

# The flavonol quercetin activates basolateral $K^+$ channels in rat distal colon epithelium

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**1** The flavonol quercetin has been shown to activate a  $Cl^-$  secretion in rat colon. Unlike the secretory activity of the related isoflavone genistein, quercetin's secretory activity does not depend on cyclic AMP; instead, it depends on  $Ca^{2+}$ . We investigated the possible involvement of  $Ca^{2+}$  dependent basolateral  $K^+$  channels using apically permeabilized rat distal colon epithelium mounted in Ussing chambers.

**2** In intact epithelium, quercetin induced an increase in short-circuit current ( $I_{sc}$ ), which was diminished by the  $Cl^-$  channel blockers NPPB and DPC, but not by glibenclamide, DIDS or anthracene-9-carboxylic acid. The effect of the flavonol was also inhibited by several serosally applied  $K^+$  channel blockers ( $Ba^{2+}$ , quinine, clotrimazole, tetrapentylammonium, 293B), whereas other  $K^+$  channel blockers failed to influence the quercetin-induced increase in  $I_{sc}$  (tetraethylammonium, charybdotoxin).

**3** The apical membrane was permeabilized by mucosal addition of nystatin and a serosally directed  $K^+$  gradient was applied. The successful permeabilization was confirmed by experiments demonstrating the failure of bumetanide to inhibit the carbachol-induced current.

**4** In apically permeabilized epithelium, quercetin induced a  $K^+$  current ( $I_K$ ), which was neither influenced by ouabain nor by bumetanide. Whereas DPC, NPPB, charybdotoxin and 293B failed to inhibit this  $I_K$ , quinine,  $Ba^{2+}$ , clotrimazole and tetrapentylammonium were effective blockers of this current.

**5** We conclude from these results that at least part of the quercetin-induced  $Cl^-$  secretion can be explained by an activation of basolateral  $K^+$  channels.

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**Abbreviations:** 293B, trans-6-cyano-4-(N-ethylsulphonyl-N-methylamino)-3-hydroxy-2,2-dimethyl-chromane; 9-AC, anthracene-9-carboxylic acid; cyclic AMP, cyclic adenosine monophosphate; CFTR, cystic fibrosis transmembrane conductance regulator; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid; DPC, diphenylamine-2-carboxylate;  $G_t$ , tissue conductance;  $I_K$ ,  $K^+$  current;  $I_{sc}$ , short-circuit current; NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoic acid; rSK4, rat small-conductance  $Ca^{2+}$ -activated  $K^+$  channel; TEA, tetraethylammonium; TPcA, tetrapentylammonium

## Introduction

The flavonol quercetin is widely distributed in higher plants and, therefore, the most abundant flavonoid in the diet of humans and herbivorous animals (Herrmann, 1988; Hertog *et al.*, 1993). We have recently demonstrated that this flavonol induces a  $Cl^-$  and  $HCO_3^-$  secretion in rat colon epithelium (Cermak *et al.*, 1998). Intestinal  $Cl^-$  secretion depends on the combined activity of several transport mechanisms. The  $Na^+/K^+$  ATPase generates the  $Na^+$  gradient, which is responsible for the combined influx of  $Na^+$ ,  $K^+$ , and  $Cl^-$  *via* basolateral  $Na^+/K^+/2 Cl^-$  cotransport. The  $Cl^-$  ions are secreted *via* the cystic fibrosis transmembrane conductance regulator (CFTR), which represents the  $Cl^-$  channel in the apical membrane of the colon mucosa. The  $K^+$  ions can recycle *via* basolateral  $K^+$  channels (Greger *et al.*, 1997). Changes in any of these mechanisms will alter epithelial  $Cl^-$  secretion.

Secretagogues principally activate two different second messenger systems. Cyclic adenosine monophosphate (cyclic AMP) activates  $Cl^-$  secretion *via* protein kinase A dependent phosphorylation of CFTR (Greger, 2000). Furthermore, cyclic AMP activates a certain class of basolateral  $K^+$  channels (Warth *et al.*, 1996). Basolateral  $K^+$  channels are mandatory for  $Cl^-$  secretion. Activation of these channels hyperpolarizes the membrane potential, thereby providing the electrochemical driving force for the apical exit of  $Cl^-$  ions (Greger *et al.*, 1997). Another second messenger is used by a variety of secretagogues such as muscarinic agonists. They increase intracellular  $Ca^{2+}$  *via*  $Ca^{2+}$  release from intracellular stores and *via*  $Ca^{2+}$  influx through cation selective channels (Frings *et al.*, 1999; Greger *et al.*, 1997). The rise in cytosolic  $Ca^{2+}$  leads to a hyperpolarization of the membrane potential due to the opening of basolateral and apical  $K^+$  channels, which are distinct from the ones activated by cyclic AMP. Hence,  $Cl^-$  secretion increases without direct involvement of CFTR (Mall *et al.*, 1998; Schultheiss & Diener, 1997; Strabel & Diener, 1995).

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In an attempt to identify the underlying mechanism of the secretory activity of quercetin, we observed in previous studies that quercetin's effect is independent of cyclic AMP. Instead, it depends on Ca<sup>2+</sup>/calmodulin. Quercetin diminishes the secretion evoked by the muscarinic agonist carbachol. However, we demonstrated that the flavonol does not act *via* muscarinic receptors (Cermak *et al.*, 1998; 2000). Since quercetin's activity is dependent on Ca<sup>2+</sup>, the flavonol should activate basolateral K<sup>+</sup> channels in a similar manner to carbachol.

The objective of the present study was to test this hypothesis. For this purpose, we used intact rat distal colon mucosa as well as apically permeabilized tissue sheets mounted in Ussing chambers.

## Methods

### Solutions

The standard buffer solution contained (in mM) NaCl, 107; KCl, 4.5; NaHCO<sub>3</sub>, 25; Na<sub>2</sub>HPO<sub>4</sub>, 1.8; NaH<sub>2</sub>PO<sub>4</sub>, 0.2; CaCl<sub>2</sub>, 1.25; MgCl<sub>2</sub>, 1; and glucose 12. The solution was gassed with 5% CO<sub>2</sub> in 95% O<sub>2</sub> and kept at 37°C; pH was adjusted to 7.4. When Ba<sup>2+</sup> was used, bicarbonate was replaced by Cl<sup>-</sup> and 10 mM HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]) was added to the solution which was gassed with 100% O<sub>2</sub>. In the experiments with apically permeabilized epithelia, a serosally directed K<sup>+</sup> gradient was applied by changing the standard solution on both sides of the epithelium. The mucosal buffer contained (in mM): KCl, 114.5; NaHCO<sub>3</sub>, 25; Na<sub>2</sub>HPO<sub>4</sub>, 1.8; NaH<sub>2</sub>PO<sub>4</sub>, 0.2; MgCl<sub>2</sub>, 1 and glucose 12; the serosal buffer contained (in mM) KCl, 4.5; N-methyl-D-glucamine (NMDG<sup>+</sup>) chloride, 110; NaHCO<sub>3</sub>, 25; Na<sub>2</sub>HPO<sub>4</sub>, 1.8; NaH<sub>2</sub>PO<sub>4</sub>, 0.2; CaCl<sub>2</sub>, 1.25; MgCl<sub>2</sub>, 1 and glucose, 12. In the experiments with Ba<sup>2+</sup> and Gd<sup>3+</sup>, bicarbonate was replaced by Cl<sup>-</sup>; 10 mM HEPES and 10 mM Tris (2-amino-2-hydroxymethyl-propan-1,3-diol) were added to the mucosal and serosal solution, respectively, which were gassed with 100% O<sub>2</sub>.

### Tissue preparation

Male Sprague Dawley rats (180–250 g) (RCC Ltd., Füllinsdorf, Switzerland) were used. The animals had free access to water and food (Eberle Nafag AG, Gossau, Switzerland) until the day of the experiment. Animals were stunned by a blow on the head and killed by exsanguination. The distal colon was taken out immediately and the serosa and muscularis were dissected free.

### Determination of electrophysiological parameters

Sheets of tissue were mounted in a modified Ussing chamber, bathed with a volume of 4 ml buffer solution on each side of the epithelium and continuously short-circuited by an automatic voltage clamp device (Aachen Microclamp, AC Copy Datentechnik, Aachen, Germany) with correction for solution resistance. Tissue conductance ( $G_t$ ) was measured by recording the voltage resulting from bipolar current pulses ( $\pm 100 \mu\text{A}$ ) applied across the tissue at one

minute intervals. The continuously applied short-circuit current ( $I_{sc}$ ) and  $G_t$  were printed out every minute. Before the addition of any drugs, there was an equilibration time of at least 60 min to stabilize basal values. When effects of blockers were tested, control experiments without the blocker were performed with tissue from the same animal. The baseline of the electrical parameters was determined as the mean over a 5 min period (five values) immediately prior to administration of a drug. The maximal changes in  $I_{sc}$  and  $G_t$  induced by a drug were expressed as the difference from the baseline ( $\Delta I_{sc}$  and  $\Delta G_t$ ).

### Drugs and statistics

Anthracene-9-carboxylic acid (9-AC), bumetanide, carbachol, clotrimazole, diphenylamine-2-carboxylate (DPC), glibenclamide, ouabain, quercetin, quinine, tetraethylammonium (TEA), and tetrapentylammonium (TPeA) were obtained from Sigma or Fluka (Buchs, Switzerland); 4,4'-diisothiocyano-stilbene-2,2'-disulphonic acid (DIDS) and 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) were from Calbiochem (Bad Soden, Germany). Charybdotoxin was purchased from Sigma and from Latoxan (Valence, France). Trans-6-Cyano-4-(N-ethylsulphonyl-N-methylamino)-3-hydroxy-2,2-dimethyl-chromane (293B) was a generous gift from Professor R Greger (University of Freiburg, Germany). Charybdotoxin was dissolved in a solution containing (mM): NaCl, 100; Tris, 10; ethylenediaminetetraacetic acid (EDTA) 1, and 0.1% (w/w) bovine albumin and stored in glass containers. TEA and TPeA were dissolved in aqueous stock solutions, quinine was dissolved in ethanol, and all other drugs were dissolved in dimethyl sulphoxide (DMSO).

Data are presented as mean values  $\pm$  s.e.mean. Comparisons between the experimental group and the respective control were done with the unpaired *t*-test. *P* values  $< 0.05$  were considered to be statistically significant.

## Results

### Blockers of CFTR inhibit quercetin's effect in intact colon mucosa

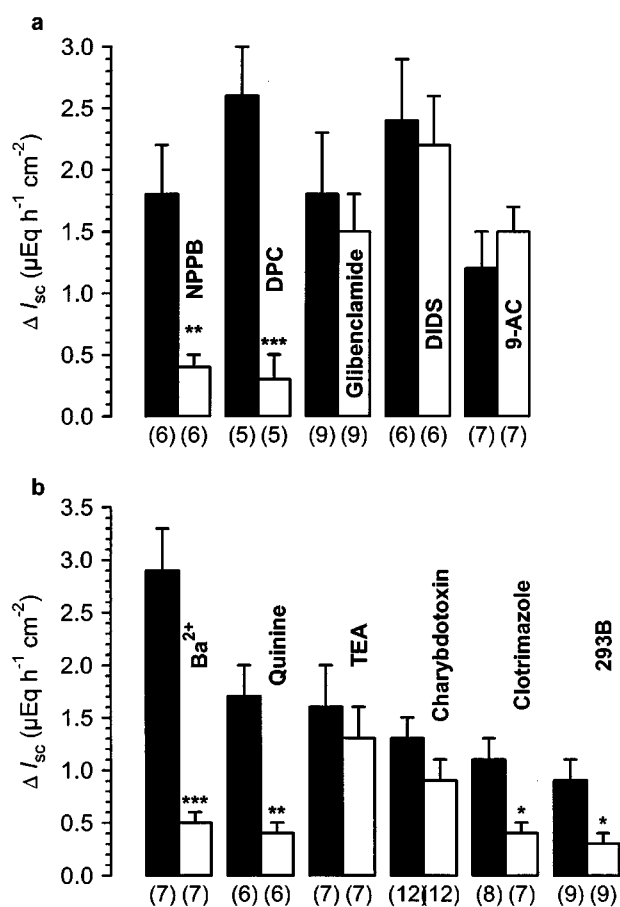
In all experiments with quercetin, the flavonol was applied at a concentration of 100  $\mu\text{M}$  to the serosal side only.

Quercetin increased  $I_{sc}$  in intact distal colon tissue sheets bathed in buffer solution as previously demonstrated (Cermak *et al.*, 1998; 2000). A first set of experiments was carried out in order to identify the apical Cl<sup>-</sup> channels involved. Since it had been demonstrated that NPPB, a compound which is able to block CFTR (Schultz *et al.*, 1999), abolishes this flavonol-induced  $I_{sc}$  in proximal colon (Cermak *et al.*, 1998), NPPB and other Cl<sup>-</sup> channel blockers were applied to the mucosal side in order to investigate their influence on the quercetin-induced secretion in the distal segment. Among the inhibitors used in this study, NPPB, glibenclamide, and DPC have been shown to block CFTR. DIDS and 9-AC, blockers of Ca<sup>2+</sup>-activated or volume-sensitive Cl<sup>-</sup> channels, respectively, are ineffective in this respect (Begenisich & Melvin, 1998; Clarke *et al.*, 1994; Diener *et al.*, 1992; Schultz *et al.*, 1999).

DPC (1 mM) significantly decreased  $I_{sc}$  by  $1.0 \pm 0.3 \mu\text{Eq h}^{-1} \text{cm}^{-2}$  ( $P < 0.05$ ;  $n = 5$ ), whereas 9-AC (1 mM), DIDS (100  $\mu\text{M}$ ), glibenclamide (100  $\mu\text{M}$ ) and NPPB (100  $\mu\text{M}$ ) had no effect on basal  $I_{sc}$ .  $G_t$  rose significantly after the administration of NPPB ( $\Delta G_t = 8.9 \pm 1.8 \text{ mS cm}^{-2}$ ,  $P < 0.001$ ,  $n = 6$ ). All other compounds did not influence this parameter. Among the Cl<sup>-</sup> channel blockers used, only NPPB and DPC were able to inhibit the quercetin-induced rise in  $I_{sc}$  (Figure 1a). The rise in  $G_t$  induced by the flavonol was not altered by any of the blockers used. These results indicate that Cl<sup>-</sup> channels other than CFTR are not involved in the secretion induced by quercetin.

#### Blockers of K<sup>+</sup> channels also inhibit quercetin's effect in intact colon mucosa

The basolateral K<sup>+</sup> channels involved in colonic Cl<sup>-</sup> secretion belong to either one of two classes. The channels



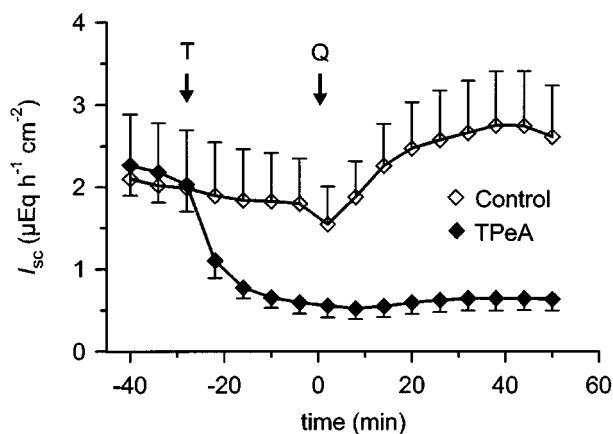
**Figure 1** Effects of Cl<sup>-</sup> (a) and K<sup>+</sup> (b) channel blockers on quercetin-induced  $I_{sc}$  in rat distal colon. The Cl<sup>-</sup> channel blockers DPC (1 mM), glibenclamide (100  $\mu\text{M}$ ), DIDS (100  $\mu\text{M}$ ), and 9-AC (1 mM) were added 20 min prior to quercetin (100  $\mu\text{M}$  serosal) to the mucosal side of the tissue; NPPB (100  $\mu\text{M}$ ) was added 40 min before the flavonol. The K<sup>+</sup> channel blockers Ba<sup>2+</sup> (10 mM) and quinine (1 mM) were added 30 min before quercetin (100  $\mu\text{M}$ ), TEA (5 mM), charybdotoxin (200 nM), clotrimazole (30  $\mu\text{M}$ ) and 293B (30  $\mu\text{M}$ ) were added 20 min prior to the flavonol to the serosal side. The experiments with Ba<sup>2+</sup> and the respective controls were performed in HEPES-buffer. Black bars represent the control experiments without the blocker. Numbers in parentheses indicate the number of experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus respective controls.

which are activated by intracellular Ca<sup>2+</sup> were recently identified in rat colonic crypts and named rSK4 due to their homology to the human small-conductance Ca<sup>2+</sup> activated K<sup>+</sup> channels hSK4 (Warth *et al.*, 1999). They are sensitive to Ba<sup>2+</sup>, quinine, and charybdotoxin, but insensitive to the K<sup>+</sup> channel blocker TEA (Bleich *et al.*, 1996; Strabel & Diener, 1995). The antifungal antibiotic, clotrimazole, is able to block the analogous human SK4 channel expressed in *Xenopus oocytes*, but is rather ineffective in preventing carbachol-induced secretion in rat colon mucosa (Warth *et al.*, 1999). The other relevant class of K<sup>+</sup> channels is activated by cyclic AMP and was also recently identified. These channels consist of two subunits in colon epithelium: KCNQ1 (or KvLQT1) and KCNE3 (Schroeder *et al.*, 2000). This conductance is specifically blocked by the chromanol 293B as well as by clotrimazole, Ba<sup>2+</sup> and quinine, but not by TEA or by charybdotoxin (Diener *et al.*, 1996; Schroeder *et al.*, 2000; Warth *et al.*, 1996).

In order to differentiate between the K<sup>+</sup> channels possibly involved in the secretory activity of quercetin, we added the above-mentioned blockers to the serosal side. Ba<sup>2+</sup> (10 mM) induced a transient increase in  $I_{sc}$  by  $3.4 \pm 0.3 \mu\text{Eq h}^{-1} \text{cm}^{-2}$  and in  $G_t$  by  $6.9 \pm 1.0 \text{ mS cm}^{-2}$  ( $P < 0.001$ ,  $n = 7$ ). Thereafter,  $I_{sc}$  decreased towards basal values. The serosal addition of quinine (1 mM) induced a similar transient  $I_{sc}$  increase by  $1.5 \pm 0.4 \mu\text{Eq h}^{-1} \text{cm}^{-2}$  ( $P < 0.01$ ,  $n = 6$ ) with a subsequent fall in the short-circuit current below basal values ( $\Delta I_{sc} = -0.6 \pm 0.3 \mu\text{Eq h}^{-1} \text{cm}^{-2}$ ,  $P > 0.05$ );  $G_t$  was not significantly altered by quinine.

The effect of quercetin on  $I_{sc}$  was blocked in the presence of Ba<sup>2+</sup> and quinine (Figure 1b). Tetraethylammonium (TEA, 5 mM) and charybdotoxin (200 nM) failed to influence the effect of the flavonol (Figure 1b). Due to our original hypothesis that quercetin activates the same Ca<sup>2+</sup> dependent K<sup>+</sup> channels as carbachol, this last result came quite unexpectedly. In order to prove the effectiveness of charybdotoxin, we checked the interaction of this compound with the effect of carbachol. Charybdotoxin itself increased  $I_{sc}$  by  $0.4 \pm 0.1 \mu\text{Eq h}^{-1} \text{cm}^{-2}$  ( $n = 14$ ,  $P < 0.001$ ). The muscarinic agonist carbachol (50  $\mu\text{M}$  serosal) induced a transient increase in  $I_{sc}$  by  $9.3 \pm 0.7 \mu\text{Eq h}^{-1} \text{cm}^{-2}$  in control tissues ( $n = 14$ ) which was partially inhibited by charybdotoxin ( $\Delta I_{sc} = 6.6 \pm 1.0 \mu\text{Eq h}^{-1} \text{cm}^{-2}$ ,  $n = 14$ ,  $P < 0.05$ ). However, in two further experimental series charybdotoxin failed to inhibit the effect of carbachol (data not shown). Thus, the effectiveness of charybdotoxin is questionable, at least under our experimental conditions. TEA increased  $I_{sc}$  by  $0.3 \pm 0.1 \mu\text{Eq h}^{-1} \text{cm}^{-2}$  ( $P < 0.01$ ,  $n = 7$ ).  $I_{sc}$  did not return towards basal values over 20 min. Clotrimazole (30  $\mu\text{M}$ ) and 293B (30  $\mu\text{M}$ ) had the opposite effect on basal  $I_{sc}$ , decreasing  $I_{sc}$  by  $0.7 \pm 0.2 \mu\text{Eq h}^{-1} \text{cm}^{-2}$  ( $P < 0.05$ ,  $n = 7$ ) and by  $0.6 \pm 0.1 \mu\text{Eq h}^{-1} \text{cm}^{-2}$  ( $P < 0.05$ ,  $n = 9$ ), respectively. Both compounds inhibited the effect of quercetin effectively (Figure 1b). Especially the result obtained with 293B may suggest an involvement of the cyclic AMP-induced conductance in the effect of the flavonol.

Tetrapentylammonium (TPEA), which is a very effective blocker of Ca<sup>2+</sup> activated K<sup>+</sup> channels in human distal colon (Mcnamara *et al.*, 1999), had a strong inhibitory effect on basal  $I_{sc}$  at a concentration of 100  $\mu\text{M}$  ( $-1.6 \pm 0.3 \mu\text{Eq h}^{-1} \text{cm}^{-2}$ ,  $P < 0.01$ ,  $n = 7$ ). Furthermore, it abolished the effect of quercetin (Figure 2).



**Figure 2** Effect of tetrapentylammonium (TPeA) on quercetin-induced  $I_{sc}$  in rat distal colon. TPeA (T, 100  $\mu$ M) or vehicle were applied at the time indicated to the serosal side. Quercetin (Q, 100  $\mu$ M serosal) was added at the time indicated to both the control group ( $n=7$ ) and to the TPeA-pretreated group ( $n=7$ ).

#### Apical permeabilization of rat distal colon epithelium

Next, we tried to bypass the apical membrane of the epithelium in order to selectively investigate electrogenic transport *via* the basolateral membrane. For this purpose, we permeabilized the apical membrane by using the polyene antibiotic nystatin. Nystatin forms channels in lipid membranes, which are permeable for monovalent cations and Cl<sup>-</sup> (selectivity for cations over Cl<sup>-</sup> is  $\sim 9:1$ ), but impermeable for divalent cations or larger molecules like ATP (Rae & Fernandez, 1991).

In preliminary experiments, we observed that the effect of nystatin was maximal at 200 mg l<sup>-1</sup>. Thus, we used this concentration in all of the following experiments. The standard buffer on the mucosal surface was replaced by the high K<sup>+</sup> solution while the serosal surface was perfused with the low K<sup>+</sup> solution. Since the concentration of all other permeable ions was the same on both sides of the tissue, this procedure yielded a K<sup>+</sup> gradient across the basolateral membrane, thereby mimicking the physiological K<sup>+</sup> gradient. Afterwards, the polyene antibiotic was added to the mucosal solution and remained throughout the rest of the experiment. Nystatin induced very large currents which led to saturation of the voltage-clamp device. Ten to 30 min after the addition of nystatin, these currents decreased and returned back to the measurable range. Similarly,  $G_t$  increased rapidly and returned afterwards slowly towards basal values. After 2 h,  $G_t$  had decreased to values that were not much higher than the pre-nystatin values. Substances were added during the phase of a constant  $I_{sc}$  decrease, usually around 80 min after the addition of nystatin.

Ouabain (1 mM), a Na<sup>+</sup>/K<sup>+</sup> ATPase inhibitor, caused a decrease in  $I_{sc}$  by  $2.8 \pm 0.5 \mu\text{Eq h}^{-1} \text{cm}^{-2}$  ( $n=10$ ,  $P<0.001$ ), demonstrating the existence of an electrogenic pump current in the nystatin-treated epithelia. The remaining current must have been carried mainly by K<sup>+</sup> ions due to the applied gradient. For this reason, it was named  $I_K$ .

In order to prove the successful permeabilization of the apical membrane, we investigated the effect of bumetanide, a blocker of Na<sup>+</sup>/K<sup>+</sup>/2 Cl<sup>-</sup> cotransport, on the  $I_K$  induced by carbachol. In intact tissue, carbachol induces a Cl<sup>-</sup> secretion

due to the activation of K<sup>+</sup> channels. Since this Cl<sup>-</sup> secretion depends on the basolateral influx of Cl<sup>-</sup> *via* the Na<sup>+</sup>/K<sup>+</sup>/2 Cl<sup>-</sup> cotransporter; bumetanide is able to block this effect (Strabel & Diener, 1995). First, we performed control experiments with intact distal colon mucosa. In these tissues, carbachol (50  $\mu$ M serosal) induced a transient increase in  $I_{sc}$  by  $11.1 \pm 0.6 \mu\text{Eq h}^{-1} \text{cm}^{-2}$  and in  $G_t$  by  $8.4 \pm 0.5 \text{mS cm}^{-2}$  ( $n=6$ ). Preincubation with serosal bumetanide (100  $\mu$ M) nearly abolished this  $I_{sc}$  transient ( $\Delta I_{sc} = 1.9 \pm 0.3 \mu\text{Eq h}^{-1} \text{cm}^{-2}$ ,  $n=6$ ;  $P<0.001$ ), whereas the carbachol-induced increase in  $G_t$  was not significantly smaller in the presence of bumetanide ( $\Delta G_t = 6.3 \pm 0.8 \text{mS cm}^{-2}$ ,  $P>0.05$ ).

In nystatin treated tissues, bumetanide (100  $\mu$ M serosal) failed to alter  $I_K$ , something which is in accordance with the electroneutral mechanism of Na<sup>+</sup>/K<sup>+</sup>/2 Cl<sup>-</sup> cotransport. In contrast to intact epithelia, bumetanide failed to influence the current induced by 50  $\mu$ M carbachol (controls:  $I_K = 0.7 \pm 0.4 \mu\text{Eq h}^{-1} \text{cm}^{-2}$ ,  $n=5$ ; bumetanide-group:  $I_K = 0.5 \pm 0.1 \mu\text{Eq h}^{-1} \text{cm}^{-2}$ ,  $n=5$ ;  $P>0.05$ ). Thus, the carbachol-induced current was solely due to a K<sup>+</sup> current *via* the basolateral membrane and did not depend on transepithelial Cl<sup>-</sup> transport as in intact tissue. This demonstrated that the apical membrane was successfully permeabilized by nystatin.

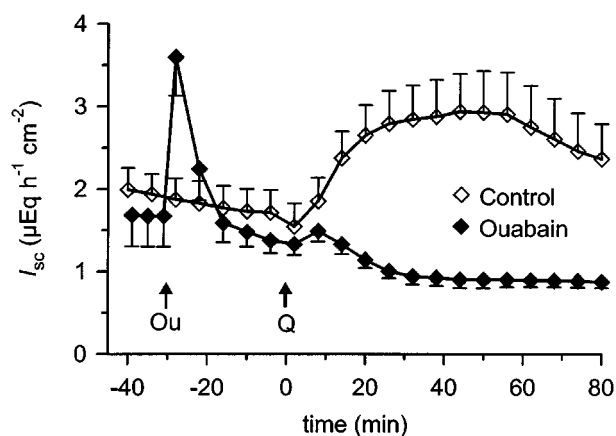
#### Basolateral Na<sup>+</sup>/K<sup>+</sup>/2 Cl<sup>-</sup> cotransport and Na<sup>+</sup>/K<sup>+</sup> ATPase are not directly involved in the activity of the flavonol

As mentioned in the introduction, transepithelial Cl<sup>-</sup> secretion depends on the cofunction of Na<sup>+</sup>/K<sup>+</sup>/2 Cl<sup>-</sup> cotransport and Na<sup>+</sup>/K<sup>+</sup> ATPase. We investigated the influence of both transport mechanisms on the effect of the flavonol.

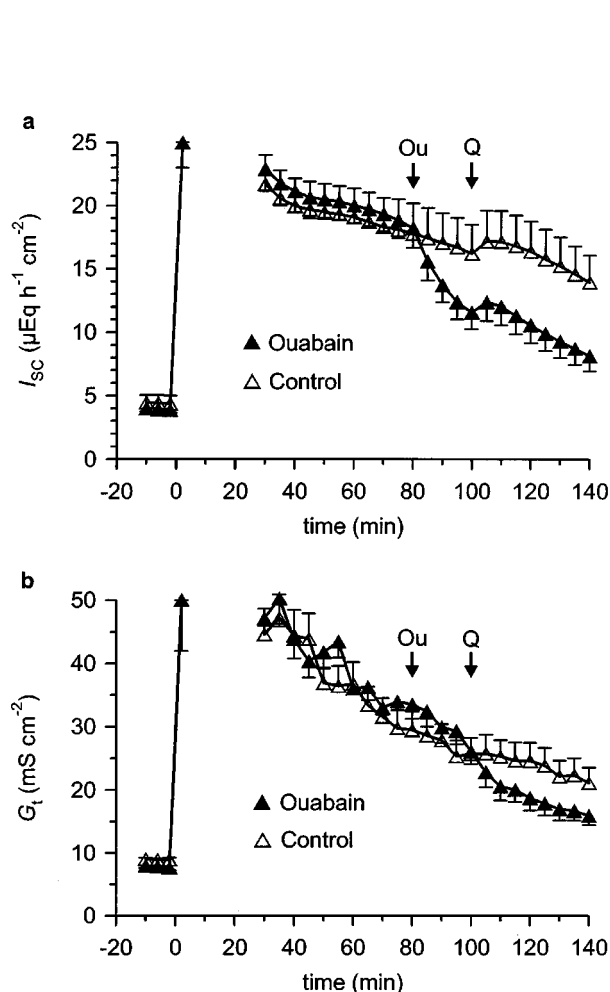
Block of the Na<sup>+</sup>/K<sup>+</sup> ATPase by ouabain (1 mM) in intact distal colon mucosa induced a transient increase in  $I_{sc}$  (Figure 3) and in  $G_t$  ( $\Delta G_t = 1.8 \pm 0.4 \text{mS cm}^{-2}$ ,  $n=7$ ; controls:  $\Delta G_t = -0.4 \pm 0.2 \text{mS cm}^{-2}$ ,  $n=6$ ,  $P<0.001$ ), something which was similar to the effect of Ba<sup>2+</sup> and quinine. The effect of quercetin on  $I_{sc}$  (Figure 3) and  $G_t$  was nearly abolished in the presence of ouabain (ouabain-group:  $\Delta G_t = 0.3 \pm 0.1 \text{mS cm}^{-2}$ , controls:  $\Delta G_t = 1.5 \pm 0.4 \text{mS cm}^{-2}$ ,  $P<0.01$ ).

In nystatin-permeabilized mucosa, ouabain induced a marked decrease in  $I_{sc}$  by  $6.6 \pm 0.3 \mu\text{Eq h}^{-1} \text{cm}^{-2}$  ( $n=7$ ) which was clearly different from the slight  $I_{sc}$  decrease in control tissues ( $\Delta I_{sc} = -1.4 \pm 0.5 \mu\text{Eq h}^{-1} \text{cm}^{-2}$ ,  $n=7$ ,  $P<0.001$ ) (Figure 4a). In control tissues, quercetin induced an increase in  $I_{sc}$  ( $\Delta I_{sc} = 1.7 \pm 0.4 \mu\text{Eq h}^{-1} \text{cm}^{-2}$ ), which was similar to the one in intact epithelium. This increase was not altered by ouabain in permeabilized tissues ( $\Delta I_{sc} = 2.0 \pm 0.3 \mu\text{Eq h}^{-1} \text{cm}^{-2}$ ). This demonstrates that the Na<sup>+</sup>/K<sup>+</sup> ATPase does not directly affect quercetin's activity.

In agreement with the model of Cl<sup>-</sup> secretion, inhibition of Na<sup>+</sup>/K<sup>+</sup>/2 Cl<sup>-</sup> cotransport diminishes quercetin-induced secretion in rat colon (Cermak *et al.*, 1998). In nystatin-treated tissues, basal  $I_K$  decreased by  $2.2 \pm 0.8 \mu\text{Eq h}^{-1} \text{cm}^{-2}$  ( $n=7$ ) in the presence of bumetanide (100  $\mu$ M serosal). This decrease in basal  $I_K$  was not different from the commonly observed decrease in control tissues ( $\Delta I_K = -3.7 \pm 0.9 \mu\text{Eq h}^{-1} \text{cm}^{-2}$ ,  $n=7$ ;  $P>0.05$ ). Due to the electroneutral nature of the cotransporter, bumetanide's lack of effect on the basal current was expected. Quercetin induced an increase in  $I_K$  which was not different between controls



**Figure 3** Effect of ouabain on quercetin-induced  $I_{sc}$  in rat distal colon. Ouabain (*Ou*, 1 mM) or vehicle were applied at the time indicated to the serosal side; quercetin (*Q*, 100  $\mu$ M serosal) was added at the time indicated to both the control group ( $n=6$ ) and to the ouabain-pretreated group ( $n=7$ ).

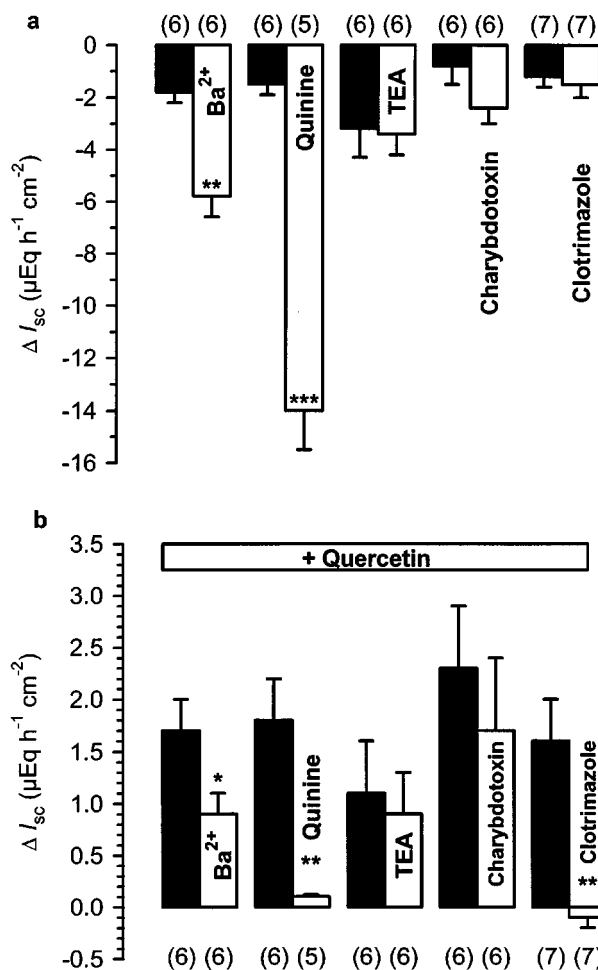


**Figure 4** Effect of ouabain on quercetin-induced  $I_k$  (a) and  $G_t$  (b) in rat distal colon mucosa after apical permeabilization with nystatin. Nystatin (200 mg  $l^{-1}$ ) was added to the mucosal side at time 0 in the presence of a serosally directed K<sup>+</sup> gradient. Since the voltage clamp was saturated directly after this manoeuvre, data up to 30 min after the addition of nystatin are not indicated. Either ouabain (*Ou*, 1 mM serosal) or vehicle was added at the time indicated. Both groups received quercetin (*Q*, 100  $\mu$ M serosal) at the time indicated.  $n=7$  for ouabain group and for control group.

( $\Delta I_K = 1.1 \pm 0.4 \mu\text{Eq h}^{-1} \text{cm}^{-2}$ ) and the bumetanide group ( $\Delta I_K = 0.9 \pm 0.4 \mu\text{Eq h}^{-1} \text{cm}^{-2}$ ,  $n=7$ ). Thus, the cotransporter is also not directly involved in the effect of the flavonol.

#### Quercetin activates basolateral K<sup>+</sup> channels

After excluding a direct effect of quercetin on Na<sup>+</sup>/K<sup>+</sup>/2 Cl<sup>-</sup> cotransport and Na<sup>+</sup>/K<sup>+</sup> ATPase in the previous experiments, we investigated a possible participation of basolateral K<sup>+</sup> channels in the effect of the flavonol. In nystatin-permeabilized mucosal sheets, the K<sup>+</sup> channel blockers Ba<sup>2+</sup> (5 mM serosal) and quinine (1 mM serosal) induced a marked decrease in basal  $I_K$  which was clearly different from the commonly observed continuous current decrease in control tissues (Figure 5a). On the other hand, TEA (5 mM serosal), charybdotoxin (200 nM serosal) and clotrimazole (30  $\mu$ M serosal) had no significant effect on basal  $I_K$ . Ba<sup>2+</sup>, quinine and clotrimazole inhibited the current induced by quercetin,



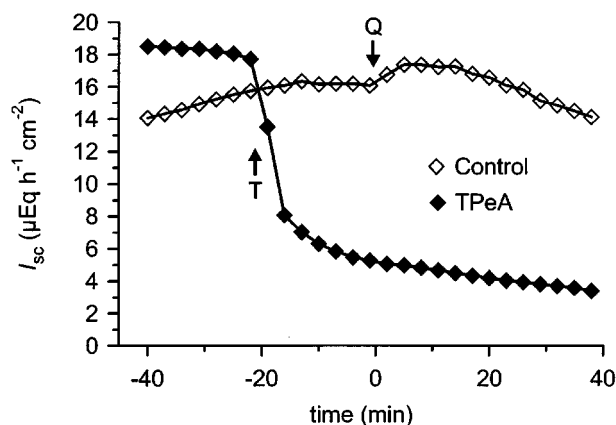
**Figure 5** (a) Effect of different blockers in nystatin-permeabilized rat distal colon (200 mg nystatin  $l^{-1}$ ). Ba<sup>2+</sup> (5 mM), quinine (1 mM), TEA (5 mM), charybdotoxin (200 nM) and clotrimazole (30  $\mu$ M) were applied to the serosal side of the tissue. (b) Effect of quercetin (100  $\mu$ M), added to the serosal bath 20 min after the blockers in the same experiments. The experiments with Ba<sup>2+</sup> and the respective controls were performed in HEPES/Tris-buffer. Black bars represent the control experiments without the blocker. Numbers in parentheses indicate the number of experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus respective controls.

whereas TEA and charybdotoxin were without effect (Figure 5b). Similar to its effect in intact colon mucosa, TPpA (100  $\mu$ M serosal) drastically decreased basal  $I_K$  by  $10.8 \pm 1.3 \mu\text{Eq h}^{-1} \text{cm}^{-2}$ ;  $n=10$ ) in comparison with controls ( $\Delta I_{sc} = -1.1 \pm 0.3 \mu\text{Eq h}^{-1} \text{cm}^{-2}$ ;  $n=10$ ;  $P < 0.001$ ). The quercetin-induced current that was visible in control tissues ( $\Delta I_{sc} = 1.8 \pm 0.6 \mu\text{Eq h}^{-1} \text{cm}^{-2}$ ) was nearly abolished by TPpA ( $\Delta I_{sc} = 0.2 \pm 0.1 \mu\text{Eq h}^{-1} \text{cm}^{-2}$ ;  $P < 0.05$ ) (Figure 6). In contrast to the experiments with intact epithelium, the chromanol 293B did not affect basal  $I_K$  or the current induced by the flavonol (Figure 7a,b).

Interestingly, the mucosally applied Cl<sup>-</sup> channel blockers DPC (1 mM) and NPPB (100  $\mu$ M) diminished basal  $I_K$  (Figure 7a). In contrast to intact colon mucosa, both blockers failed to significantly alter the quercetin-induced current (Figure 7b). In a previous study, we showed that Gd<sup>3+</sup>, a lanthanide which is able to inhibit the carbachol-induced Ca<sup>2+</sup> influx into colon mucosa (Frings *et al.*, 1999), had no effect on the short-circuit current induced by quercetin (Cermak *et al.*, 2000). In distal colon epithelium permeabilized with nystatin, the serosal addition of Gd<sup>3+</sup> (100  $\mu$ M) affected neither basal  $I_K$  nor the current induced by the flavonol (Figure 7a,b). The latter experiments were conducted in HEPES/Tris buffer due to the fact that Gd<sup>3+</sup> ions can be effectively bound by CO<sub>3</sub><sup>2-</sup> and PO<sub>4</sub><sup>3-</sup> anions (Caldwell *et al.*, 1998).

## Discussion

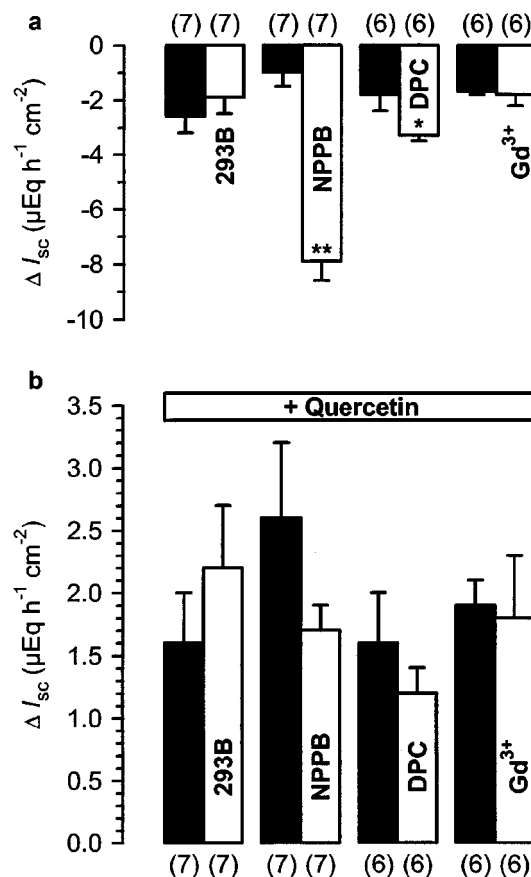
Intestinal chloride secretion depends on the coordinated activity of apical Cl<sup>-</sup> channels as well as on basolateral Na<sup>+</sup>/K<sup>+</sup> ATPases, Na<sup>+</sup>/K<sup>+</sup>/2 Cl<sup>-</sup> cotransporters and K<sup>+</sup> channels. Inhibition of one of these transport mechanisms leads to the cessation of secretion. Accordingly, the short-circuit current induced by quercetin, which is indicative of the Cl<sup>-</sup> secretion induced by this flavonol, is inhibited by several manoeuvres: block of the Na<sup>+</sup>/K<sup>+</sup> pump with ouabain, inhibition of Na<sup>+</sup>/K<sup>+</sup>/2 Cl<sup>-</sup> cotransport (Cermak *et al.*, 1998), block of basolateral K<sup>+</sup> channels, and inhibition of



**Figure 6** Effect of tetrapentylammonium (TPpA) on quercetin-induced  $I_{sc}$  in a mucosal sheet of rat distal colon after apical permeabilization with nystatin. TPpA (T, 100  $\mu$ M) was applied at the time indicated to the serosal side; the control tissue received only vehicle. Quercetin (Q, 100  $\mu$ M serosal) was added at the time indicated to both the control tissue and the TPpA-pretreated tissue.

CFTR. In intestinal mucosa, additional Ca<sup>2+</sup> activated Cl<sup>-</sup> channels do not play a role in Cl<sup>-</sup> secretion, something which is different from other secretory epithelia (Clarke *et al.*, 1994; Greger, 2000; Strabel & Diener, 1995). Accordingly, a blocker of Ca<sup>2+</sup> activated Cl<sup>-</sup> channels, DIDS (Begenisich & Melvin, 1998; Clarke *et al.*, 1994; Schultz *et al.*, 1999), as well as a blocker of volume-sensitive Cl<sup>-</sup> channels, 9-AC (Begenisich & Melvin, 1998; Diener *et al.*, 1992), failed to inhibit the quercetin-induced current. On the other hand, NPPB and DPC, which are able to block CFTR at the concentrations used in this study (Schultz *et al.*, 1999), diminished quercetin's effect. However, another blocker of CFTR, namely glibenclamide (Schultz *et al.*, 1999), failed to inhibit the flavonol-induced secretion in our study. We do not have an explanation for the latter observation at present.

Several K<sup>+</sup> channel blockers were also able to block the secretion induced by quercetin in intact mucosa. At present, two classes of K<sup>+</sup> channels have been identified which take part in chloride secretion in the rat colon: Ca<sup>2+</sup> dependent rSK4 channels and cyclic AMP-activated KCNQ1/KCNE3 channels (Schroeder *et al.*, 2000; Warth *et al.*, 1999). All of



**Figure 7** (a) Effect of different blockers in nystatin-permeabilized rat distal colon (200 mg nystatin l<sup>-1</sup>). 293B (30  $\mu$ M) and Gadoliniumchloride (100  $\mu$ M) were applied to the serosal side, NPPB (100  $\mu$ M) and DPC (1 mM) to the mucosal side of the tissue. (b) Effect of quercetin (100  $\mu$ M), added to the serosal bath 20 min after the blockers in the same experiments. The experiments with Gd<sup>3+</sup> and the respective controls were performed in HEPES/Tris-buffer. Black bars represent the control experiments without the blocker. Numbers in parentheses indicate the number of experiments. \* $P < 0.05$ , \*\* $P < 0.001$  versus respective controls.

the inhibitors which block the cyclic AMP-activated K<sup>+</sup> conductance, especially the chromanol 293B, were also effective in diminishing the quercetin-induced  $I_{sc}$  in intact mucosa. On the other hand, charybdotoxin, which is specific for the Ca<sup>2+</sup> dependent K<sup>+</sup> conductance, failed to alter the effect of the flavonol.

At first sight, these results seem to be contrary to our previous studies in which we could clearly demonstrate that the secretion evoked by quercetin is independent of cyclic AMP, but dependent on Ca<sup>2+</sup> (Cermak *et al.*, 1998; 2000). One possible explanation is that quercetin acts on KCNQ1, which might also be activated by Ca<sup>2+</sup> other than cyclic AMP (Warth *et al.*, 1999).

However, the failure of charybdotoxin to block the effect of quercetin could have been due to the tissue preparation we used. In contrast to its marked effect on Ca<sup>2+</sup> dependent K<sup>+</sup> channels in isolated epithelial cells, charybdotoxin seems to be rather ineffective in epithelial preparations with several submucosal cell layers present. This is indicated by its weak effect on the carbachol-induced  $I_{sc}$  in our experiments. Another blocker of Ca<sup>2+</sup> activated K<sup>+</sup> channels, TPcA (Mcnamara *et al.*, 1999), nearly abolished the effect of the flavonol. In addition, 293B is also known to block CFTR at the concentration that we used in our experiments (Bachmann *et al.*, 2001). It is possible that the chromanol could have blocked the quercetin-induced current independently of cyclic AMP-activated K<sup>+</sup> channels.

Therefore, we went on to examine the ion transport mechanisms involved in the effect of the flavonol more selectively. By using nystatin-permeabilized epithelium, we bypassed the apical Cl<sup>-</sup> conductance in order to specifically investigate the possible involvement of the basolateral Na<sup>+</sup>/K<sup>+</sup> pump, Na<sup>+</sup>/K<sup>+</sup>/2 Cl<sup>-</sup> cotransport and K<sup>+</sup> channels. In agreement with a recent study that used the same approach (Schultheiss & Diener, 1997), we also found that a major part of the observed current under this condition was due to the electrogenic Na<sup>+</sup>/K<sup>+</sup> pump. The remaining current was carried by K<sup>+</sup> ions due to the applied transepithelial gradient.

In apically permeabilized colon mucosa, quercetin induced a current which was independent of the Na<sup>+</sup>/K<sup>+</sup> pump and Na<sup>+</sup>/K<sup>+</sup>/2 Cl<sup>-</sup> cotransport. This observation indicated that the current induced by the flavonol must have been solely due to an activation of basolateral K<sup>+</sup> channels ( $I_K$ ). This assumption was further supported by the observation that the Cl<sup>-</sup> channel blockers NPPB and DPC, which nearly abolished the quercetin-induced  $I_{sc}$  in intact epithelium, failed to alter this current in permeabilized mucosa. Thus, apical Cl<sup>-</sup> channels were bypassed.

The results obtained with several inhibitors of K<sup>+</sup> channels in nystatin-treated tissue were similar to our previous experiments in intact epithelium. Interestingly, the only K<sup>+</sup> channel blocker which acted differently in intact and permeabilized mucosa was 293B. The chromanol affected

neither basal  $I_K$  nor the current increase induced by quercetin in nystatin-treated epithelium. This observation very likely rules out a participation of cyclic AMP-activated KCNQ1/KCNE3 channels in the effect of the flavonol. Thus, the effect of 293B observed in intact epithelium can be explained by an inhibition of CFTR, which was absent in the nystatin experiments (Bachmann *et al.*, 2001).

Basal  $I_K$  in nystatin-treated colon mucosa was sensitive to Ba<sup>2+</sup> and quinine.  $I_K$  was insensitive to TEA which is in accordance with another study (Schultheiss & Diener, 1997). Surprisingly, the Cl<sup>-</sup> channel blockers NPPB and DPC inhibited a part of the basal  $I_K$  as well. Since this cannot be explained by block of a residual Cl<sup>-</sup> current due to incomplete permeabilization (otherwise the quercetin-induced current in the same experiments would have been affected as well, which was not the case), we suggest that this was an unspecific effect of NPPB and DPC on K<sup>+</sup> channels (Chang & Dawson, 1988; Illek *et al.*, 1992).

Because carbachol induces a Ca<sup>2+</sup> influx through Gd<sup>3+</sup>-sensitive cation channels in rat colon crypts (Frings *et al.*, 1999), we additionally investigated if quercetin might activate a similar mechanism. Gd<sup>3+</sup> neither affected basal  $I_K$  nor did it influence the current induced by the flavonol, observations which are consistent with a previous study performed with intact epithelium (Cermak *et al.*, 2000).

Illek & Fischer (1998) suggested that quercetin directly interacts with CFTR in airway epithelium in a similar manner to the related isoflavone genistein. The recent demonstration of a direct interaction of genistein and quercetin with the second nucleotide-binding fold of CFTR strongly supports this assumption (Randak *et al.*, 1999). In our experiments, we could not determine if quercetin acts additionally on the apical Cl<sup>-</sup> conductance. In contrast to monolayers of cultured cell lines, permeabilization of the basolateral membrane by nystatin was not feasible in our preparation. This was probably due to the presence of subepithelial tissue. Based on the published data, a direct interaction of quercetin with CFTR might be an additional means of inducing Cl<sup>-</sup> secretion in colon mucosa. This has to be investigated in future studies.

In summary, we have demonstrated that the flavonol quercetin activates basolateral K<sup>+</sup> channels in rat distal colon mucosa. Cyclic AMP-activated K<sup>+</sup> channels of the KCNQ1/KCNE3 type do not seem to be involved in the effect of the flavonol. The K<sup>+</sup> conductance altered by quercetin is likely activated by Ca<sup>2+</sup>. Activation of such K<sup>+</sup> channels is probably the major mechanism by which quercetin induces chloride secretion in rat colon.

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