



Cyclic AMP-dependent regulation of K^+ transport in the rat distal colon

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1 The effect of agonists of the cyclic AMP pathway and of 293B, a chromanole-derived K^+ channel blocker, on K^+ transport in the rat distal colon was studied by measuring unidirectional fluxes, uptake, and efflux of Rb^+ in mucosa-submucosa preparations and by patch-clamp of crypt epithelia from isolated crypts.

2 293B concentration-dependently inhibited basal and forskolin-stimulated short-circuit current. In isolated crypts 293B blocked a basal K^+ conductance but had no effect on cyclic AMP-evoked depolarization induced by the opening of apical Cl^- channels. When the effect of cyclic AMP on Cl^- conductance was prevented by substituting Cl^- with gluconate, an inhibition of total cellular K^+ conductance by forskolin and a membrane-permeable cyclic AMP analogue was unmasked.

3 Unidirectional ion flux measurements revealed that 293B suppressed the increase in J_{sm}^{Rb} induced by forskolin. This, together with the inhibition of cyclic AMP-induced anion secretion indicates that the drug blocks K^+ channels, presumably both in the apical and the basolateral membrane. Forskolin caused not only inhibition of K^+ absorption, but also stimulation of K^+ secretion. The inhibition was diminished, but not blocked, in the presence of inhibitors of the apical $H^+-K^+-ATPase$, vanadate and ouabain. Forskolin stimulated serosal, bumetanide-sensitive Rb^+ uptake, whereas mucosal, ouabain/vanadate-sensitive uptake remained unaffected.

4 Efflux experiments revealed that forskolin caused a redistribution of cellular K^+ efflux reducing the ratio of basolateral versus apical Rb^+ efflux.

5 These results suggest that intracellular cyclic AMP exerts its effects on K^+ transport by several mechanisms: an increase in the driving force for K^+ efflux due to the depolarization induced by opening of Cl^- channels, a stimulation of the basolateral uptake of K^+ via the $Na^+-K^+-Cl^-$ -cotransporter, and a decrease of the ratio of basolateral versus apical K^+ conductance leading to an enhanced efflux of K^+ into the lumen and a reduced K^+ efflux to the serosal compartment.

Keywords: 293B; cyclic AMP; Cl^- secretion; electrolyte transport; $H^+-K^+-ATPase$; K^+ channels; K^+ secretion; rat distal colon

Introduction

The distal colon is able both to absorb and to secrete K^+ ions. The current, generally accepted model of K^+ absorption by the colon (Binder & Sandle, 1994) assumes that K^+ ions are absorbed from the colonic lumen by an energy-consuming step, i.e. a $H^+-K^+-ATPase$. In the rat distal colon this enzyme is restricted to the apical membrane of the differentiated cells at the surface and the upper fifth of the colonic crypts (Jaisser *et al.*, 1993) as shown by *in situ* hybridization, whereas in the rabbit distal colon biochemical studies indicate a different distribution of two distinct enzymes along the crypt-surface axis (Abrahamse *et al.*, 1995). Functionally, the $H^+-K^+-ATPase(s)$ in the rat colon comprises a ouabain-sensitive and a ouabain-resistant, but vanadate-sensitive component (Del Castillo *et al.*, 1991; Tabuchi *et al.*, 1992). Basolateral exit of K^+ in the rabbit distal colon may be mediated by K^+ channels as shown by the inhibition of K^+ absorption by serosal Ba^{2+} (Halm & Frizzell, 1986). Potassium secretion is driven by the intracellular accumulation of K^+ ions which enter by the basolateral $Na^+-K^+-Cl^-$ -cotransporter and the $Na^+-K^+-ATPase$. K^+ leaves the cell via apical K^+ channels (for review, see Binder & Sandle, 1994). In addition, there is a passive flux of K^+ across the epithelium via the (paracellular) shunt pathway (McCabe *et al.*, 1986).

Potassium transport is under the control of adenosine 3': 5'-cyclic monophosphate (cyclic AMP). A stimulation of K^+ secretion by cyclic AMP (Foster *et al.*, 1983) or agents which

increase intracellular cyclic AMP like β -receptor agonists (Smith & McCabe, 1984), is well known. Hormones, which decrease intracellular cyclic AMP such as neuropeptide Y or somatostatin, inhibit K^+ secretion and stimulate K^+ absorption (Strabel & Diener, 1995b).

Recently, a new class of K^+ channel blockers based on 1,4-sulphonylaminochromanole, was developed (Lohrmann *et al.*, 1995). The most potent of them, 293B (*trans*-6-cyano-4-[N-ethylsulphonyl-N-methylamino]-3-hydroxy-2,2-dimethyl-chromanole), inhibited cyclic AMP-induced short-circuit current (I_{sc}) in rabbit distal colon with a high potency (IC_{50} 7×10^{-7} mol l^{-1}). Microelectrode experiments revealed that the drug inhibited a cyclic AMP-stimulated K^+ conductance (Lohrmann *et al.*, 1995). The present experiments were carried out in order to investigate the effects of cyclic AMP on K^+ transport across the rat distal colon using 293B as a tool. The effect of agonists of the cyclic AMP-pathway and K^+ transport inhibitors on I_{sc} , unidirectional Rb^+ fluxes, Rb^+ uptake, and Rb^+ efflux in the intact mucosa and membrane potential and membrane currents in isolated colonic crypts was studied in order to elucidate the mechanisms of cyclic AMP-induced stimulation of K^+ secretion and cyclic AMP-induced inhibition of K^+ absorption.

Methods

Solutions

For the experiments with isolated crypts the following buffers were used. The EDTA solution for the crypt isolation con-

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tained (mmol l⁻¹): NaCl 107, KCl 4.5, NaH₂PO₄ 0.2, Na₂HPO₄ 1.8, NaHCO₃ 25, EDTA (ethylene diamino tetraacetic acid) 10, glucose 12, with 0.1% bovine serum albumin (w/v). The pH was adjusted to 7.4 by Tris-base (tris(hydroxymethyl)aminomethane). The high K⁺ Tyrode solution for the storage of the crypts consisted of (mmol l⁻¹): K⁺ gluconate 100, KCl 30, NaCl 20, CaCl₂ 1.25, MgCl₂ 1, HEPES (N-(2-hydroxyethyl)piperazine-N'-2-ethansulphonic acid) 10, glucose 12, Na pyruvate 5, 0.1% bovine serum albumin (w/v). The solution was adjusted with KOH to a pH of 7.4. The medium for the superfusion of the crypts was a 140 mmol l⁻¹ Ringer solution containing (mmol l⁻¹): NaCl 140, KCl 5.4, CaCl₂ 1.25, MgSO₄ 1, HEPES 10. For the Cl⁻-free buffer, NaCl was replaced by Na⁺ gluconate; the Ca²⁺ concentration was elevated to 10 mmol l⁻¹ in order to compensate for the Ca²⁺-chelating properties of gluconate (Kenyon & Gibbons, 1977). For superfusion experiments with vasoactive intestinal polypeptide (VIP), the solutions also contained bovine serum albumin (0.1%; w/v) in order to prevent adsorption of peptide to the perfusion system.

In the Ussing chamber experiments a Parsons solution was used containing (mmol l⁻¹): NaCl 107, KCl 4.5, NaHCO₃ 25, Na₂HPO₄ 1.8, NaH₂PO₄ 0.2, CaCl₂ 1.25, MgSO₄ 1 and glucose 12. The solution was gassed with carbogen (5% CO₂ in 95% O₂); pH was 7.4. When fluxes of ⁸⁶Rb⁺ were measured, KCl was substituted by RbCl. For the Cl⁻-free buffer, NaCl was replaced with Na⁺ gluconate (elevating the Ca²⁺-concentration to 5.75 mmol l⁻¹).

Most of the whole-cell experiments were performed with a K⁺ gluconate/KCl pipette solution, which contained (mmol l⁻¹): K⁺ gluconate 100, KCl 30, NaCl 10, MgCl₂ 2, EGTA (ethyleneglycol bis-(β-aminoethylether) N,N,N',N'-tetraacetic acid) 0.1, Tris 10, ATP (adenosine 5'-triphosphate disodium salt) 5; the pH was 7.2. For the Cl⁻-free experiments, KCl and NaCl were replaced by K⁺ gluconate.

Tissue preparation

Female SIVZ-50 rats (Institut für Labortierkunde, Universität Zürich, Switzerland) were used with a weight of 180–220 g. The animals had free access to water and food until the day of the experiment. Animals were killed by a blow on the head followed by exsanguination. The serosa and muscularis propria were stripped away to obtain a mucosa-submucosa preparation of the distal part of the colon descendens. Briefly, the colon was placed on a small plastic rod with a diameter of 5 mm. A circular incision was made near the anal end with a blunt scalpel and the serosa together with the lamina propria were gently removed in a proximal direction (Andres *et al.*, 1985).

Crypt isolation

The mucosa-submucosa was fixed on a plastic holder with tissue adhesive and transferred for 8 min in the EDTA solution. The mucosa was vibrated once for 30 s in order to isolate intact crypts. They were collected in an intracellular like high K⁺ Tyrode buffer (Böhme *et al.*, 1991). The mucosa was kept at 37°C during the isolation procedure. All further steps including the patch experiments were carried out at room temperature.

Patch-clamp experiments

The crypts were pipetted into the experimental chamber, which comprised a silicon ring attached to a glass slide. The volume of the chambers used was 0.4 ml. The crypts were fixed to the glass bottom of the chamber with the aid of poly-L-lysine (0.02%, w/v). The preparation was superfused hydrostatically throughout the experiment at a perfusion rate of about 1 ml min⁻¹. The chamber was mounted on the stage of an inverted microscope (Olympus IMT2-F). Patch pipettes were pulled from thick-walled borosilicate glass capillaries (Jencons

Scientific Ltd., Bedfordshire, UK, outer diameter 2 mm, inner diameter 1 to 1.25 mm) on a two-stage puller (H. Ochotzki, Homburg/Saar, Germany). After fire-polishing, the tips had resistances of 5 to 10 MΩ, when filled with the standard pipette solution. For conventional whole-cell recording the membrane patch under the tip of the pipette was broken by a stronger suction pulse after formation of the seal. In the experiments with VIP, when chemically perforated patches were used, the pipette tip was pre-filled with the normal pipette solution by dipping it into this solution for 8–10 s. The pipette was then backfilled with a solution containing nystatin (300 μg ml⁻¹) using a syringe (Böhme *et al.*, 1991). Perforation of the patch was indicated by an increase of the capacitance, a decrease of the resistance and a stable membrane potential under current-clamp conditions. Seal resistances were 2 to 10 GΩ. The capacitance of the patch membrane was compensated for.

Patch-clamp currents were recorded on a RK-400 amplifier (Biologics, Meylan, France). Current and voltage signals were digitized at 48 kHz and stored on a modified digital audio recorder (DTR-1200, Biologics, Meylan, France). The reference point for the patch potentials was the extracellular side of the membrane, which was assumed to have zero potential. Current-voltage (*I-V*) curves were obtained by clamping the cell at a holding potential of -80 mV and giving a stepwise

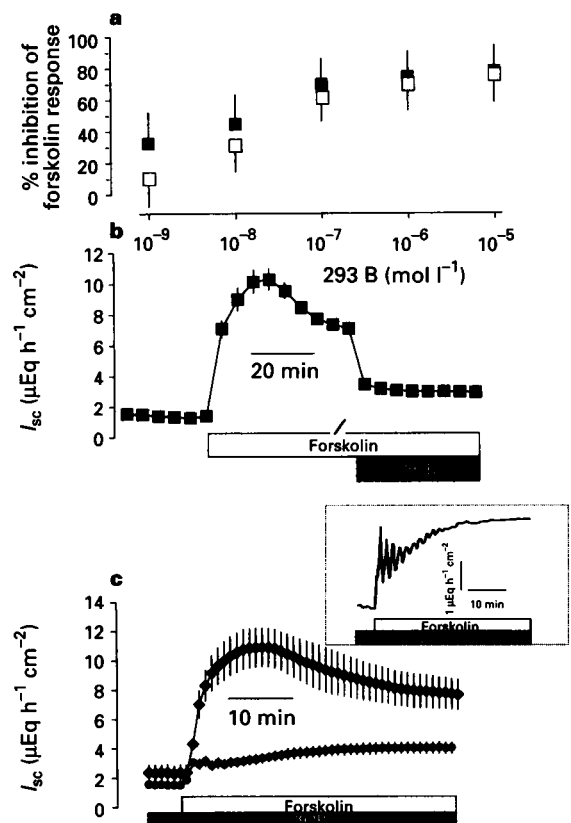


Figure 1 (a) Effect of 293B, administered cumulatively to the serosal (□) or the mucosal (■) side, on the *I*_{sc} response induced by forskolin (5 × 10⁻⁶ mol l⁻¹, administered to the serosal and the mucosal side). Values are means ± 1 s.e.mean, *n* = 7–8. (b) Time-course of the effect of 293B (10⁻⁶ mol l⁻¹, administered to the serosal and the mucosal side) on the *I*_{sc} response induced by forskolin (5 × 10⁻⁶ mol l⁻¹, administered to the serosal and the mucosal side). Values are means ± 1 s.e.mean, *n* = 15. (c) Effect of forskolin (5 × 10⁻⁶ mol l⁻¹, administered to the serosal and the mucosal side) alone (◆) and after pretreatment with 293B (10⁻⁶ mol l⁻¹, administered to the serosal and the mucosal side; ●). Values are means ± 1 s.e.mean, *n* = 6. In the inset, a representative tissue showing the oscillations of the *I*_{sc} response induced by forskolin in the presence of 293B is shown, which is lost in Figure 1c due to the averaging.

depolarization of 10 mV for 30 ms. The amplitude of the voltage step was augmented after each pulse by 10 mV until a final clamping voltage of +60 mV. After each depolarization step, the cell was clamped again to the holding potential for 1 s. For statistical comparison of membrane currents, outward current was measured at the end of a pulse depolarizing the cell for 30 ms from -80 to +60 mV, inward current was measured at the holding potential of -80 mV just before application of the first voltage step, i.e. just before depolarization from -80 to -70 mV.

Short-circuit current measurement

The mucosa-submucosa preparation was fixed in a modified Ussing chamber, bathed with a volume of 4 ml on each side of the mucosa (Diener & Rummel, 1989). The tissue was incubated at 37°C in Parsons solution and short-circuited by a voltage clamp (Aachen Microclamp, AC Copy Datentechnik, Aachen, Germany) with correction for solution resistance. Short-circuit current (I_{sc}) was continuously recorded on a chart-recorder. In addition, I_{sc} and tissue conductance (Gt) were printed every min by a computer printer. When the transient effects of a drug on I_{sc} are described such as the response to carbachol, the maximal increase in I_{sc} induced by the drug is given. In the tables describing the unidirectional ion flux or the uptake experiments, the electrical parameters were averaged over the total 20 min period, in which fluxes or uptake were measured. I_{sc} was expressed as charge transfer per time and area ($\mu\text{Eq h}^{-1} \text{cm}^{-2}$) in order to allow a direct comparison of ion fluxes with I_{sc} .

Unidirectional flux measurements

In most cases (as indicated in the text), tissues were pretreated with indomethacin (administered before application of the radioisotopes) in order to block the on-going spontaneous production of cyclic AMP by prostaglandins released from the submucosa (Craven & DeRubertis, 1983). Ion flux studies were performed as previously described (Diener *et al.*, 1994). After an equilibration period of 60 min, ^{22}Na (59 kBq) and ^{36}Cl (29 kBq), or ^{86}Rb (74 kBq) was added to one side (= labelled side) of the epithelium. After an additional 20 min to allow isotope fluxes to reach a steady state, unidirectional ion fluxes were determined over 2 or 3 sequential 20 min periods starting 20 min after administration of the drug to be studied or anion substitution, respectively. A 20 min delay between drug administration and the flux measurement was selected in order to allow ion fluxes to reach a new steady state as indicated by the new stable I_{sc} .

From the measured unidirectional fluxes net ion fluxes were calculated according to: $J_{net} = J_{ms} - J_{sm}$. Residual ion flux, i.e. the sum of the movement of all ions other than Na^+ and Cl^- , was calculated according to: $J_{net}^R = I_{sc} - (J_{net}^{Na} - J_{net}^{Cl})$. A positive J_{net}^R indicates either the absorption of a cation or the secretion of an anion.

Uptake experiments

For the measurement of Rb^+ uptake, $^{86}\text{Rb}^+$ (22 kBq) was added to either the mucosal or the serosal side of the Ussing

chamber. Tissues were pretreated with indomethacin ($10^{-6} \text{mol l}^{-1}$ at the mucosal and the serosal side) in order to inhibit spontaneous production of prostaglandins and tetraethylammonium ($5 \times 10^{-3} \text{mol l}^{-1}$ at the mucosal side) to reduce loss of cellular Rb^+ by apical K^+ channels (cf. Table 3). When the uptake was measured in the presence of drugs (e.g. forskolin, vanadate, ouabain), they were administered 5 min prior to the addition of $^{86}\text{Rb}^+$ with the exception of bumetanide, which was administered 30 min prior to administration of the radiotracer in order to stabilize I_{sc} to a new plateau.

Twenty min after administration of $^{86}\text{Rb}^+$, standards were taken from the labelled side and the uptake was stopped by washing the chamber with 20 ml of fresh, unlabelled buffer solution on both sides. The 20 min period was selected to allow direct comparison with the unidirectional flux experiments. The tissue was removed from the chamber, blotted on filter paper and the wet weight determined. This procedure took 1–2 min. The tissue was solubilized in 1 ml 0.1 N HNO_3 for 16 h at 70°C (Venglarik *et al.*, 1990). After neutralization with 0.1 ml 1 N NaOH, the radioactivity in the sample was determined in a liquid scintillation counter. Results were calculated both as uptake per area mucosa (nmol cm^{-2}) and uptake per wet weight (nmol mg^{-1}). Because both procedures gave similar results, only the uptakes per area are presented.

Efflux experiments

The tissue was loaded with $^{86}\text{Rb}^+$ (74 kBq at both sides of the chamber) for 90 min in the presence of indomethacin ($10^{-6} \text{mol l}^{-1}$ at the mucosal and the serosal side). Then the serosal and the mucosal compartments were washed twice at 5 min intervals with 20 ml fresh Parsons solution. Release of $^{86}\text{Rb}^+$ into the mucosal and the serosal compartment was determined by taking $2 \times 0.5 \text{ml}$ aliquots at 6 min intervals. All aliquots were replaced by unlabelled buffer solution and ap-

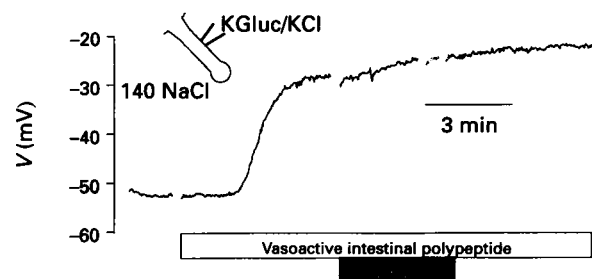


Figure 2 Effect of 293B ($10^{-6} \text{mol l}^{-1}$, solid bar) on the membrane potential of a crypt cell after stimulation Cl^- secretion with VIP ($10^{-8} \text{mol l}^{-1}$, open bar). The experiment was performed in the presence of Cl^- ions. The cell was located at the middle of an isolated crypt as indicated by the schematic (nystatin-permeabilized patch). The tracing is representative of 2 experiments with VIP and 6 experiments with forskolin as agonist of cyclic AMP-dependent Cl^- secretion. The interruptions in the voltage tracing line are caused by the measurements of I - V relations in the voltage-clamp mode.

Table 1 Effect of 293B on unidirectional $^{22}\text{Na}^+$ and $^{36}\text{Cl}^-$ fluxes

	J_{ms}^{Na}	J_{sm}^{Na}	J_{net}^{Na} ($\mu\text{Eq h}^{-1} \text{cm}^{-2}$)	J_{ms}^{Cl}	J_{net}^{Cl}	I_{sc}	J_{net}^R	Gt (mS cm^{-2})	
Indomethacin	14.6 ± 2.9	7.4 ± 1.1	7.1 ± 3.1	17.7 ± 2.1	12.9 ± 1.2	4.8 ± 2.4	0.9 ± 0.2	-1.4 ± 3.9	12.9 ± 2.1
Forskolin	8.4 ± 1.4*	8.6 ± 1.0	-0.2 ± 1.7	11.2 ± 2.0*	17.5 ± 1.2*	-6.3 ± 2.3	8.8 ± 0.4*	2.7 ± 2.9	17.0 ± 1.6*
293B	9.2 ± 1.4	7.9 ± 1.0	1.4 ± 1.7	12.1 ± 1.5	11.9 ± 1.2*	0.2 ± 1.9	3.0 ± 0.2*	1.8 ± 2.6	18.6 ± 2.4

Ion fluxes were measured in 3×20 -min periods in the subsequent presence of indomethacin ($10^{-6} \text{mol l}^{-1}$ at the mucosal and the serosal side), forskolin ($5 \times 10^{-6} \text{mol l}^{-1}$ at the mucosal and the serosal side) and 293B ($10^{-6} \text{mol l}^{-1}$ at the mucosal and the serosal side). Flux periods started 20 min after the administration of each drug. I_{sc} and Gt values were averaged over the 20 min flux periods. Values are means ± 1 s.e. mean. $n = 8-9$. * $P < 0.05$ versus preceding period.

appropriate correction for this replacement solution was performed. At the end of the experiment, the resting amount of ⁸⁶Rb⁺ in the tissue was determined as described for the uptake experiments. Release was expressed as efflux of the actual amount of radioactivity in the tissue per minute (Mandel *et al.*, 1986).

Drugs

Trans-6-cyano-4-(*N*-ethylsulphonyl-*N*-methylamino)-3-hydroxy-2,2-dimethyl-chromane (293B), (5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB; both drugs gift from R. Greger, Physiologisches Institut, Freiburg, Germany), nystatin, and ouabain were dissolved in dimethylsulphoxide (DMSO; final maximal concentration 0.5%, v/v). Bumetanide, forskolin, indomethacin, and quinine were dissolved in ethanol (final maximal concentration 0.25%, v/v). All other drugs including 8-(4-chlorophenylthio)-adenosine 3',5' cyclic monophosphate (CPT-cAMP) and sodium orthovanadate (Anawa, Wangen, Switzerland) were dissolved in aqueous stock solutions diluted in salt buffer just before use. Tetraethylammonium (TEA) was added as chloride salt. If not indicated differently, drugs were from Sigma, Buchs, Switzerland.

Radioisotopes were obtained from NEN, Dreieich, Germany. Specific activities were 3.9 TBq g⁻¹ ²²Na and 550 MBq g⁻¹ ³⁶Cl; the initial activity of ⁸⁶Rb amounted to 411 GBq g⁻¹.

Statistics

Values are given as means ± one standard error of the mean (s.e.mean). When the means of several groups had to be

compared, first an analysis of variances was performed. If the analysis of variances indicated significant differences between the groups investigated, further comparison was carried out by a Student's *t* test or by the U test. An *F* test decided which test method was to be used. Both paired and unpaired two-tailed Student's *t* tests were used as indicated. *P* < 0.05 was considered to be statistically significant.

Standard errors for calculated values, i.e. net ion transport, were calculated according to the law of error propagation from the errors J_{ms} and J_{sm} (Sachs, 1982). Statistical comparisons between net transports were performed by means of the test of Scheffé for comparison of linear contrasts (Sachs, 1982).

Results

Effect of 293B on the forskolin response

Forskolin (5 × 10⁻⁶ mol l⁻¹) induced an increase in I_{sc} of 5.1 ± 0.7 μEq h⁻¹ cm⁻² above baseline (*P* < 0.05, *n* = 15). When 293B was administered in increasing concentrations during the plateau phase of the forskolin response, a concentration-dependent decrease in I_{sc} was observed (Figure 1a). The inhibition was maximal at concentrations ≥ 10⁻⁶ mol l⁻¹. No statistically significant difference was observed in the effectiveness of the drug to the mucosal or the serosal side, probably due to the lipophilic nature of 293B (see Discussion). In order to avoid a decrease in the effective concentration by unilateral administration due to diffusion into the opposite compartment, for all subsequent experiments the drug was administered to both sides of the preparation.

Inhibition of the forskolin effect by 293B was rapid. Within

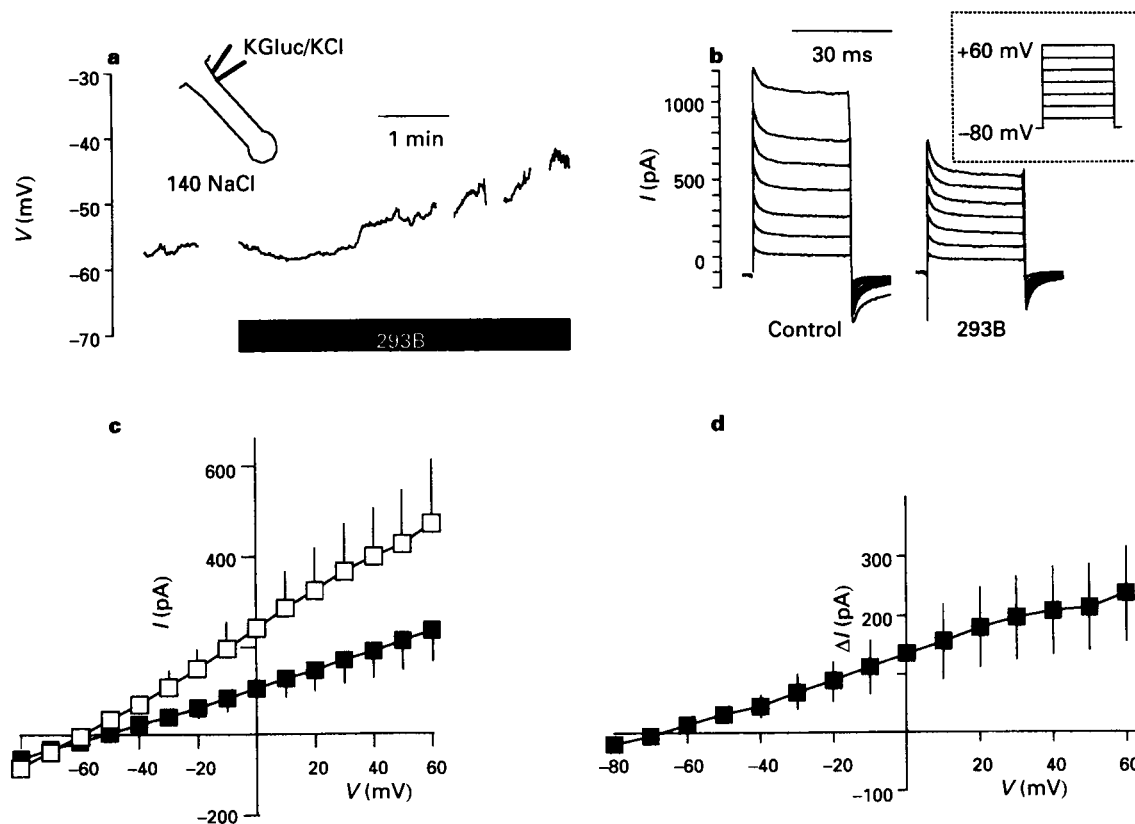


Figure 3 (a) Effect of 293B (10⁻⁶ mol l⁻¹; solid bar) on the membrane potential of a crypt cell measured with the whole-cell patch-clamp technique. The experiment was performed in the presence of Cl⁻ ions. The cell was located at the upper third of an isolated crypt as indicated by the schematic (conventional whole-cell recording). The interruptions in the voltage tracing line are caused by the measurements of *I-V* relations in the voltage-clamp mode. The tracing is representative of 7 experiments (from different crypts) with similar results. (b) Original current tracings during superfusion with control solution (left) and 293B-containing solution (right). For graphical clarity, only each second pulse of the 14 pulse voltage protocol (inset) is depicted. (c) *I-V* relation under control conditions (□) and in the presence of 293B (■) pooled from 7 cells at different positions along the crypt axis. Values are means ± 1 s.e.mean. (d) 293B-inhibited current (obtained after subtraction of the current during the control period from the data in c).

5 min after administration of 293B (10^{-6} mol l⁻¹), I_{sc} fell to a new stable plateau (Figure 1b). Unidirectional flux measurements showed that this decrease in I_{sc} was due to an inhibition of forskolin-induced Cl⁻ secretion. Forskolin caused an inhibition of the mucosa to serosa fluxes of Na⁺ and Cl⁻ (J_{ms}^{Na} and J_{ms}^{Cl}) and an increase in the serosa to mucosa flux of Cl⁻ (J_{sm}^{Cl} ; Table 1). 293B (10^{-6} mol l⁻¹) suppressed the increase in J_{sm}^{Cl} induced by forskolin but had no effect on the mucosa to serosa fluxes of Na⁺ or Cl⁻ (Table 1).

Inhibition of the forskolin-induced rise in I_{sc} was also observed, when 293B was applied in pretreatment (Figure 1c). When the tissues were pretreated with 293B, forskolin often induced oscillations in I_{sc} during the first minutes after administration of the drug (Figure 1c, inset). The nature of these oscillations was not examined further due to their transient nature.

In contrast, 293B had no effect on the response to carbachol, which induces Cl⁻ secretion in the rat colon by opening of Ca²⁺-dependent basolateral K⁺ channels (Böhme *et al.*, 1991; Strabel & Diener, 1995a). In the presence of 293B (10^{-6} mol l⁻¹) carbachol (5×10^{-5} mol l⁻¹, administered at the serosal side) induced a transient increase in I_{sc} of 9.2 ± 1.0 μ Eq h⁻¹ cm⁻² above baseline ($P < 0.05$, $n = 5$), which was not significantly different from that of untreated control tissue (increase of 8.5 ± 1.6 μ Eq h⁻¹ cm⁻², $n = 6$). This is in agreement with recent Ussing chamber experiments reported by Ecke *et al.* (1995).

Effect of 293B on baseline I_{sc}

When 293B (10^{-6} mol l⁻¹) was administered to untreated mucosa, the drug induced a prompt decrease in I_{sc} from

3.0 ± 0.2 μ Eq h⁻¹ cm⁻² to 1.2 ± 0.1 μ Eq h⁻¹ cm⁻² ($P < 0.05$, $n = 20$). This decrease was markedly attenuated, when the tissue was pretreated with indomethacin (10^{-6} mol l⁻¹). In the presence of indomethacin, which itself reduced basal I_{sc} to 1.2 ± 0.2 μ Eq h⁻¹ cm⁻² ($n = 14$), 293B caused a decrease in I_{sc} of only 0.6 ± 0.6 μ Eq h⁻¹ cm⁻² ($P < 0.05$ versus response in the absence of indomethacin, $n = 14$).

Effect of 293B on the membrane potential

The above results, i.e. inhibition of basal and forskolin-stimulated secretion could be due to an inhibition of Cl⁻ channels or of K⁺ channels. In order to distinguish between these possibilities, the effect of 293B on the membrane potential of isolated crypts was studied. Basal membrane potential (pooled from cells at different positions along the crypt axis) was -49.0 ± 4.4 mV ($n = 8$). An increase in the intracellular cyclic AMP concentration evoked by VIP or forskolin has been shown to cause a depolarization due to opening of apical Cl⁻ channels (Böhme *et al.*, 1991; Diener, 1994). In the presence of VIP (10^{-8} mol l⁻¹) or forskolin (5×10^{-6} mol l⁻¹), respectively, the cells depolarized to -29.3 ± 4.6 mV ($P < 0.05$, $n = 8$; pooled for both agonists with $n = 2$ for VIP and $n = 6$ for forskolin). When 293B (10^{-6} mol l⁻¹) was administered during the plateau phase of cyclic AMP-induced depolarization, the drug induced only a slight, insignificant further depolarization of 1.7 ± 1.2 mV ($n = 8$, Figure 2).

When 293B (10^{-6} mol l⁻¹) was applied under control conditions, the drug caused a depolarization, which amounted to 13.2 ± 4.4 mV ($P < 0.05$, $n = 7$; Figure 3a). This depolarization was associated with a decrease in membrane current (Figure 3b and c). Membrane outward current (measured at +60 mV)

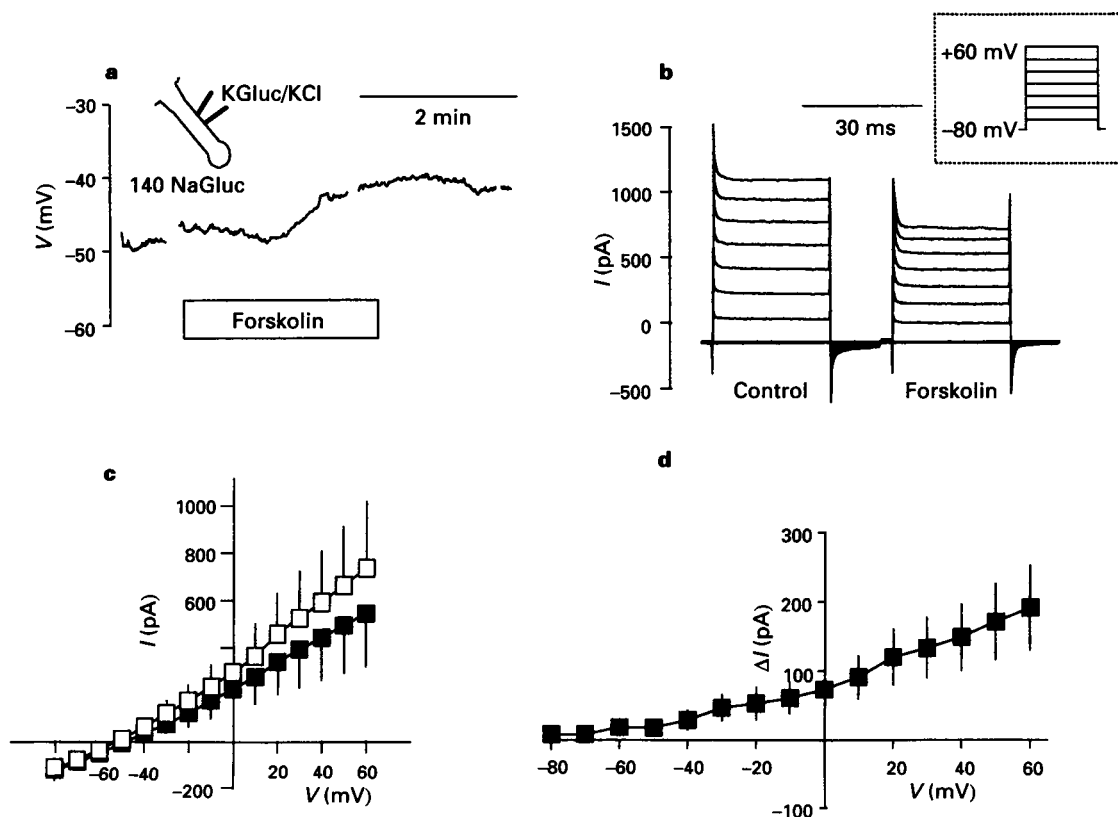


Figure 4 (a) Effect of forskolin (5×10^{-6} mol l⁻¹; open bar) on the membrane potential of a crypt cell measured with the whole-cell patch-clamp technique under Cl⁻-free conditions. The cell was located at the middle of an isolated crypt as indicated by the schematic (conventional whole-cell recording). The interruptions in the voltage tracing line are caused by the measurements of $I-V$ relations in the voltage-clamp mode. The tracing is representative of 7 experiments (from different crypts) with similar results. (b) Original current tracings during superfusion with control solution (left) and forskolin-containing solution (right). For graphical clarity, only each second pulse of the 14 pulse voltage protocol (inset) is depicted. (c) $I-V$ relation under control conditions (□) and in the presence of forskolin (■) pooled from 7 cells at different positions along the crypt axis. Values are means ± 1 s.e.mean. (d) Forskolin-inhibited current (obtained after subtraction of the current during the control period from the data in c).

decreased from 474.0 ± 139.8 pA to 227.7 ± 65.4 pA ($P < 0.05$, $n = 7$), whereas membrane inward current (measured at -80 mV) was not altered significantly (decrease from -71.4 ± 21.3 pA to -58.5 ± 19.3 pA, $n = 7$). The I - V relation of the current inhibited by 293B had a reversal potential of -72.9 ± 6.0 mV (Figure 3d), which was not statistically different from the K⁺ reversal potential (-84.3 mV with 130 mmol l⁻¹ K⁺ in the pipette and 5.4 mmol l⁻¹ in the superfusion solution) indicating that 293B inhibits a K⁺ conductance. The effect of 293B did not depend on the location of the cell along the longitudinal axis of a crypt.

For the rabbit distal colon it has been shown that 293B inhibits a cyclic AMP-stimulated K⁺ conductance (Lohrmann *et al.*, 1995). In order to find out whether the drug exerts its inhibitory action in the rat colon by a similar mechanism, the effect of agonists of the cyclic AMP-pathway was tested under Cl⁻-free conditions. However, forskolin (5×10^{-6} mol l⁻¹) did not induce a hyperpolarization under Cl⁻-free conditions. Instead, the drug induced a depolarization, which amounted to 4.2 ± 0.5 mV ($P < 0.05$, $n = 7$); Figure 4a). This depolarization was associated with a decrease in membrane outward current from 735.4 ± 278.8 pA to 544.3 ± 224.3 pA ($P < 0.05$, $n = 7$; Figure 4b and c). The current inhibited by forskolin had a reversal potential of -75.8 ± 7.1 mV ($n = 7$; Figure 4d), which was not significantly different from the K⁺ reversal potential

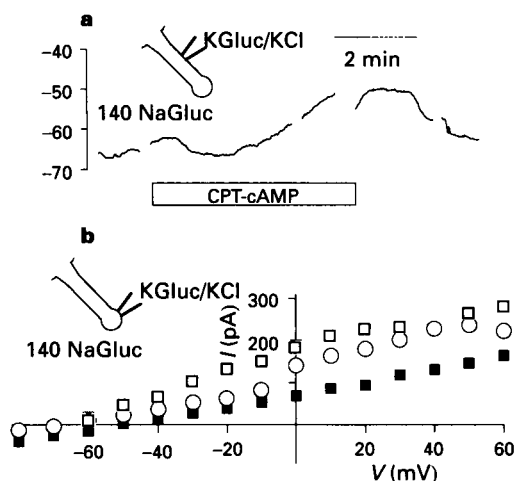


Figure 5 (a) Effect of CPT-cAMP (10^{-4} mol l⁻¹; open bar) on the membrane potential of a crypt cell measured with the whole-cell patch-clamp technique under Cl⁻-free conditions. The cell was located at the middle of an isolated crypt as indicated by the schematic (conventional whole-cell recording). The interruptions in the voltage tracing line are caused by measurement of I - V relations in the voltage-clamp mode. (b) I - V relation of the same cell in the absence (□), in the presence (■) and after washout of CPT-cAMP (○).

(-86.2 mV with 140 mmol l⁻¹ K⁺ in the pipette and 5.4 mmol l⁻¹ in the superfusion solution). The same was observed with a membrane-permeable cyclic AMP-derivative, CPT-cAMP (10^{-4} mol l⁻¹, Figure 5). Under Cl⁻-free conditions, CPT-cAMP induced a depolarization of 5.8 ± 2.0 mV ($P < 0.05$, $n = 9$) concomitant with a decrease in membrane outward current from 973.9 ± 211.6 pA (at $+60$ mV) to 741.6 ± 159.0 pA ($P < 0.05$, $n = 9$). Consequently, these results suggest that in the rat distal colon, in the absence of Cl⁻, total cellular K⁺ conductance decreases when the intracellular cyclic AMP concentrations rises.

Effect of 293B on basal K⁺ transport

In order to discover the localization of the K⁺ conductance sensitive to 293B, the effect of the drug on unidirectional Rb⁺ fluxes was measured. Under control conditions, J_{ms}^{Rb} exceeded J_{sm}^{Rb} , leading to a net Rb⁺ absorption (J_{net}^{Rb}) of 0.35 ± 0.15 μ Eq h⁻¹ cm⁻² ($P < 0.05$ versus zero; test of Scheffé). 293B (10^{-6} mol l⁻¹) had no effect on J_{sm}^{Rb} and caused a small, but insignificant decrease in J_{ms}^{Rb} (Table 2). When the experiments were repeated in the presence of indomethacin (10^{-6} mol l⁻¹) J_{net}^{Rb} appeared to increase due to an increase in J_{ms}^{Rb} and a decrease in J_{sm}^{Rb} (Table 2), although none of these indomethacin-induced changes reached statistical significance. However, also under these conditions the small decrease in J_{ms}^{Rb} induced by 293B did not reach statistical significance indicating that the basal K⁺ conductance inhibited by 293B, although obviously important to keep the driving force for basal and cyclic AMP-stimulated Cl⁻ secretion, does not contribute quantitatively to transepithelial K⁺ transport.

Stimulation of K⁺ secretion by cyclic AMP

All further experiments were carried out in the presence of indomethacin (10^{-6} mol l⁻¹) in order to decrease basal prostaglandin-mediated, cyclic AMP-dependent secretion. Forskolin (5×10^{-6} mol l⁻¹) caused a decrease in J_{ms}^{Rb} and an increase in J_{sm}^{Rb} (Table 3). When the results from several experimental series were pooled, forskolin decreased J_{ms}^{Rb} from 1.18 ± 0.14 μ Eq h⁻¹ cm⁻² to 0.76 ± 0.09 μ Eq h⁻¹ cm⁻² ($P < 0.05$, $n = 29$) and increased J_{sm}^{Rb} from 0.32 ± 0.02 μ Eq h⁻¹ cm⁻² to 0.49 ± 0.03 μ Eq h⁻¹ cm⁻² ($P < 0.05$, $n = 30$). The result was a decrease in J_{net}^{Rb} from 0.86 ± 0.14 μ Eq h⁻¹ cm⁻² to 0.27 ± 0.10 μ Eq h⁻¹ cm⁻² ($P < 0.05$; test of Scheffé), which was still significantly different from zero (test of Scheffé).

When 293B (10^{-6} mol l⁻¹) was administered during the forskolin-induced secretion, it caused a prompt decrease in I_{sc} and Gt (Table 3). This was concomitant with an inhibition of the forskolin-stimulated increase in J_{sm}^{Rb} , whereas J_{ms}^{Rb} was not affected significantly (Table 3) suggesting that 293B inhibits apical K⁺ channels involved in K⁺ secretion. In accordance to previous results by Sweiry & Binder (1990), cyclic AMP-stimulated K⁺ secretion was blocked by mucosal administration of a K⁺ channel blocker, TEA (5×10^{-3} mol l⁻¹),

Table 2 The effect of 293B on unstimulated unidirectional ⁸⁶Rb⁺ fluxes

	J_{ms}^{Rb}	J_{sm}^{Rb} (μ Eq h ⁻¹ cm ⁻²)	J_{net}^{Rb}	I_{sc}	Gt (mS cm ⁻²)
Control	0.85 ± 0.14	0.49 ± 0.06	0.35 ± 0.15	3.0 ± 0.2	9.6 ± 0.6
293B	0.73 ± 0.12	0.50 ± 0.06	0.22 ± 0.13	$1.2 \pm 0.1^*$	$8.6 \pm 0.7^*$
Indomethacin	1.00 ± 0.32	0.39 ± 0.04	0.61 ± 0.33	1.2 ± 0.2	14.4 ± 1.9
293B	0.95 ± 0.24	0.44 ± 0.06	0.51 ± 0.24	$0.7 \pm 0.1^*$	$17.1 \pm 2.6^*$

Ion fluxes were measured in 2×20 -min periods. Flux periods started 20 min after the administration of each drug. Concentrations of drugs were: 293B (10^{-6} mol l⁻¹ at the mucosal and the serosal side), indomethacin (10^{-6} mol l⁻¹ at the mucosal and the serosal side). I_{sc} and Gt values were averaged over the 20 min flux periods. Values are means ± 1 s.e.mean $n = 7-10$. * $P < 0.05$ versus preceding period.

or by serosal administration of an inhibitor of the Na⁺-K⁺-Cl⁻-cotransporter, bumetanide (10⁻⁴ mol l⁻¹; Table 3), confirming that K⁺ secretion is mediated by a basolateral Na⁺-K⁺-Cl⁻-cotransporter and by apical K⁺ channels.

The question arises how cyclic AMP causes an increase in J^{Rb_{sm}}, although total cellular K⁺ conductance decreases (Figure 4). One possible explanation may be that the opening of apical Cl⁻ channels by cyclic AMP, which causes a membrane depolarization, will indirectly stimulate K⁺ secretion by increasing the driving force for K⁺ exit, provided that there are spontaneously open apical K⁺ channels. In order to test this hypothesis, in a first attempt NPPB, a Cl⁻ channel blocker (for review see Cabantchik & Greger, 1992), was used. NPPB (10⁻⁴ mol l⁻¹) at a concentration which blocks cyclic AMP-stimulated Cl⁻ secretion (Diener & Rummel, 1989), did not inhibit the increase in J^{Rb_{sm}} induced by forskolin. Instead, the drug further reduced J^{Rb_{ms}}, which was already inhibited by forskolin (Table 3). This result may be explained by the known effect of NPPB on basolateral K⁺ channels (Illek et al., 1992).

Because of these side effects of NPPB, another approach was used to investigate the role of the apical membrane depolarization for K⁺ secretion. After measurement of J^{Rb_{sm}} under control conditions, i.e. with Cl⁻ ions present on both sides of the tissue, apical NaCl was replaced by Na⁺ gluconate. This replacement should cause a depolarization of the apical membrane if the membrane possesses a Cl⁻ permeability by shifting the Cl⁻ equilibrium potential to more positive values. The depolarization did indeed cause a significant increase in J^{Rb_{sm}} (Table 4). When the tissue was preincubated with indomethacin, and the manoeuvre was repeated, the increase in J^{Rb_{sm}} was attenuated by 67% (*P* < 0.05 versus increase in J^{Rb_{sm}} in the absence of indomethacin, *n* = 9; Table 4), suggesting that even after inhibition of the production of prostaglandins the epithelium contains spontaneously open apical K⁺ channels.

Inhibition of K⁺ absorption by cyclic AMP

An increase in the intracellular cyclic AMP concentration does not only stimulate K⁺ secretion but also inhibits K⁺ absorption. In order to investigate the underlying mechanism of cyclic AMP-mediated inhibition of J^{Rb_{ms}}, experiments with blockers were performed. Vanadate (10⁻⁴ mol l⁻¹), an inhibitor of the apical H⁺-K⁺-ATPase (Sweiry & Binder, 1990; Del Castillo et al., 1991; Tabuchi et al., 1992), caused a decrease in J^{Rb_{ms}} by 47%, whereas J^{Rb_{sm}} was not altered significantly (Table 5). In the presence of vanadate, forskolin (5 × 10⁻⁶ mol l⁻¹) caused a further decrease in J^{Rb_{ms}}. The same was observed, when va-

nadate was combined with ouabain (10⁻³ mol l⁻¹), another inhibitor of the colonic H⁺-K⁺-ATPase (Sweiry & Binder, 1990; Del Castillo et al., 1991; Tabuchi et al., 1992). The combined presence of both inhibitors caused a decrease in J^{Rb_{ms}} by 73%. Nevertheless, forskolin further decreased J^{Rb_{ms}} (Table 5), suggesting that forskolin exerts an action at a site distinct from the H⁺-K⁺-ATPase. In order to find out whether forskolin might act at a basolateral K⁺ channel responsible for K⁺ exit to the serosal side, two K⁺ channel blockers, TEA (5 × 10⁻³ mol l⁻¹) and quinine (10⁻³ mol l⁻¹) were administered to the serosal side. However, none of these blockers had an effect on J^{Rb_{ms}} or on the inhibition of J^{Rb_{ms}} by forskolin (Table 5).

Effect of forskolin on K⁺ uptake

The inhibition of K⁺ absorption by forskolin might be due to an inhibition of apical entry or due to an inhibition of basolateral exit of K⁺ ions. In order to distinguish between these possibilities, the uptake of Rb⁺ from the mucosal compartment was tested under short-circuit conditions in the presence of indomethacin (10⁻⁶ mol l⁻¹) and mucosal TEA (5 × 10⁻³ mol l⁻¹) in order to inhibit basal stimulation of K⁺ secretion by endogenous prostaglandins and to reduce loss of

Table 4 Unidirectional ⁸⁶Rb⁺ fluxes: the effect of apical depolarization on K⁺ secretion

	J ^{Rb_{sm}} (μEq h ⁻¹ cm ⁻²)	I _{sc}	Gt (mS cm ⁻²)
Control	0.38 ± 0.04	5.7 ± 0.5	10.8 ± 0.4
Cl ⁻ -free mucosal	0.56 ± 0.06*	4.2 ± 0.4*	8.9 ± 0.4*
Indomethacin	0.27 ± 0.02	0.5 ± 0.1	15.9 ± 2.7
Cl ⁻ -free mucosal	0.34 ± 0.02*	-0.7 ± 0.2*	12.0 ± 1.8*

Ion fluxes were measured in 2 × 20-min periods under Cl⁻-containing and subsequently under Cl⁻-free conditions. Flux periods started 20 min after the anion substitution. Indomethacin was used in a concentration of 10⁻⁶ mol l⁻¹ (at the mucosal and the serosal side). Anion substitution was performed only at the mucosal side of the tissue. I_{sc} and Gt values were averaged over the 20 min flux periods. Values are means ± 1 s.e.mean. *n* = 8–9. **P* < 0.05 versus preceding period.

Table 3 Unidirectional ⁸⁶Rb⁺ fluxes: studies on the stimulation of K⁺ secretion by cyclicAMP

	J ^{Rb_{ms}}	J ^{Rb_{sm}} (μEq h ⁻¹ cm ⁻²)	J ^{Rb_{net}}	I _{sc}	Gt (mS cm ⁻²)
Indomethacin	1.21 ± 0.23	0.31 ± 0.04	0.89 ± 0.23	1.4 ± 0.2	7.3 ± 0.5
Forskolin	0.74 ± 0.21*	0.54 ± 0.06*	0.20 ± 0.21	8.7 ± 0.4*	11.2 ± 0.5*
293B	0.78 ± 0.14	0.36 ± 0.06*	0.42 ± 0.15	3.0 ± 0.3*	10.4 ± 0.7*
Indomethacin	0.89 ± 0.36	0.34 ± 0.06	0.56 ± 0.36	1.5 ± 0.2	11.3 ± 1.2
Forskolin	0.67 ± 0.22	0.61 ± 0.06*	0.06 ± 0.23	7.6 ± 0.7*	16.9 ± 1.9*
TEA mucosal	0.64 ± 0.18	0.42 ± 0.05*	0.23 ± 0.19	5.9 ± 0.5*	17.0 ± 1.9
Indomethacin	1.13 ± 0.24	0.29 ± 0.03	0.84 ± 0.24	2.7 ± 0.3	8.7 ± 0.5
Forskolin	0.73 ± 0.17*	0.42 ± 0.05	0.31 ± 0.18	8.5 ± 0.5*	12.4 ± 1.0*
Bumetanide	1.06 ± 0.18*	0.30 ± 0.04*	0.76 ± 0.19	2.7 ± 0.2*	12.2 ± 1.0
Indomethacin	1.48 ± 0.29	0.34 ± 0.05	1.14 ± 0.29	2.5 ± 0.3	8.7 ± 0.8
Forskolin	0.91 ± 0.16*	0.40 ± 0.04	0.50 ± 0.16	8.3 ± 0.7*	12.4 ± 1.0*
NPPB	0.45 ± 0.09*	0.41 ± 0.08	0.03 ± 0.12	4.5 ± 0.5*	11.5 ± 1.1*

Ion fluxes were measured in 3 × 20-min periods in the presence of the drugs indicated. Flux periods started 20 min after the administration of each drug. Concentrations of drugs were: 293B (10⁻⁶ mol l⁻¹ at the mucosal and the serosal side), bumetanide (10⁻⁴ mol l⁻¹ at the serosal side), forskolin (5 × 10⁻⁶ mol l⁻¹ at the mucosal and the serosal side), indomethacin (10⁻⁶ mol l⁻¹ at the mucosal and the serosal side), NPPB (10⁻⁴ mol l⁻¹ at the mucosal side), TEA (5 × 10⁻³ mol l⁻¹ at the mucosal side). I_{sc} and Gt values were averaged over the 20 min flux periods. Values are means ± 1 s.e.mean. *n* = 7–8. **P* < 0.05 versus preceding period.

Rb⁺ via apical K⁺ channels. Forskolin (5×10^{-6} mol l⁻¹) did not inhibit the mucosal uptake of Rb⁺ (Table 6), although experiments with inhibitors revealed that the mucosal Rb⁺ uptake was inhibited by 45% in the presence of vanadate and ouabain, i.e. was mediated by the H⁺-K⁺-ATPase. This, together with the unidirectional flux measurements (Table 5) supports the hypothesis that the apical H⁺-K⁺-ATPase is not under the control of cyclic AMP.

In contrast, when the serosal uptake of Rb⁺ was measured, forskolin caused a significant increase by 23% (Table 6). Although bumetanide had no significant effect on basal serosal Rb⁺ uptake, it prevented completely the stimulation of serosal Rb⁺ uptake by forskolin (Table 6). Consequently, forskolin leads to an activation of the basolateral Na⁺-K⁺-Cl⁻-co-transporter as shown already for HT29 cells (Kim *et al.*, 1988), an effect, which will contribute to the induction of K⁺ secretion by the drug.

Effect of forskolin on K⁺ efflux

In a final series of experiments the effect of forskolin on the efflux of Rb⁺ in tissues preloaded with this tracer was tested. Under control conditions, efflux from the tissue to the serosal side (1.76 ± 0.10 % min⁻¹, $n=8$) exceeded that to the mucosal side (0.80 ± 0.04 , % min⁻¹, $n=8$) by a factor of 2.2 ± 0.2 ($P < 0.05$ versus unity, $n=8$). After administration of forskolin, efflux to the serosal compartment rose transiently but fell within 15 min to the former control levels (Figure 6). In contrast, there was a delayed increase in the efflux of Rb⁺ to the mucosal compartment. Total Rb⁺ efflux was constantly elevated in the presence of forskolin (Figure 6).

Discussion

The chromanole compound, 293B, is a potent antisecretory agent in the rat colon. It exerts a maximal antisecretory action at a concentration (10^{-6} mol l⁻¹; Figure 1a), which is about ten times lower than in the rabbit distal colon, where a concentration of 10^{-5} mol l⁻¹ was necessary to induce a maximal effect (Lohrmann *et al.*, 1995). Whole-cell patch-clamp experiments revealed that 293B did indeed act as a K⁺ channel blocker. When applied under control conditions, 293B inhibited a K⁺ current and induced a membrane depolarization (Figure 3). However, when administered during the cyclic

AMP-induced depolarization, it had no effect on membrane potential (Figure 2), probably because this potential (-29.3 ± 4.6 mV) was already very close to the Cl⁻ equilibrium potential (-32.5 mV with 44 mmol l⁻¹ Cl⁻ in the pipette and 149.9 mmol l⁻¹ in the superfusion medium) so that any decrease in K⁺ conductance cannot depolarize the cell further. This is in contrast to the effect of 293B in the rabbit colon (Lohrmann *et al.*, 1995) or in dexamethasone-treated rats (Ecke *et al.*, 1995), where 293B causes a depolarization after stimulation of the crypts with forskolin due to inhibition of a cyclic AMP-activated K⁺ conductance. The activation of a 130 pS K⁺ channel by cyclic AMP (and intracellular Ca²⁺) has been demonstrated in the rabbit distal colon (Loo & Kaunitz, 1989). In the rat distal colon, however, no activation of a K⁺ conductance by cyclic AMP was observed. Under Cl⁻-free conditions, neither forskolin (Figure 4) nor CPT-cAMP caused a hyperpolarization, which one should expect during opening of a K⁺ conductance, but instead a depolarization was observed. This depolarization was associated with an apparently paradoxical decrease in K⁺ current indicating a decrease in total cellular K⁺ conductance. In colonic adenocarcinoma cell lines such as HT29-cl.19A or T84-cells either no increase (Bajnath *et al.*, 1991) or even a decrease in basolateral K⁺ conductance after stimulation of cyclic AMP-production have been reported (Reenstra, 1993).

Measurement of unidirectional fluxes of Rb⁺ revealed that 293B suppressed the increase in J_{sm}^{Rb} induced by forskolin (Table 3). J_{sm}^{Rb} is also inhibited by the K⁺ channel blocker, TEA (Sweiry & Binder, 1989) (see also Table 3), suggesting that a part of the 293B-sensitive K⁺ conductance is localized in the apical membrane. Inhibition of forskolin-induced I_{sc} , however, by inhibition of K⁺ channels is only possible, if the drug in addition blocks a basolateral K⁺ conductance and thereby reduces the driving force for Cl⁻ exit across apical Cl⁻ channels. Inhibition of apical K⁺ channels alone would also reduce the driving force for Cl⁻ secretion, but it would not cause a decrease in I_{sc} . Unfortunately, due to the lipophilic nature of the drug, no clear sidedness of the actions of 293B was observed (Figure 1a). The basolateral K⁺ conductance sensitive to 293B does not, however, contribute quantitatively to basolateral K⁺ efflux during K⁺ absorption as shown by the absence of an effect of 293B on J_{ms}^{Rb} (Table 2). The same was observed with two other K⁺ channel blockers, TEA and quinine (Table 5). Only high concentrations of TEA (30 mmol l⁻¹) or Ba²⁺ (5 mmol l⁻¹) have been shown up to

Table 5 Unidirectional ⁸⁶Rb⁺ fluxes: studies on the inhibition of K⁺ absorption by cyclic AMP

	J_{ms}^{Rb}	J_{sm}^{Rb} ($\mu\text{Eq h}^{-1} \text{cm}^{-2}$)	J_{net}^{Rb}	I_{sc}	Gt (mS cm^{-2})
Indomethacin	1.07 ± 0.17	0.26 ± 0.06	0.81 ± 0.18	1.3 ± 0.3	12.4 ± 3.6
Vanadate	$0.57 \pm 0.08^*$	0.31 ± 0.06	0.26 ± 0.10	$0.9 \pm 0.3^*$	12.2 ± 3.4
Forskolin	0.35 ± 0.10	0.56 ± 0.16	-0.21 ± 0.19	$7.2 \pm 0.8^*$	$17.4 \pm 4.0^*$
Indomethacin	0.96 ± 0.20	0.34 ± 0.03	0.62 ± 0.20	2.1 ± 0.3	10.8 ± 1.0
Vanadate + Ouabain	$0.26 \pm 0.04^*$	0.40 ± 0.05	-0.14 ± 0.06	$1.4 \pm 0.2^*$	$9.5 \pm 1.0^*$
Forskolin	$0.17 \pm 0.04^*$	$0.62 \pm 0.06^*$	-0.45 ± 0.07	$6.7 \pm 0.4^*$	$15.2 \pm 1.4^*$
Indomethacin	0.90 ± 0.22	0.36 ± 0.03	0.54 ± 0.22	1.6 ± 0.3	12.7 ± 2.0
TEA serosal	0.83 ± 0.18	0.39 ± 0.06	0.44 ± 0.19	1.7 ± 0.3	15.0 ± 3.1
Forskolin	$0.49 \pm 0.11^*$	$0.61 \pm 0.09^*$	-0.12 ± 0.14	$9.0 \pm 1.4^*$	$21.0 \pm 4.1^*$
Indomethacin	0.89 ± 0.14	0.36 ± 0.03	0.53 ± 0.14	1.7 ± 0.3	12.0 ± 1.9
Quinine	0.92 ± 0.14	0.31 ± 0.05	0.61 ± 0.15	$1.2 \pm 0.5^*$	12.4 ± 2.6
Forskolin	0.75 ± 0.16	$0.53 \pm 0.09^*$	0.23 ± 0.18	$4.5 \pm 0.4^*$	$16.1 \pm 3.2^*$

Ion fluxes were measured in 3×20 -min periods in the absence of the drugs indicated. Flux periods started 20 min after the administration of each drug. Concentrations of drugs were: 293B (10^{-6} mol l⁻¹ at the mucosal and the serosal side), forskolin (5×10^{-6} mol l⁻¹ at the mucosal and the serosal side), indomethacin (10^{-6} mol l⁻¹ at the mucosal and the serosal side), ouabain (10^{-3} mol l⁻¹ at the mucosal side), quinine (10^{-3} mol l⁻¹ at the serosal side), TEA (5×10^{-3} mol l⁻¹ at the serosal side), vanadate (10^{-4} mol l⁻¹ at the mucosal side). I_{sc} and Gt values were averaged over the 20 min flux periods. Values are means ± 1 s.e.mean. $n=7-10$. * $P < 0.05$ versus preceding period.

Table 6 Mucosal and serosal ⁸⁶Rb⁺-uptake

	Mucosal uptake (nmol cm ⁻²)	I _{sc} (μEq h ⁻¹ cm ⁻²)	Gt (mS cm ⁻²)
Indomethacin	252.1 ± 33.8	0.6 ± 0.1	13.1 ± 3.0
Forskolin	277.6 ± 49.2	7.9 ± 1.1*	18.1 ± 2.4
Vanadate + ouabain	138.0 ± 17.6*	0.8 ± 0.2	13.9 ± 2.4
	Serosal uptake (nmol cm ⁻²)	I _{sc} (μEq h ⁻¹ cm ⁻²)	Gt (mS cm ⁻²)
Indomethacin	551.8 ± 35.9	0.4 ± 0.1	10.5 ± 2.9
Forskolin	676.6 ± 36.4*	7.3 ± 0.6*	15.1 ± 2.1
Bumetanide	520.1 ± 50.3	0.6 ± 0.1	11.0 ± 2.2
Bumetanide + forskolin	529.4 ± 35.1	2.1 ± 0.1 [#]	11.8 ± 1.5

Uptake was measured in the presence of indomethacin (10⁻⁶ mol l⁻¹ at the mucosal and the serosal side) and TEA (5 × 10⁻³ mol l⁻¹ at the mucosal side). Bumetanide (10⁻⁴ mol l⁻¹ at the serosal side), forskolin (5 × 10⁻⁶ mol l⁻¹ at the mucosal and the serosal side), ouabain (10⁻³ mol l⁻¹ at the mucosal side), vanadate (10⁻⁴ mol l⁻¹ at the mucosal side). I_{sc} and Gt values were measured just at the end of the 20 min uptake period. Values are means ± 1 s.e.mean. n = 6–7. *P < 0.05 versus indomethacin group, [#]P < 0.05 versus bumetanide group.

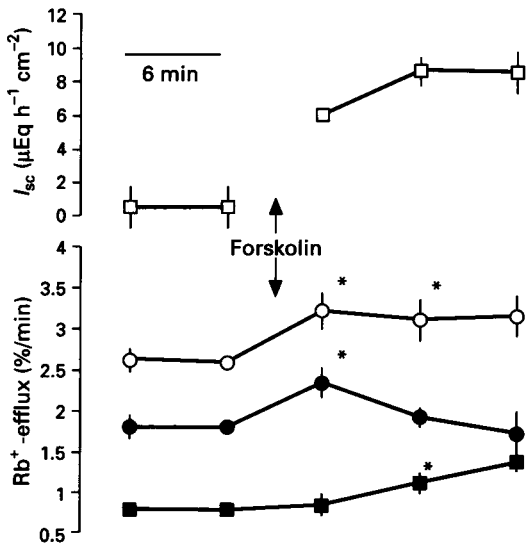


Figure 6 Effect of forskolin on the efflux of ⁸⁶Rb⁺: (■) efflux on the mucosal side, (●) efflux on the serosal side; (○) total efflux. In the upper part of the figure the increase in I_{sc} induced by forskolin during the efflux experiments is given. Values are means ± 1 s.e.mean, n = 8. * P < 0.05 versus mean of the control period.

now to reduce J^{Rb}_{ms} in the rat colon (Sweiry & Binder, 1990) raising the question concerning of what the nature of the putative basolateral K⁺ channels involved in K⁺ absorption may be (for further discussion see Binder & Sandler, 1994).

An unexpected result in the present investigations was the obvious decrease in cellular K⁺ conductance induced by cyclic AMP as unmasked by whole-cell patch-clamp experiments under Cl⁻-free conditions (Figure 4). It has to be kept in mind, however, that the whole-cell configuration allows only the measurement of total K⁺ conductances. An increase in apical K⁺ conductance is not an a priori necessity for the induction of K⁺ secretion provided that the epithelium possesses spontaneously open apical K⁺ channels. An increase in intracellular cyclic AMP concentration has been shown to cause a stimulation of the basolateral Na⁺-K⁺-Cl⁻-cotransporter in the colonic tumour cell line, HT29 (Kim *et al.*, 1988; Slotki *et al.*, 1993). A similar activation of basolateral Rb⁺ uptake, which can be prevented by bumetanide, was observed in the rat colon (Table 6). Forskolin stimulated total serosal Rb⁺ uptake by 23% (P < 0.05, n = 7; Table 6), and bumetanide-sensitive serosal Rb⁺ uptake increased from 31.7 ± 35.9 nmol cm⁻² to

156.5 ± 36.4 nmol cm⁻² (P < 0.05, n = 7), i.e. by a factor of 4.9. Suppression by bumetanide confirmed that this effect reflected the stimulation of the basolateral Na⁺-K⁺-Cl⁻-cotransporter (Table 6). This stimulation is responsible for the furosemide-sensitive cell swelling induced by forskolin (Diener, 1994). In accordance with previous observations (Halm & Frizzell, 1986; Sweiry & Binder, 1989), bumetanide also suppressed the stimulation of J^{Rb}_{ms} by forskolin (Table 3). Consequently, a stimulation of basolateral K⁺ uptake via the Na⁺-K⁺-Cl⁻-cotransporter is involved in the induction of K⁺ secretion by cyclic AMP. Surprisingly, bumetanide also restored J^{Rb}_{ms} previously inhibited by forskolin (Table 3), a result already observed in the rabbit distal colon by Halm & Frizzell (1986) and in the rat distal colon by Sweiry & Binder (1989). The reason for this effect is unknown; it might indicate that stimulation of the Na⁺-K⁺-Cl⁻-cotransporter causes some 'recycling' of absorbed K⁺ ions back from the basolateral compartment into the cell.

Another mechanism, which will contribute to the induction of K⁺ secretion by cyclic AMP is the membrane depolarization caused by the opening of apical Cl⁻ channels after cyclic AMP-dependent phosphorylation. The reverse action, i.e. the indirect stimulation of Cl⁻ secretion by opening of basolateral K⁺ channels is well known and underlies carbachol-induced Cl⁻ secretion in the rat colon, for example (Strabel & Diener, 1995a). As was expected, a depolarization of the apical membrane, induced by replacing Cl⁻ by an impermeable anion, gluconate, caused an increase in J^{Rb}_{ms}. This increase was attenuated but not abolished in the presence of indomethacin (Table 4). Whether this inhibition is due to the reduction of apical Cl⁻ permeability or due to the reduction of apical K⁺ permeability after blockade of the spontaneous production of prostaglandins cannot be determined. However, an alternative explanation of these experiments cannot be excluded. Omission of mucosal HCO₃⁻ will inhibit the apical Cl⁻/HCO₃⁻ exchanger and thereby induce an increase in pH_i, which could stimulate pH-sensitive apical K⁺ channels and thereby induce an increase in J^{Rb}_{ms}.

The action of cyclic AMP on K⁺ transport includes not only the induction of K⁺ secretion but also the inhibition of K⁺ absorption. In principle, two action sites may underlie this effect: an action on the apical entry of K⁺ ions or on the basolateral K⁺ exit step. The H⁺-K⁺-ATPase from the rat colon has meanwhile been cloned (Crowson & Shull, 1992). The enzyme, a P-type ATPase, possesses a 63% homology to the three α-subunit isoforms of the Na⁺-K⁺-ATPase, which is known to be regulated by cyclic AMP-dependent phosphorylation in several tissues (Beguin *et al.*, 1994). Therefore, the assumption of a cyclic AMP-dependent inhibition of the colonic H⁺-K⁺-ATPase seems plausible. However, forskolin still

decreased J_{ms}^{Rb} in the presence of vanadate or ouabain, two inhibitors of the H⁺-K⁺-ATPase (Table 5). Forskolin had no inhibitory effect upon mucosal Rb⁺ uptake, but the latter was attenuated (~45%) by vanadate and ouabain (Table 6). Consequently, these results suggest that the regulation of K⁺ absorption by cyclic AMP does not take place at the apical entry step of K⁺ ions. Nonspecific effects of vanadate, e.g. inhibition of protein-phosphotyrosine phosphatase (Gordon, 1991), cannot be excluded at this stage.

Forskolin had a profound effect on Rb⁺ efflux. The ratio of serosal to mucosal Rb⁺ efflux, normally 2.2 ± 0.2 , decreased to 1.3 ± 0.2 (not significantly different from unity) at the end of the forskolin period (Figure 6). In other words, during forskolin stimulation there is a redistribution of cellular K⁺ conductance from a predominantly basolateral one during control conditions, to a nearly equal efflux across both cell poles following elevation of intracellular cyclic AMP. Interestingly, Rb⁺ efflux to the serosal and the mucosal compartment showed a different time-course. In the first 3 min after administration of forskolin, there was a significant increase in serosal efflux, while mucosal efflux was steady. This increase in serosal efflux may be caused by increasing the driving force for K⁺ exit across basolateral K⁺ channels due to the forskolin-induced depolarization and due to the activation of the Na⁺-K⁺-Cl⁻-cotransporter. Subsequently, serosal efflux decreased and reached the former control level despite the increased driving force for K⁺ exit. This effect can be explained only if the basolateral K⁺ conductance decreases, an effect, which was observed in patch-clamp experiments (Figure 4). Mucosal Rb⁺ efflux showed a continuous increase after administration of forskolin, which might be caused by a delayed opening of apical K⁺ channels and/or the closure of basolateral K⁺ channels.

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