forskolin-induced chloride secretion were determined in intact isolated colon. Sulphonylureas inhibit ATP-regulated K^+ channels (Ashcroft & Ashcroft, 1990) and quaternary ammonium ions are effective blockers of calcium-activated K^+ channels (Villarroel *et al.* 1988). The possible involvement of these classes of K^+ channels in the secretory response was tested using tolbutamide and tetrapentylammonium (TPeA), respectively. We selected a high dose of tolbutamide, 100 μM, to ensure we had maximally inhibited K_{ATP} channels. Pre-treatment of the basolateral side of the colonic epithelium with tolbutamide resulted in a reduction in the steady-state I_{SC} from $12{\cdot}7 \pm 0{\cdot}9$ to $2{\cdot}1 \pm 0{\cdot}4\ \mu A\ cm^{-2}$ ($n = 6$), but did not prevent the chloride secretory response to forskolin ($\Delta I_{SC} = 61 \pm 2{\cdot}4\ \mu A\ cm^{-2}$ compared with control $\Delta I_{SC} = 58{\cdot}4 \pm 3{\cdot}1\ \mu A\ cm^{-2}$, $n = 6$, $P = 0{\cdot}27$). A representative experiment is shown in Fig. 3. Subsequent basolateral addition of TPeA (10 μM) during the peak forskolin response caused a rapid reduction in I_{SC} from $62{\cdot}9 \pm 3{\cdot}2$ to $2{\cdot}7 \pm 0{\cdot}8\ \mu A\ cm^{-2}$ ($n = 6$) (Fig. 3). At the high concentration of tolbutamide used, other classes of potassium channel may have been inhibited. However, since at this concentration of tolbutamide most K_{ATP} channels would be inactive, these results indicate a minor role for sulphonylurea-sensitive K^+ channels in the secretory response. The complete inhibition of the forskolin-induced chloride secretion using TPeA indicates an absolute requirement for activation of a basolateral calcium-dependent K^+ channel to sustain the secretory response. This hypothesis was further tested by pre-treating the epithelium with TPeA prior to forskolin addition (Fig. 4). Pre-treatment with TPeA (10 μM) did not change the steady-state short-circuit current (control $I_{SC} = 11{\cdot}9 \pm 1{\cdot}8\ \mu A\ cm^{-2}$, +TPeA $I_{SC} = 11{\cdot}7 \pm 0{\cdot}9\ \mu A\ cm^{-2}$, $n = 6$). However, the normal chloride secretory response to forskolin was completely prevented by TPeA (TPeA + forskolin $I_{SC} = 11{\cdot}4 \pm 0{\cdot}8\ \mu A\ cm^{-2}$, $n = 6$). The forskolin effect on chloride secretion was sensitive to another quaternary ammonium ion, tetraethylammonium (TEA) and to Ba^{2+}, a general K^+ channel blocker. The concentration dependence for these K^+ channel blockers is shown in comparison with TPeA in Fig. 5. Half-maximal inhibition (IC_{50}) for TPeA was obtained at 5·1 μM ($n = 6$), with 10 μM TPeA producing a maximum inhibition of chloride secretion. TEA and Ba^{2+} were less potent inhibitors of chloride secretion with IC_{50} values of 4·3 ($n = 6$) and 5·6 mM ($n = 6$), respectively.

Effect of prostaglandin E_2

Prostaglandin E_2 (PGE_2) is a secretagogue known to act by increasing intracellular cAMP levels. Addition of PGE_2 (10 μM) to the basolateral membrane of this preparation produced a rapid and sustained increase in I_{SC} consistent with chloride secretion. This I_{SC} was inhibited by bumetanide and DIDS (basal $I_{SC} = 9{\cdot}3 \pm 1{\cdot}8\ \mu A\ cm^{-2}$, PGE_2 I_{SC} 43·6 ±

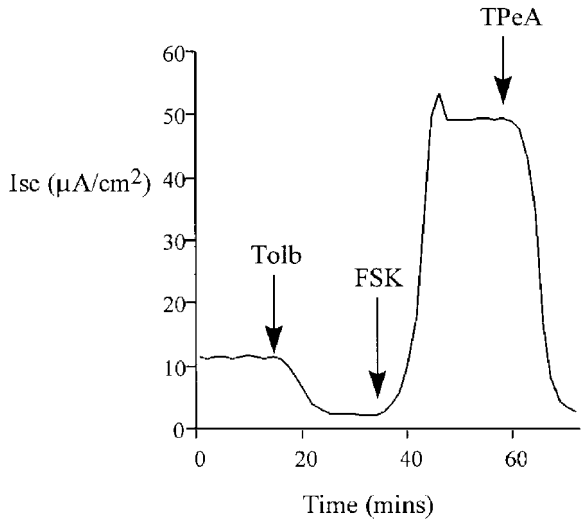

Figure 3

Pre-treatment with tolbutamide (Tolb, 100 μM) decreased baseline I_{SC} but did not reduce the forskolin-induced chloride secretion in isolated colon. The forskolin-induced transepithelial current was TPeA (10 μM) sensitive ($n = 6$).

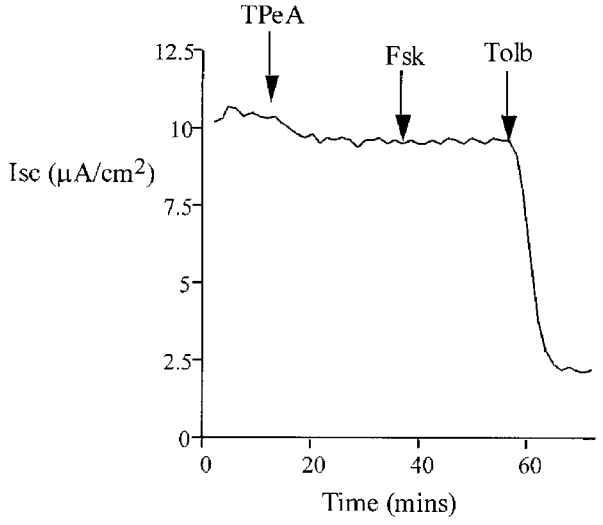

Figure 4

Pre-treatment with TPeA (10 μM) did not affect baseline current, and subsequent addition of basolateral forskolin (20 μM) failed to increase I_{SC}. The remaining current was sensitive to tolbutamide (100 μM) ($n = 6$).

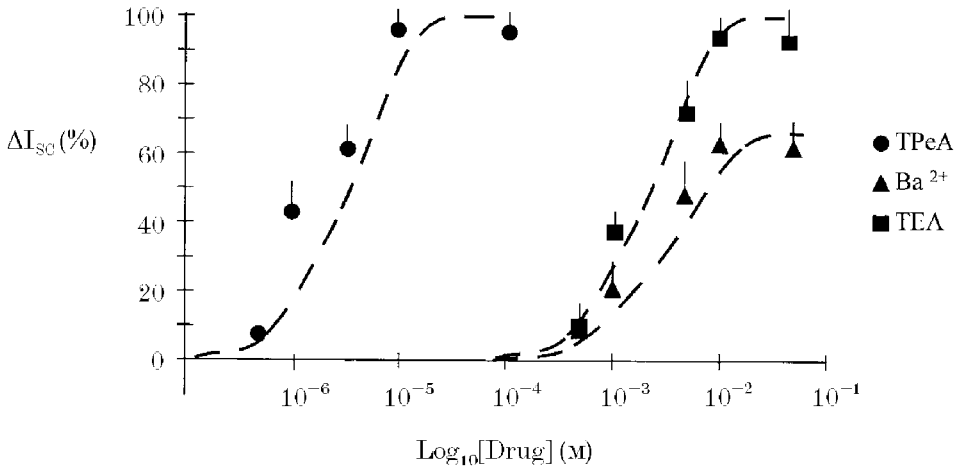

Figure 5. Concentration–inhibition characteristics for TPeA, TEA and Ba^{2+} effects on forskolin-activated Cl^- secretion

Responses are shown as means + S.E.M. ($n = 6$). The forskolin-activated I_{SC} was almost completely inhibited by TPeA at a concentration of 10 μM and by TEA at a concentration of 10 mM. Ba^{2+} produced maximal inhibition at 10 mM. ●, TPeA; ■, TEA; ▲, Ba^{2+}. Best fit for an equation analagous to the Michaelis–Menten equation shown with dashed line.

6·1 μA cm^{-2}, PGE_2 + bumetanide $I_{SC} = 17·4 \pm 4·1$ μA cm^{-2}, PGE_2 + bumetanide + DIDS $I_{SC} = 4·2 \pm 1·1$ μA cm^{-2}, $n = 6$). The PGE_2-activated current showed similar sensitivity to the forskolin-activated current to treatment of the basolateral membrane with potassium channel blockers (PGE_2 $I_{SC} = 39·3 \pm 5·8$ μA cm^{-2}, PGE_2 + 100 μM tolbutamide $I_{SC} = 38·6 \pm 6·1$ μA cm^{-2}, PGE_2 + 100 μM tolbutamide + 10 μM TPeA $I_{SC} = 5·4 \pm 4·1$ μA cm^{-2}).

Basolateral K^+ transport in the nystatin-perforated colon

We investigated basolateral K^+ transport using nystatin perforation of the apical membrane. Treatment of the apical membrane of mammalian colonic epithelium with nystatin has previously been shown to remove the electromotive force and resistance generated by the apical membrane. This allows investigation of the basolateral membrane in isolation (Wills *et al.* 1979). In the presence of high mucosal [K^+] and low [Ca^{2+}] (10 nM), the addition of nystatin produced an immediate increase in transepithelial current (from 22·3 ± 0·8 to 51·0 ± 1·2 μA cm^{-2}, corresponding to a mean increase in current (ΔI_t) = 28·7 ± 1·1 μA cm^{-2}, $n = 40$), with membrane potential clamped to 0 mV. This was associated with a fall in transepithelial resistance (control $R_t = 326 \pm 9$ Ω cm^2, forskolin $R_t = 166 \pm 12$ Ω cm^2, $n = 40$, $P < 0·01$). The K^+ dependence of this current was tested by graded replacement of all potassium salts with sodium salts in the apical bath (Fig. 6). The nystatin-induced current was abolished following total replacement of apical K^+ by Na^+ and a linear correlation ($r = 0·89$, $P < 0·01$) with zero intercept was found for the relationship between transepithelial current and apical [K^+] over the range 0–120 mM. Therefore, the nystatin-induced transepithelial current reflects channel-mediated K^+ flow (K^+ channel activity) across the basolateral membrane and is hereafter referred to as trans-basolateral K^+ current (I_K). Further proof for this conclusion was obtained from the effects of K^+ channel inhibitors on the nystatin-induced current. There was a small transepithelial HCO_3^- gradient generated by the solutions used for our nystatin method. This did not result in a significant diffusion potential (the HCO_3^- gradient was maintained during the sodium substitution protocol; when the K^+ gradient was zero there was no measurable I_{SC} despite the maintained HCO_3^- gradient).

Under conditions designed to preserve the intracellular ionic environment (apical bath containing high [K^+] (120 mM), low [Cl^-] (20 mM) and low [Ca^{2+}] (10 nM)), the current

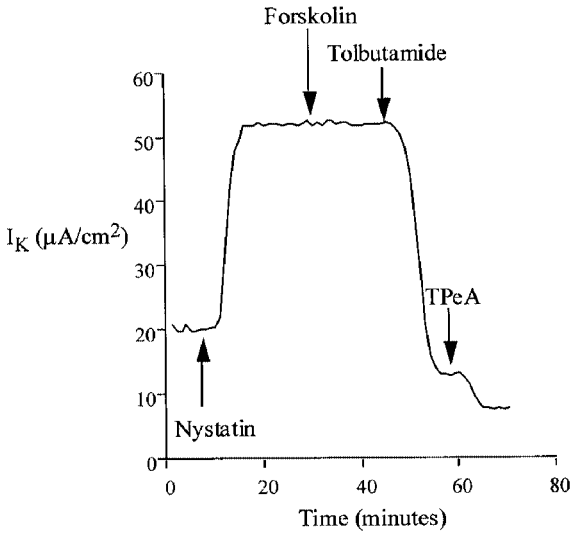

Figure 6. Potassium dependence of the trans-basolateral current in the nystatin-perforated colon

Replacement of potassium gluconate with equimolar sodium gluconate in the apical bath over the concentration range 0–120 mM.

present under nystatin-permeabilized conditions was almost completely inhibited by basolateral tolbutamide and, to a much lesser extent, by TPeA (Fig. 7). This sensitivity profile of I_K to these K^+ channel inhibitors was independent of the order of addition of drugs (data not shown). Tolbutamide reduced the K^+-dependent current by $47{\cdot}5 \pm 1{\cdot}2\ \mu A\ cm^{-2}$ ($n = 20$), corresponding to a 93% inhibition of the total I_K, whereas TPeA inhibited the K^+-dependent current by only $6{\cdot}3 \pm 0{\cdot}8\ \mu A\ cm^{-2}$ (5% of total I_K) ($n = 20$). The current remaining after combined tolbutamide and TPeA treatment was insensitive to barium and may be generated by paracellular K^+ leak from mucosa to serosa, since it was abolished by elimination of the transepithelial K^+ gradient (mucosal Na^+ substitution for K^+). These results imply that under low intracellular [Ca^{2+}], basolateral K^+ transport occurs through a sulphonylurea-sensitive K^+ conductance pathway.

Under experimental conditions with approximately 1 μM free calcium, the addition of nystatin produced an immediate increase in K^+-dependent transepithelial current (from $18{\cdot}4 \pm 1{\cdot}8\ \mu A\ cm^{-2}$, $\Delta I_K = 33{\cdot}6 \pm 2{\cdot}5\ \mu A\ cm^{-2}$, $n = 40$). Under these conditions of high $[Ca^{2+}]_i$, the I_K was almost completely abolished by TPeA or TEA with relatively low sensitivity to tolbutamide (Fig. 8). TPeA (10 μM) reduced the I_K by $45{\cdot}2 \pm 3{\cdot}8\ \mu A\ cm^{-2}$ (control nystatin $I_K = 52{\cdot}6 \pm 3{\cdot}2\ \mu A\ cm^{-2}$, nystatin + TPeA $I_K = 7{\cdot}4 \pm 0{\cdot}6\ \mu A\ cm^{-2}$, $n = 20$), corresponding to an 86% inhibition of I_K, whereas tolbutamide (100 μM) inhibited the I_K by only $5{\cdot}4 \pm 0{\cdot}7\ \mu A\ cm^{-2}$, corresponding to 10% of total I_K ($n = 20$) (nystatin $I_K = 55{\cdot}1 \pm 2{\cdot}7\ \mu A\ cm^{-2}$, nystatin + tolbutamide $I_K = 49{\cdot}7 \pm 3{\cdot}5\ \mu A\ cm^{-2}$). This high sensitivity of I_K, under conditions of elevated $[Ca^{2+}]_i$, to quaternary ammonium ions and low sensitivity to sulphonylureas is in contrast to the pharmacological profile observed under low $[Ca^{2+}]_i$. The IC_{50} values for TPeA (5·5 μM), TEA (5·1 mM) and Ba^{2+} (4·4 mM) are very similar to the half-maximal effective concentration of these drugs on forskolin-induced chloride secretion observed in the intact tissue (Fig. 5). The results indicate that under low physiological levels of intracellular Ca^{2+}, a sulphonylurea-sensitive K^+ channel generates the basolateral membrane current. When intracellular [Ca^{2+}] is increased, or when chloride secretion is activated by forskolin, the tolbutamide-sensitive channel is inhibited and basolateral K^+ current is carried predominantly by a TPeA-sensitive K^+ channel. It has been shown in other tissues that activation of one conductance class downregulates the activity of the other (Germann *et al.* 1986).

With 50 nM Ca^{2+} in the apical bath, the I_K was generated by both tolbutamide- and TPeA-sensitive channels (nystatin $I_K = 55{\cdot}4 \pm 6{\cdot}6\ \mu A\ cm^{-2}$, nystatin + TPeA $I_K = 41{\cdot}7 \pm 4{\cdot}6\ \mu A\ cm^{-2}$, nystatin + TPeA + tolbutamide $I_K = 6{\cdot}4 \pm 2{\cdot}1\ \mu A\ cm^{-2}$, $n = 6$). This implies that under conditions close to physiological levels of intracellular calcium (~100 nM), approximately 70% of the I_K is generated by tolbutamide-sensitive channels, and the remaining current is generated mainly by TPeA-sensitive channels.

Forskolin effects in nystatin-perforated colon

The colonic crypt is a heterogeneous functional epithelium. Chloride secretion in mammalian colon is believed to occur in cells located in the middle third of the crypt. If nystatin failed to penetrate deep into the crypt, to perforate the

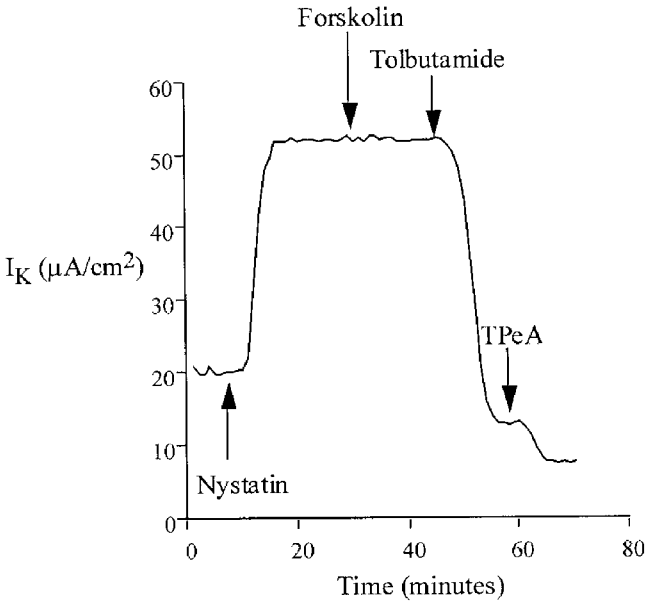

Figure 7

Following permeabilization of the apical membrane with nystatin, treatment of the basolateral membrane with forskolin did not alter serosal K^+ transport under low apical [Ca^{2+}] conditions. However, the remaining current was almost completely inhibited by basolateral addition of tolbutamide (100 μM).

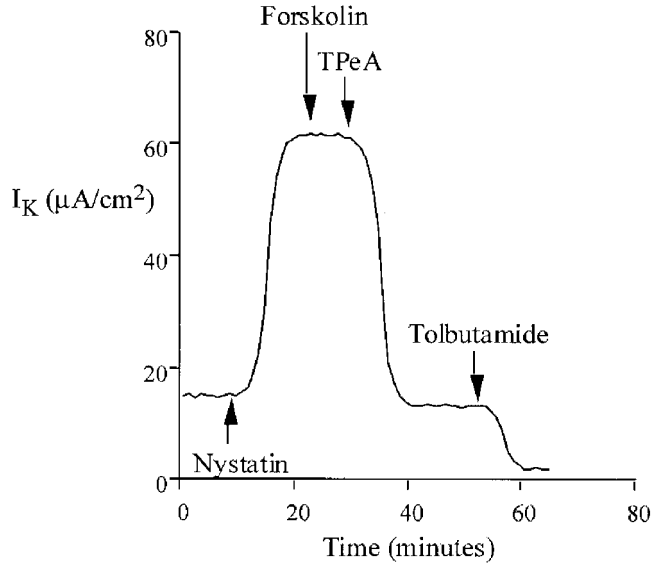

Figure 8

Inhibition by TPeA (10 μM) of basolateral K^+ current in the nystatin-perforated colon under conditions of high intracellular calcium (1 μM Ca^{2+} in the apical bathing solution). Forskolin (20 μM) did not produce a change in basolateral K^+ current in the nystatin-perforated colon at high intracellular [Ca^{2+}].

apical membrane, the forskolin-induced chloride secretory current should remain intact. We found no response to forskolin following nystatin treatment under conditions designed to measure chloride secretion (physiological Krebs solution in both apical and basolateral baths; nystatin $I_{SC} = 0{\cdot}7 \pm 0{\cdot}2$ μA cm^{-2}, nystatin + forskolin $I_{SC} = 0{\cdot}8 \pm 0{\cdot}1$ μA cm^{-2}, $n = 6$, $P = 0{\cdot}25$).

The nystatin-induced current has been shown to be essentially generated by a tolbutamide-inhibitable K^+ channel under low [Ca^{2+}] conditions and via a TPeA-inhibitable K^+ channel with a high [Ca^{2+}]. After treatment with nystatin, forskolin failed to affect transepithelial current under conditions of 10 nM, 50 nM, 100 nM or 1 μM Ca^{2+} in the external bathing solution; 10 nM and 1 μM experiments are shown in Figs 7 and 8, respectively (nystatin + 10 nM Ca^{2+} $I_K = 49{\cdot}7 \pm 2{\cdot}2$ μA cm^{-2}, +forskolin $I_K = 48{\cdot}4 \pm 1{\cdot}9$ μA cm^{-2}, $n = 6$, $P = 0{\cdot}84$; nystatin + 50 nM Ca^{2+} $I_K = 51{\cdot}3 \pm 1{\cdot}9$ μA cm^{-2}, +forskolin $I_K = 51{\cdot}1 \pm 1{\cdot}9$ μA cm^{-2}, $n = 6$, $P = 0{\cdot}63$; nystatin + 100 nM Ca^{2+} $I_K = 50{\cdot}8 \pm 3{\cdot}1$ μA cm^{-2}, +forskolin $I_K = 50{\cdot}4 \pm 3{\cdot}0$ μA cm^{-2}, $n = 6$, $P = 0{\cdot}68$; nystatin + 1 μM Ca^{2+} $I_K = 53{\cdot}7 \pm 3{\cdot}9$ μA cm^{-2}, +forskolin $I_K = 53{\cdot}1 \pm 4{\cdot}2$ μA cm^{-2}, $n = 6$, $P = 0{\cdot}42$).

Intracellular calcium measurements

Basal [Ca^{2+}]$_i$ was 131 ± 29 nM ($n = 12$ crypts). Forskolin did not increase or decrease basal [Ca^{2+}]$_i$ of human colonic crypts over 20 min at concentrations of 1–20 μM ($n = 12$, $P > 0{\cdot}3$). [Ca^{2+}]$_i$ was 137 ± 31 nM ($n = 12$ crypts) 20 min after exposure to 20 μM forskolin.

DISCUSSION

The aim of this study was to determine the role of basolateral K^+ channels in cAMP-activated secretion in human colon. We found that forskolin activated an immediate and sustained increase in chloride-dependent short-circuit current consistent with chloride secretion. This current was sensitive to inhibition of basolateral Na^+–K^+–$2Cl^-$ cotransport with bumetanide and to a lesser extent to inhibition of Cl^-–HCO_3^- exchange produced by DIDS. Since the maximal sensitivity of human colonic Cl^-–HCO_3^- exchange is approximately 60% with 1 mM DIDS (Mahajan *et al.* 1996), experiments were also performed in the nominal absence of CO_2/HCO_3^- in order to completely inhibit Cl^-–HCO_3^- exchange. We found that the Na^+–K^+–$2Cl^-$ cotransporter is responsible for two-thirds of cAMP-dependent chloride

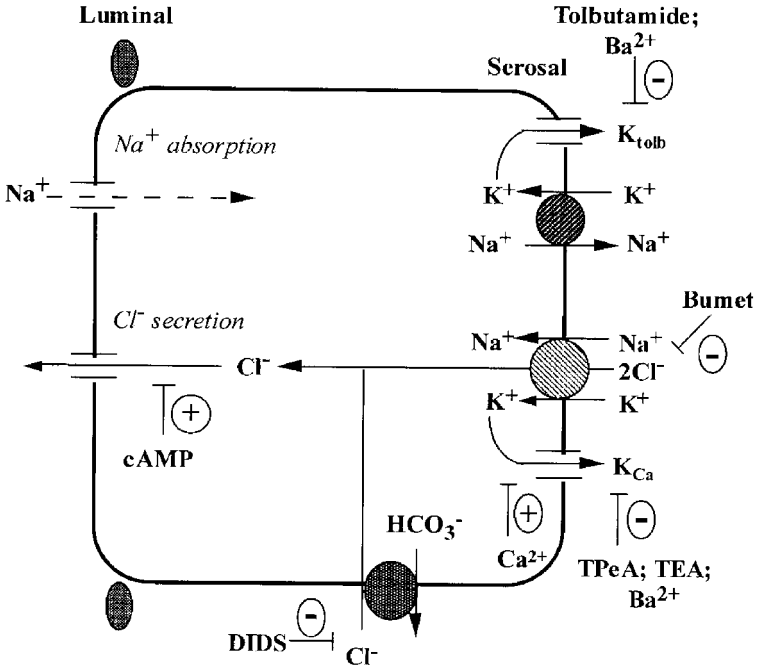

Figure 9. Cell model of the membrane transport processes in the human distal colon

The basolateral Na^+–K^+–$2Cl^-$ cotransporter and Cl^-–HCO_3^- exchanger contribute two-thirds and one-third, respectively, of transepithelial chloride secretion. The cAMP-activated chloride secretion is dependent on co-stimulation of basolateral calcium-dependent, quaternary ammonium-sensitive K^+ channels. A tolbutamide-sensitive K^+ channel (K_{tolb}) is dominant under basal conditions and is inactivated following forskolin-induced Cl^- secretion or under conditions of high intracellular calcium. The K_{tolb} channel shares pharmacological properties with ATP-regulated K^+ channels and is most probably involved in basolateral K^+ recycling under steady-state and aldosterone-dependent sodium reabsorption conditions. K_{Ca} channels and K_{tolb} channels may be localized in separate cell types (e.g. K_{tolb} channels in surface Na^+ reabsorbing cells and K_{Ca} channels in chloride-secreting crypt cells) or the two channel types may co-exist in the same pluripotent crypt cell.

secretion under physiological buffer conditions, and that Cl^-–HCO_3^- exchange activity contributes the remaining one-third to the secretory process (Fig. 9). We could not detect any alteration in amiloride-sensitive Na^+ transport following forskolin treatment. These results are not reliable because of the small amount ($\sim 2\ \mu A\ cm^{-2}$) of basal amiloride-sensitive current. Forskolin has been shown to inhibit amiloride-sensitive Na^+ transport in rat colon (Ecke *et al.* 1996) and further experiments are required to elucidate the exact effect of cAMP on Na^+ transport in intact human colonic epithelium.

Apical chloride transport must be coupled to basolateral potassium transport if a favourable gradient for chloride secretion is to be maintained (Mandel *et al.* 1986). There are believed to be two signal transduction pathways involved in human colonic secretion (Weyer *et al.* 1985; Dharmsathaphorn & Pandol, 1987). Agonists such as carbachol increase intracellular calcium while PGE_2 acts through cAMP. In this study we investigated basolateral potassium transport under conditions of high and low cellular $[Ca^{2+}]$ and during cAMP-activated chloride secretion in human colon.

Basolateral K^+ conductance was shown to be essential in maintaining a secretory response to forskolin. The potassium channel inhibitor Ba^{2+} produced an immediate inhibition of the forskolin-activated current. This chloride secretory current was also inhibited by TPeA and TEA at similar doses to those which inhibited basolateral calcium-dependent K^+ channels in nystatin-perforated colon. The sulphonylurea tolbutamide, an inhibitor of ATP-dependent K^+ channels, had a much smaller effect on forskolin-induced chloride secretion. These results imply a dominant role for a calcium-dependent K^+ conductance in cAMP-mediated chloride secretion with a minimal role for tolbutamide-inhibitable K^+ channels in the secretory response (Fig. 9). We also found that Ba^{2+} only blocked 60 % of the Ca^{2+}-dependent K^+ conductance, while TPeA and TEA produced $< 90\,\%$ inhibition of the Ca^{2+}-dependent K^+ conductance. This suggests either that this current is carried by a single channel which is only partially inhibited by Ba^{2+} or that the current is carried by two channels, one of which is insensitive to Ba^{2+}. Previous patch-clamp experiments on isolated human colonic crypts have identified a 23 pS, Ba^{2+}-sensitive and calcium-activated K^+ channel on the basolateral membrane of human colonic epithelium (Sandle *et al.* 1994). Our results suggest that this channel may play an important role in maintaining chloride secretion in human colonic epithelium.

In nystatin-perforated colon, forskolin had no effect on basolateral K^+ conductance under the following conditions: (1) at low levels of intracellular calcium when both K_{ATP} and K_{Ca} channels were active, and (2) when K_{Ca} channels were activated by increasing the level of intracellular calcium. This was a surprising result because exposure to cAMP activates Ca^{2+}-activated K^+ channels in the basolateral membrane of human colonic crypts (Sandle *et al.* 1994). Forskolin or exposure to cAMP also activates Ca^{2+}-activated K^+ channels in the apical membrane of cultured kidney cells (Guggino *et al.* 1985), and basolateral K^+ channels in rat (Böhme *et al.* 1991; Siemer & Gögelein, 1992, 1993; Greger *et al.* 1997*b*) and rabbit (Loo & Kaunitz, 1989) colonic crypts and T84 cells (Baró *et al.* 1994). There are two possible explanations for this surprising result. Firstly, our results may be an artefact of using nystatin perforation to investigate K^+ transport across the basolateral membrane, and measurements of macroscopic K^+ current may not be sensitive enough to measure direct cAMP activation of basolateral K^+ channels. This would particularly apply if, as in rat colon, cAMP actually inhibited one group of K^+ channels. Secondly, the activation of basolateral K^+ transport which supports chloride secretion may occur as a result of an indirect mechanism which is disrupted by nystatin perforation of the apical membrane. Two such signals are membrane potential and intracellular calcium. The evidence does not support a role for intracellular calcium. Carbachol activates chloride secretion and basolateral K^+ channels by increasing intracellular Ca^{2+}. We have found that carbachol also activates basolateral K^+ conductance in nystatin-perforated human colon using an identical protocol (Winter *et al.* 1996). Moreover, we found no detectable change in the levels of intracellular calcium measured in isolated colonic crypts exposed to forskolin. However, membrane potential could act as this 'cross-talk' signal. Activation of apical chloride channels would depolarize the cell and increase the electrical driving force for basolateral K^+ efflux. A possible role for membrane potential in driving basolateral K^+ efflux during forskolin-induced chloride secretion in human colon is supported by micro-impalement studies on the human colonic cell line HT-29. Here cAMP does not directly activate basolateral K^+ transport but cAMP-activated chloride secretion is associated with significant depolarization (Bajnath *et al.* 1991). There also appears to be an important interspecies difference between rat and human colon. In rat colon increasing intracellular cAMP activates chromanol-sensitive K^+ channels while inhibiting a Ca^{2+}-dependent channel (Greger *et al.* 1997*a*, *b*; Nielsen *et al.* 1998), and this results in a fall in macroscopic K^+ conductance (Schultheiss & Diener, 1997) .

cAMP-mediated chloride secretion is an important component of inflammatory and infective colonic disease (Binder *et al.* 1991). Understanding the mechanism of secretion in intact native human colon is important for developing therapeutic strategies for the medical treatment of diseases of the large intestine. We have shown that during cAMP-mediated chloride secretion chloride enters the cell through two distinct pathways. We have also shown that potassium recycling across the basolateral membrane is an essential component of cAMP-mediated chloride secretion (Fig. 9). This potassium recycling occurs through a Ca^{2+}-activated and TPeA-sensitive pathway; this has not previously been demonstrated in native human colon. We were unable to show direct activation of this pathway by cAMP.

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