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Activation of Apical K^+ Conductances by Muscarinic Receptor Stimulation in Rat Distal Colon: Fast and Slow Components

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Abstract. In the epithelium of rat distal colon the acetylcholine analogue carbachol induces a transient increase of short-circuit current (I_{sc}) via stimulation of cellular K^+ conductances. Inhibition of the turnover of inositol-1,4,5-trisphosphate (IP_3) by LiCl significantly reduced both the amplitude and the duration of this response. When the apical membrane was permeabilized with nystatin, LiCl nearly abolished the carbachol-induced activation of basolateral K^+ conductances. In contrast, in epithelia, in which the basolateral membrane was bypassed by a basolateral depolarization, carbachol induced a biphasic increase in the K^+ current across the apical membrane consisting of an early component carried by charybdotoxin- and tetraethylammonium-sensitive K^+ channels followed by a sustained plateau carried by channels insensitive against these blockers. Only the latter was sensitive against LiCl or inhibition of protein kinases. In contrast, the stimulation of the early apical K^+ conductance by carbachol proved to be resistant against inhibition of phospholipase C or protein kinases. However, apical dichlorobenzamil, an inhibitor of Na^+/Ca^{2+} exchangers, or a Ca^{2+}-free mucosal buffer solution significantly reduced the early component of the carbachol-induced apical K^+ current. The presence of an apically localized Na^+/Ca^{2+}-exchanger was proven immunohistochemically. Taken together these experiments reveal divergent regulatory mechanisms for the stimulation of apical Ca^{2+}-dependent K^+ channels in this secretory epithelium, part of them being activated by an inflow of Ca^{2+} across the apical membrane.

Key words: IP_3 — K^+ conductances — Na^+/Ca^{2+} exchanger — Rat distal colon — Secretion

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Introduction

A prerequisite for Cl^--secretion across epithelial tissues are Cl^--channels in the apical membrane and a sufficient driving force for Cl^--extrusion across these channels. In the rat distal colon the latter is maintained by an increase of the cellular K^+ conductance induced by Ca^{2+}-dependent secretagogues (for review *see* Binder & Sandle, 1994). Binding of the acetylcholine analogue carbachol at muscarinic receptors of the M3 subtype initiates a Ca^{2+}-dependent Cl^--secretion (Lindqvist et al., 1998) involving the activation of phospholipase C (PLC). Inositol-1,4,5-trisphosphate (IP_3), which is released by the PLC reaction, binds to specific receptors located on intracellular Ca^{2+}-stores to induce an increase of the cytoplasmic Ca^{2+} concentration (for review *see* e.g., Taylor & Broad, 1998). The consequence is the activation of a Ca^{2+}-dependent K^+ conductance (Böhme, Diener, & Rummel, 1989) leading to a hyperpolarization of the membrane, which increases the driving force for apical Cl^--exit.

Carbachol-stimulated K^+ conductances are not only restricted to the basolateral side of the cell, since the cholinergic agonist also evokes a K^+ current across the apical membrane (Schultheiss & Diener, 1997), concomitant with a transepithelial K^+ secretion (Heinke, Hörger & Diener, 1998) and a stimulation of the apical K^+ efflux, as shown by experiments with $^{86}Rb^+$, a tracer for K^+ (Heinke, Hörger & Diener, 1998). Ca^{2+}-dependent apical K^+ channels, which are sensitive against K^+-channel blockers such as quinidine, Ba^{2+} or tetraethylammonium (TEA), have indeed been observed in inside-out patch-clamp recordings from colonic surface cells (Butterfield et al., 1997). The aim of the present study was to investigate the mechanism of activation of the apical K^+ conductance. The current model states that the stimulation of phospholipase C results in an elevated IP_3

concentration; the subsequent binding of IP_3 to its receptor liberates Ca^{2+} from internal stores, which then activates the K^+ channels in the cell membrane(s). In order to find out whether this model holds for the apical K^+ conductance, the IP_3 turnover was inhibited by LiCl, which blocks the myo-inositol-1-phosphatase (Berridge & Irvine, 1989; Jenkinson, Nahorski & Challiss, 1994; Wolfson et al., 1998). The results of these experiments suggest that at least 2 different types of carbachol-stimulated K^+ conductances are present in the apical membrane: one that is suppressed after preincubation with LiCl, and a second one that is dependent on the presence of mucosal Ca^{2+}, probably entering the cell via an apical Na^+/Ca^{2+} exchanger.

Materials and Methods

Solutions

The Ussing-chamber experiments were carried out in a bathing solution containing (mmol · l^{-1}): NaCl 107, KCl 4.5, $NaHCO_3$ 25, Na_2HPO_4 1.8, NaH_2PO_4 0.2, $CaCl_2$ 1.25, $MgSO_4$ 1 and glucose 12. The solution was gassed with carbogen (5% CO_2/95%O_2) and kept at a temperature of 37°C; pH was 7.4. In order to apply a mucosally to serosally directed K^+ gradient, the KCl concentration in this buffer was increased to 13.5 mmol · l^{-1} while reducing equimolarly the NaCl concentration in order to maintain isosmolarity. For the depolarization of the basolateral membrane, a 111.5 mmol · l^{-1} KCl solution was used on the basolateral side, in which NaCl was equimolarly replaced by KCl; in one set of experiments, in addition NaCl at the mucosal side was equimolarly substituted by K gluconate (KGluc).

For the experiments with the trivalent cations La^{3+} or Gd^{3+} a Tyrode solution was used instead of the standard bathing solution to prevent binding of the lanthanides by CO_3^{2-}- and PO_4^{3-}-anions present in this solution (Caldwell, Clemo, & Baumgarten, 1998). This buffer contained (mmol · l^{-1}): NaCl 140, KCl 5.4, $CaCl_2$ 1.25, $MgCl_2$ 1, HEPES (N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid) 10, glucose 12; it was kept at a temperature of 37°C, a pH of 7.4 and was gassed with O_2. For basolateral depolarization, the NaCl in this solution was equimolarly substituted by KCl.

For the experiments with isolated crypts the following buffers were used. The EDTA (ethylenediamino-tetraacetic acid) solution for the crypt isolation contained (mmol · l^{-1}): NaCl 107, KCl 4.5, NaH_2PO_4 0.2, Na_2HPO_4 1.8, $NaHCO_3$ 25, EDTA 10, glucose 12, with 1 g · l^{-1} bovine serum albumin. The solution was gassed with carbogen (5% CO_2/95%O_2) and kept at a temperature of 37°C, the pH was adjusted to 7.4 by Tris-base (tris(hydroxymethyl)-aminomethane). The high-K^+ Tyrode for the storage of the crypts consisted of (mmol · l^{-1}): K gluconate 100, KCl 30, NaCl 20, $CaCl_2$ 1.25, $MgCl_2$ 1, HEPES (N-(2-hydroxyethyl)piperazine-N′-2-ethansulfonic acid) 10, glucose 12, Na pyruvate 5, and 1 g · l^{-1} bovine serum albumin. The solution was adjusted with KOH to a pH of 7.4. The fura-2 experiments were performed using a Tyrode solution of the following composition (mmol · l^{-1}): NaCl 140, KCl 5.4, $CaCl_2$ 1.25, $MgCl_2$ 1, glucose 12, HEPES 10; pH of 7.4.

Tissues and Crypt Isolation

Crypts were prepared as described in detail elsewhere (Schultheiss, Kocks & Diener, 2002). In short, Wistar rats were used with a weight of 120–220 g. The animals had free access to water and food until the day of the experiment. Animals were stunned by a blow on the head and killed by exsanguination (approved by Regierungspräsidium Gießen, Gießen, Germany). The serosa and muscularis propria of the distal colon were stripped away by hand to obtain the mucosa-submucosa preparation. 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 muscularis propria was gently removed in a proximal direction. For the isolation of intact crypts, the mucosa-submucosa was fixed on a plastic holder with tissue adhesive and transferred for about 8 min in the EDTA solution. The mucosa was vibrated once for 30 s in order to obtain crypts. They were collected in an intracellular-like high-K^+ Tyrode buffer (Böhme, Diener & Rummel, 1989). The mucosa was kept at 37°C during the isolation procedure. All further steps, including the fura-2 experiments, were carried out at room temperature.

Short-Circuit Current Measurement

The tissue was fixed in a modified Ussing chamber, bathed with a volume of 3.5 ml on each side of the mucosa and short-circuited by a computer-controlled voltage-clamp device (Mußler Ingenieurbüro für Mess- und Datentechnik, Aachen, FRG) with correction for solution resistance. The exposed surface of the tissue was 1 cm^2. Short-circuit current (I_{sc}) was continuously recorded and tissue conductance (G_t) was measured every min by applying a current pulse of ±50 μA · cm^{-2}. The baseline in electrical parameters was determined as mean over 3 min just before administration of a drug.

Measurement of Apical and Basolateral K^+ Currents

The apical membrane was permeabilized with nystatin (100 μg · ml^{-1} at the mucosal side) dissolved in dimethylsulfoxide (DMSO; final concentration 2 ml · l^{-1}). Nystatin was kept under light protection and was ultrasonified immediately before use. For measuring K^+ currents across the apical membrane, a K^+ gradient of 3:1 was administered (13.5 mmol · l^{-1} at the mucosal and 4.5 mmol · l^{-1} at the serosal side).

In order to depolarize the basolateral membrane, the tissue was exposed to a high-K^+ buffer (111.5 mmol · l^{-1} KCl) at the serosal side (Fuchs, Larsen & Lindemann, 1977).

Fura-2 Experiments

Relative changes in the intracellular Ca^{2+} concentration were measured using the Ca^{2+}-sensitive fluorescent dye fura-2 (Grynkiewicz, Poenie & Tsien, 1985), as described previously (Frings, Schultheiss & Diener, 1999). The crypts were pipetted into the experimental chamber with a volume of about 3 ml and were fixed to the glass bottom of the chamber with the aid of poly-L-lysine (0.1 g · l^{-1}). They were loaded for 60 min with $2.5 \cdot 10^{-6}$ mol · l^{-1} fura-2 acetoxymethylester (fura-2/AM) in the presence of 0.05 g · l^{-1} Pluronic®. Then the fura-2/AM was washed away. The preparation was superfused hydrostatically throughout the experiment with 140 mmol · l^{-1} NaCl Tyrode. Perfusion rate was about 1 ml · min^{-1}.

Experiments were carried out on an inverted microscope (Olympus IX-50) equipped with an epifluorescence setup and an image analysis system (Till Photonics, Martinsried, Germany). The emission above 470 nm was measured from several regions of interest, each with the size of about one cell. The cells were excited alternatively at 340 and 380 nm and the ratio of the emission signal at both excitation wavelengths was calculated. Data were sampled

at 0.2 Hz. The baseline in the fluorescence ratio of fura-2 was measured during several min before drugs were administered.

Immunofluorescence

The tissue was fixed in 4% formaldehyde for 2 h, dehydrated in a graded series of ethanol and embedded in paraffin wax. Sections (7 μm) were cut parallel to the mucosal surface to achieve a view of the enterocytes around the crypts. Sections were stained with a polyclonal antibody against the cardiac form (NCX1) of the sodium-calcium exchanger (Swant, Bellinzona, Switzerland) raised in rabbit. Visualization was performed with the peroxidase-diaminobenzidine method.

Reagents

Dichlorobenzamil (Molecular Probes, Leiden, The Netherlands), fura-2 acetoxymethylester (fura-2/AM, from Molecular Probes, Leiden, The Netherlands), nystatin, staurosporine, and U-73122 (Calbiochem, Bad Soden, Germany) were dissolved in dimethylsulfoxide (DMSO; final maximal concentration 2.5 ml $\cdot$ l^{-1}). Pluronic® (BASF, Weyandotte, NJ) was dissolved in DMSO as a 200 g $\cdot$ l^{-1} stock solution (final maximal DMSO concentration 2.5 ml $\cdot$ l^{-1}). Indomethacin and ionomycin were dissolved in ethanol (final maximal concentration 2.5 ml $\cdot$ l^{-1}). Carbachol, propranolol, ruthenium red (Alfa Aesar, Karlsruhe, Germany), ryanodine (Calbiochem, Bad Soden, Germany), and tetraethylammonium (TEA) were dissolved in aqueous stock solutions. Charybdotoxin (Alomone Labs, Jerusalem, Israel) was dissolved in an aqueous stock solution containing 1 g $\cdot$ l^{-1} bovine serum albumin. N-Ω-nitro-L-arginine (L-NNA; Tocris Cookson, Bristol, UK) was dissolved in a stock solution containing 1 mol $\cdot$ l^{-1} HCl. If not indicated differently, drugs were from Sigma, Deisenhofen, Germany.

Statistics

Results are given as means ± one standard error of the mean (SEM). For the comparison of two groups, either a Student's *t*-test or a Mann-Whitney *U*-test was applied. An *F*-test decided which test method had to be used. Both paired and unpaired two-tailed Student's *t*-tests were applied, as appropriate. $P < 0.05$ was considered to be statistically significant.

Results

Effect of LiCl on Carbachol-induced Cl^- Secretion

Under control conditions the cholinergic agonist carbachol ($5 \cdot 10^{-5}$ mol $\cdot$ l^{-1} at the serosal side) induced a biphasic increase in I_{sc}. The current increased by a maximal value (ΔI_{max}) of 5.0 ± 0.7 μEq $\cdot$ h^{-1} $\cdot$ cm^{-2}, which after 10 min (ΔI_{10}) had decayed to a value of 2.4 ± 0.6 μEq $\cdot$ h^{-1} $\cdot$ cm^{-2} above the former baseline ($P < 0.05$ versus I_{sc} prior to administration of carbachol, $n = 6$; Fig. 1). This response usually consists of a monophasic increase in I_{sc} followed by a biphasic decay, which can be described by 2 exponential functions (Strabel & Diener, 1995). The increase in I_{sc} was concomitant with an increase in G_t. The increase in G_t (ΔG_t) amounted to 2.5 ± 0.6 mS $\cdot$ cm^{-2} at the point where the current reached its maximum, and 3.6 ± 1.1 mS $\cdot$ cm^{-2} ten min after administration of carbachol ($P < 0.05$ versus G_t prior to administration of carbachol, $n = 6$).

Equimolar substitution of 10^{-2} mol $\cdot$ l^{-1} NaCl by LiCl on both sides of the tissue led to an increase in I_{sc} of 0.7 ± 0.2 μEq $\cdot$ h^{-1} $\cdot$ cm^{-2} ($P < 0.05$ to baseline, $n = 7$; Fig. 1), while G_t increased by 1.0 ± 0.3 mS $\cdot$ cm^{-2} ($P < 0.05$). When carbachol was administered in the presence of LiCl (pretreatment time: 20 min), both the time course and the duration of the I_{sc} response were altered. The monophasic increase in I_{sc} was converted into a biphasic one (observed in 6 of 7 tissues tested) consisting of a fast and transient increase of 0.6 ± 0.1 μEq $\cdot$ h^{-1} $\cdot$ cm^{-2} within 0.3 ± 0.02 min ($P < 0.05$ versus current prior to administration of carbachol; Fig. 1), which 0.7 ± 0.1 min after carbachol administration was followed by a steep decrease in I_{sc} below baseline by 1.1 ± 0.2 μEq $\cdot$ h^{-1} $\cdot$ cm^{-2} ($P < 0.05$ to baseline).[1] Within 1.7 ± 0.7 min the I_{sc} changed again direction and increased to 2.4 ± 0.7 μEq $\cdot$ h^{-1} $\cdot$ cm^{-2} above baseline ($P < 0.05$ versus current prior to administration of carbachol, $n = 7$). The amplitude of the maximal current response was significantly lower compared to the carbachol response in the absence of LiCl ($P < 0.05$, $n = 6$–7). Also the duration of the carbachol-induced current was shortened. 10 min after administration of carbachol in the presence of LiCl, the I_{sc} had already fallen below the former baseline by 0.6 ± 0.2 μEq $\cdot$ h^{-1} $\cdot$ cm^{-2} ($P < 0.05$ versus response to carbachol under LiCl-free conditions, $n = 6$–7). Also the increase in G_t evoked by carbachol was reduced in the presence of LiCl. Near the point where the current reached its maximum, carbachol evoked only an increase in G_t by 0.9 ± 0.1 mS $\cdot$ cm^{-2} (compared to a ΔG_t of 2.5 ± 0.6 mS $\cdot$ cm^{-2} in the absence of LiCl, $P < 0.05$, $n = 6$–7), whereas 10 min after administration of the cholinergirc agonist G_t was increased by only 0.6 ± 0.4 mS $\cdot$ cm^{-2} (compared to a ΔG_t of 3.6 ± 1.1 mS $\cdot$ cm^{-2} in the absence of LiCl, $P < 0.05$, n = 6–7). Due to the fact that G_t was determined only once per min (for details *see* Materials and Methods), it was not possible to exactly match the fast biphasic change in I_{sc} evoked by carbachol with the changes in G_t.

The effect of LiCl was not caused by a reduction of the extracellular Na^+ concentration. When Li^+ was replaced by the impermeant cation N-methyl-D-glucamine, the G_t- and I_{sc}-responses induced by carbachol were not altered compared to a time-dependent control ($n = 6$; *data not shown*).

[1] This negative I_{sc} probably represents the transient activation of an apical K^+ conductance, which proved to be resistant against preincubation with LiCl (*see* below).

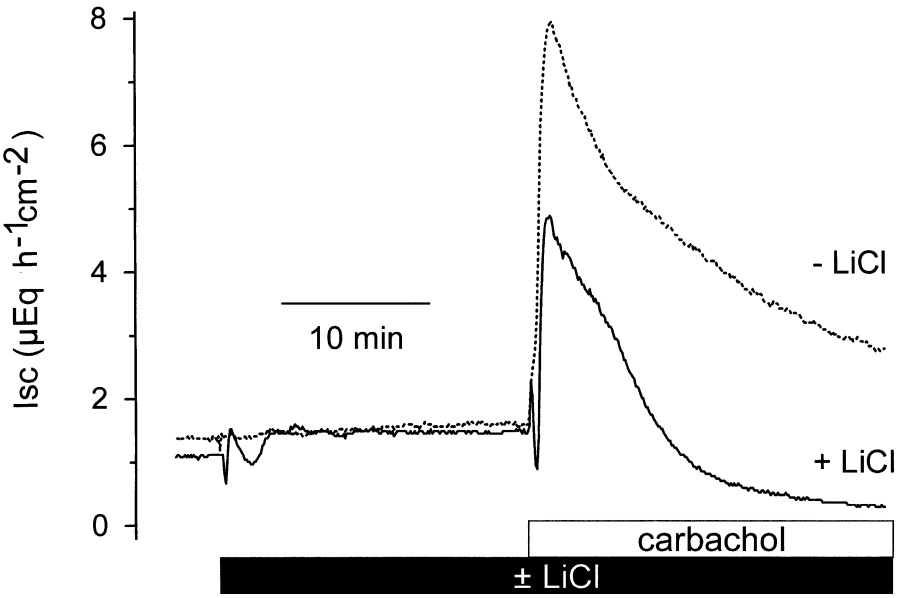

Fig. 1. Response to carbachol ($5 \cdot 10^{-5}$ mol $\cdot$ l^{-1} at the serosal side; *white bar*) in the absence (– LiCl; *dashed line*) and presence (+ LiCl; *solid line*) of LiCl (10^{-2} mol $\cdot$ l^{-1} at the mucosal and serosal side; *black bar*). Typical recording for $n = 6$–7; for statistics, *see* text.

LiCl Inhibits the Activation of Basolateral K^+ Conductances by Carbachol

In order to investigate the necessity of IP_3 turnover for the stimulation of basolateral K^+ channels by carbachol, the apical membrane was permeabilized by nystatin (100 µg $\cdot$ ml^{-1}) and a mucosal to serosal K^+ gradient of 3:1 (13.5 mmol $\cdot$ l^{-1} K^+ at the mucosal and 4.5 mmol $\cdot$ l^{-1} K^+ at the serosal side) was applied in order to drive a K^+ current across basolateral K^+ channels (as indicated by the schematic inset in Fig. 2). Under these conditions, nystatin induced an increase in I_{sc}, which amounted to 16.0 ± 1.8 µEq $\cdot$ h^{-1} $\cdot$ cm^{-2}, paralleled by an increase in G_t by 22.9 ± 4.0 mS $\cdot$ cm^{-2} ($P < 0.05$ for both G_t and I_{sc}, $n = 5$). This I_{sc} decayed slowly, as reported earlier, and represents a cation current driven across basolateral K^+ channels and the basolateral Na^+-K^+-ATPase (Schultheiss & Diener, 1997). Carbachol was administered in the decaying phase of the nystatin response either after pretreatment with 10^{-2} mol $\cdot$ l^{-1} LiCl (pretreatment time: 20 min) or 10^{-2} mol $\cdot$ l^{-1} NaCl as an osmotic control. Under control conditions, i.e., in the absence of LiCl, carbachol ($5 \cdot 10^{-5}$ mol $\cdot$ l^{-1} at the serosal side) induced a fast increase in I_{sc}, which consisted of 2 steps: a fast transient first increase (1st peak) and a subsequent second increase (2nd peak) reaching a maximal value of 10.2 ± 1.5 µEq $\cdot$ h^{-1} $\cdot$ cm^{-2} above baseline (Fig. 2; for changes in G_t *see* Table 1). 10 min after carbachol administration (I_{10}) the I_{sc} had fallen to a value of 1.3 ± 0.7 µEq $\cdot$ h^{-1} $\cdot$ cm^{-2} above the former baseline. In the presence of LiCl (10^{-2} mol $\cdot$ l^{-1} on both sides; $n = 5$), both phases of the carbachol-induced I_{sc} across the basolateral membrane were strongly inhibited (Fig. 2, Table 1).

Table 1. Effect of LiCl on carbachol-induced K^+ currents and conductance across the basolateral membrane

	ΔI_{sc} (µEq $\cdot$ h^{-1} $\cdot$ cm^{-2})	
	−LiCl	+LiCl
1st Peak	2.3 ± 0.4*	0.9 ± 0.3*#
2nd Peak	10.2 ± 1.5*	0.9 ± 0.4
I_{10}	1.3 ± 0.7	−1.3 ± 0.3*#
ΔG_t (mS $\cdot$ cm^{-2})		
G_{max}	5.6 ± 2.4*	2.9 ± 3.0
G_{10}	2.6 ± 1.7	1.5 ± 5.3

Effect of carbachol ($5 \cdot 10^{-5}$ mol $\cdot$ l^{-1} at the serosal side) on K^+ currents across the basolateral membrane and on tissue conductance in the absence (−LiCl) or presence (+LiCl) of Li^+ (10^{-2} mol $\cdot$ l^{-1} at the mucosal and the serosal side). All tissues were permeabilized by nystatin (100 µg $\cdot$ ml^{-1} at the mucosal side) in the presence of a mucosal-to-serosal K^+ gradient (13.5 mmol $\cdot$ l^{-1} at the mucosal and 4.5 mmol $\cdot$ l^{-1} at the serosal side). Carbachol was administered in the decaying phase of the nystatin-induced I_{sc}. I_{10} or G_{10} were measured 10 min after administration of carbachol. In contrast to I_{sc}, G_t data were obtained only every minute (*see* Material and Methods). Therefore, fast changes in G_t could not be recorded. Values are given as difference to the baseline in the absence of carbachol (ΔI_{sc} or ΔG_t, respectively) and are mean ± SEM, $n = 5$ for both sets of experiments. *$P < 0.05$ versus I_{sc} prior to administration of carbachol, #$P < 0.05$ versus response in the absence of LiCl.

LiCl Inhibits the Increases of the Intracellular Ca^{2+} Concentration Induced by Carbachol

It is well accepted that the stimulation of basolateral K^+ channels by carbachol is caused by an increase in the intracellular Ca^{2+} concentration mediated initially by a release of Ca^{2+} from intracellular stores

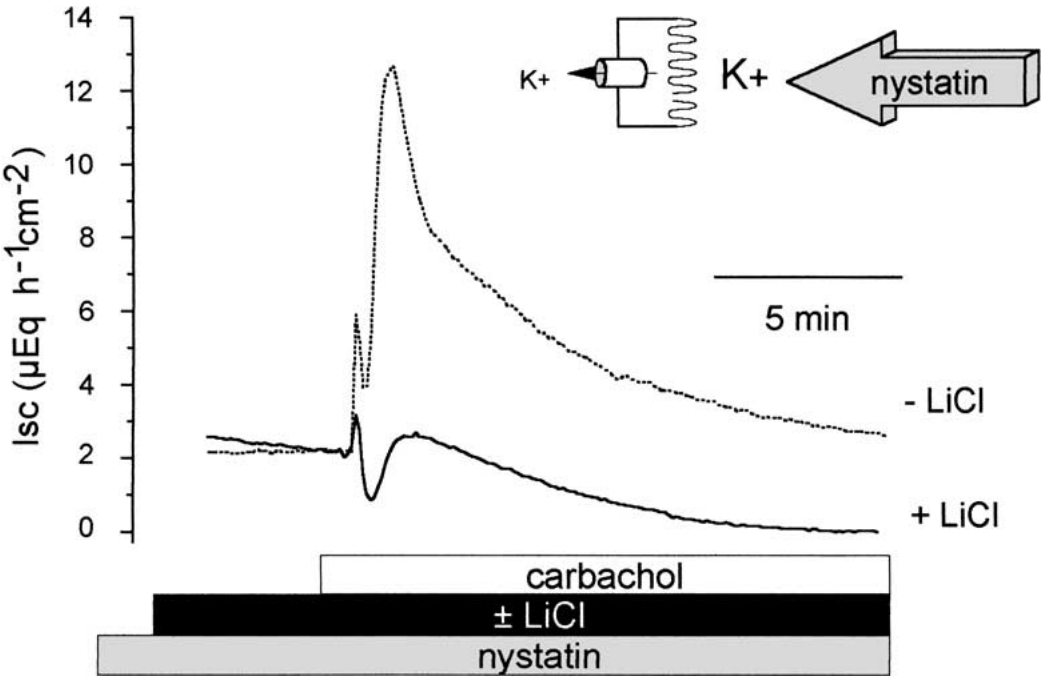

Fig. 2. Effect of carbachol ($5 \cdot 10^{-5}$ mol $\cdot$ l^{-1} at the serosal side; *white bar*) on I_{sc} across the basolateral membrane in the absence (−LiCl; *dashed line*) and presence (+ LiCl; *solid line*) of LiCl (10^{-2} mol $\cdot$ l^{-1} at the mucosal and serosal side; *black bar*). The apical membrane was permeabilized with nystatin (100 µg $\cdot$ ml^{-1} at the mucosal side; *gray bar*) and a mucosally to serosally directed K^+ gradient (13.5 mmol $\cdot$ l^{-1} and 4.5 mmol $\cdot$ l^{-1} in the mucosal and serosal bathing solution, respectively) was used to drive a K^+ current across the basolateral membrane (indicated in the schematic drawing). The figure shows a typical recording for $n = 5$; for statistics, *see* Table 1.

via IP_3 receptors. Therefore, we investigated whether LiCl interferes with the increase in the intracellular Ca^{2+} concentration evoked by carbachol in fura-2-loaded crypts. In the absence of LiCl, carbachol ($5 \cdot 10^{-5}$ mol $\cdot$ l^{-1}) caused an increase of the fura-2 fluorescence ratio. Within 3 min, the ratio had increased from a value of 1.6 ± 0.12 to 2.0 ± 0.14 ($P < 0.05$, $n = 29$). In the presence of LiCl (10^{-2} mol $\cdot$ l^{-1}; pretreatment time: 20 min), carbachol only induced a very small increase in the ratio from 1.03 ± 0.04 to 1.07 ± 0.05 ($P < 0.05$ versus response in the absence of LiCl, $n = 25$). These data confirm the general model of Ca^{2+}-dependent Cl^- secretion, in which carbachol induces an IP_3-mediated increase of the cytoplasmic Ca^{2+} concentration, leading to the activation of basolateral K^+ conductances, a hyperpolarization of the membrane and an increase in the driving force for Cl^- secretion.

Effect of Carbachol on the Apical Membrane

In order to study the necessity of IP_3 turnover for the activation of apical K^+ channels by carbachol, the technique of basolateral depolarization was chosen in which—by increasing the K^+ concentration of the serosal bathing solution from 4.5 to 111.5 mmol $\cdot$ l^{-1}—the basolateral membrane was electrically eliminated (as indicated by the schematic drawing in Fig. 3) (Fuchs, Larsen & Lindemann, 1977; Schultheiss & Diener, 1997). The chemical gradient for K^+ from the serosal to the mucosal side then drives a K^+ current across the apical membrane, which can be blocked by K^+ channel inhibitors applied at the mucosal compartment (Schultheiss & Diener, 1997). However, in contrast to the experiments with nystatin, the electrical resistance of the basolateral membrane is probably not abolished.

Exposure of the tissue to the K^+-rich buffer solution at the serosal side evoked a steep fall of I_{sc} from 2.7 ± 0.3 to −1.2 ± 0.3 µEq $\cdot$ h^{-1} $\cdot$ cm^{-2}, whereas G_t increased from 14.8 ± 1.3 to 21.4 ± 2 mS $\cdot$ cm^{-2} ($P < 0.05$, $n = 6$). Under these conditions, carbachol ($5 \cdot 10^{-5}$ mol $\cdot$ l^{-1} at the serosal side) induced a biphasic negative I_{sc} consisting of an early transient component (−1.5 ± 0.2 µEq $\cdot$ h^{-1} $\cdot$ cm^{-2}; $P < 0.05$ to baseline prior administration of carbachol, $n = 6$; Fig. 3. For G_t *see* Table 2) followed by a long-lasting 2nd phase (−0.4 ± 0.1 µEq $\cdot$ h^{-1} $\cdot$ cm^{-2}; $P <$ 0.05 versus baseline prior to administration of carbachol, $n = 6$; Fig. 3, Table 2). In the presence of LiCl (10^{-2} mol $\cdot$ l^{-1} at the mucosal and the serosal side; pretreatment time: 20 min; for the effect of LiCl and all other inhibitors used on basal I_{sc} and G_t, *see* Table 3), the carbachol-induced current during the 1st phase was unaffected, whereas the 2nd phase of the current response was suppressed (Fig. 3, Table 2).

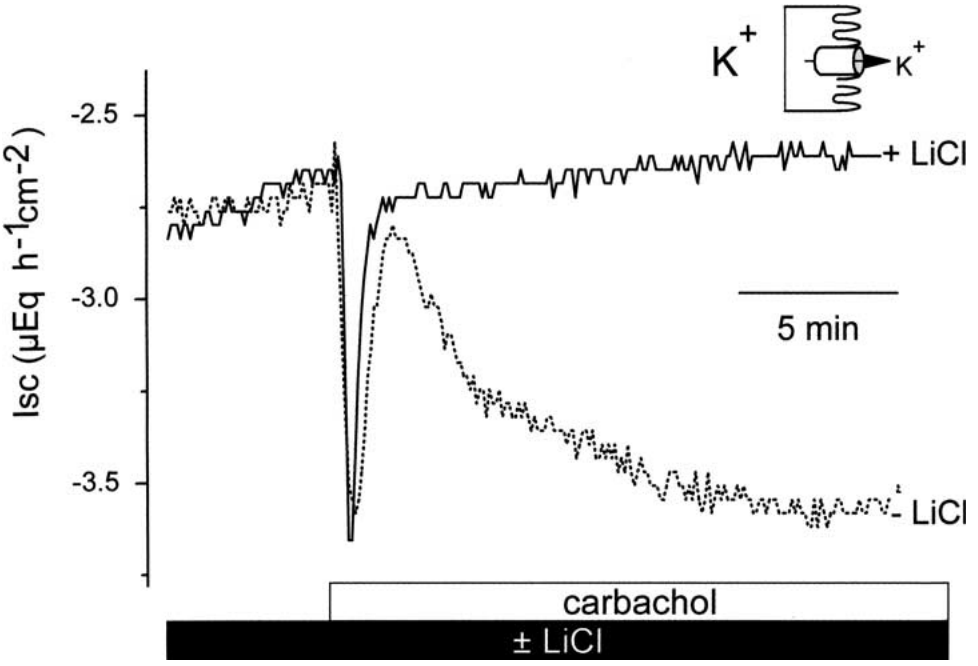

Fig. 3. Effect of carbachol ($5 \cdot 10^{-5}$ mol $\cdot$ l^{-1} at the serosal side; *white bar*) on I_{sc} across the apical membrane in the absence (−LiCl; *dashed line*) and presence (+LiCl; *solid line*) of LiCl (10^{-2} mol $\cdot$ l^{-1} at the mucosal and serosal side; *black bar*). The basolateral membrane was depolarized by a serosal bathing solution containing a high K^+ concentration (111.5 mmol $\cdot$ l^{-1} KCl, as indicated by the schematic inset). Typical tracing for $n = 6$; for statistics, *see* Table 2.

Table 2. Effect of LiCl on carbachol-induced K^+ currents and conductance across the apical membrane

	−LiCl		+LiCl	
	ΔI_{sc} (µEq $\cdot$ h^{-1} $\cdot$ cm^{-2})	ΔG_t (mS $\cdot$ cm^{-2})	ΔI_{sc} (µEq $\cdot$ h^{-1} $\cdot$ cm^{-2})	ΔG_t (mS $\cdot$ cm^{-2})
1st Phase	−1.5 ± 0.2*	−0.5 ± 0.3	−1.3 ± 0.3*	0.5 ± 0.3
2nd Phase	−0.4 ± 0.1*	5.3 ± 1.0*	−0.1 ± 0.0*#	3.5 ± 1.6

Effect of carbachol ($5 \cdot 10^{-5}$ mol $\cdot$ l^{-1} at the serosal side) on K^+-currents across the apical membrane and conductance (G_t) in the absence (−LiCl) or presence (+LiCl) of Li^+ (10^{-2} mol $\cdot$ l^{-1} at the mucosal and the serosal side). Tissues were depolarized by serosal bathing solution containing 111.5 mmol $\cdot$ l^{-1} KCl. The first phase of the carbachol response was measured at the maximum of the negative I_{sc} induced by carbachol (with the corresponding G_t at the same time); the second phase was measured 10 min after administration of carbachol (I_{10} or G_{10}, respectively). Values are given as difference to the baseline in absence of carbachol (ΔI_{sc} or ΔG_t, respectively) and are means ± SEM, $n = 6$ for both sets of experiments. *$P < 0.05$ versus I_{sc} prior to administration of carbachol, #$P < 0.05$ versus response in the absence of LiCl.

Effect of K^+-Channel Blockers on the Carbachol-induced Current Across the Apical Membrane

In the subsequently described experiments the protocol of a basolateral depolarization (*see* above) was chosen in order to investigate the interference of putative inhibitors with the I_{sc} induced by carbachol ($5 \cdot 10^{-5}$ mol $\cdot$ l^{-1} at the serosal side) across the apical membrane. The 1st phase of the carbachol-induced current is carried by an ion movement across apical K^+ conductances, as demonstrated by the inhibitory effect of different K^+-channel blockers. In the presence of charybdotoxin ($2 \cdot 10^{-7}$ mol $\cdot$ l^{-1} at the mucosal side), a scorpion toxin, which selectively blocks Ca^{2+}-dependent K^+ channels (*see* e.g., Cook & Quast, 1990), the 1st phase of the carbachol-induced current was reduced by more than 50% (Fig. 4, Table 4), whereas the second, long-lasting phase was unaffected. Similar results were obtained by tetraethylammonium ($5 \cdot 10^{-3}$ mol $\cdot$ l^{-1} at the mucosal side, Table 4). These data are in accordance with the assumption that carbachol activates apical Ca^{2+}-dependent K^+-channels.

Effect of Signal Transduction Blockers on Carbachol-induced Apical Current

The partial resistance against LiCl suggests that at least the fast transient activation of a K^+ conductance during the 1st phase of the carbachol response is not mediated by the IP_3 pathway. Indeed, inhibition

Table 3. Effects of putative inhibitors on I_{sc} and G_t in basolaterally depolarized tissues

	ΔI_{sc} (μEq $\cdot$ h^{-1} $\cdot$ cm^{-2}	ΔG_t (mS $\cdot$ cm^{-2})	n
Charybdotoxin	0.3 ± 0.1*	−0.5 ± 0.5	7
Dichlorobenzamil	0.2 ± 0.1*	−0.8 ± 0.5	7
Gd^{3+}	0.3 ± 0.1*	−0.2 ± 0.6	7
Indometacin	0.1 ± 0.1	−2.2 ± 1.2	6
La^{3+}	0.2 ± 0.1*	−0.3 ± 0.3	6
LiCl	−0.2 ± 0.3*	−2.0 ± 1.1	6
L-NNA	0.3 ± 0.1*	−0.4 ± 0.5	8
Propranolol	−0.6 ± 0.3*	4.1 ± 0.4*	6
Ruthenium red	0.3 ± 0.1	−1.8 ± 1.1	6
Ryanodine	−0.1 ± 0.1	1.4 ± 0.7	7
Staurosporine	0.0 ± 0.0	0.1 ± 0.5	6
TEA	0.6 ± 0.2*	−0.4 ± 0.7	6
U-73122	−0.1 ± 0.1	−0.2 ± 0.1	8

Effect of putative inhibitors on I_{sc} and G_t in basolaterally depolarized tissues (serosal bathing solution containing 111.5 mmol $\cdot$ l^{-1} KCl). Experiments with lanthanides were performed in a HEPES-buffered, HCO_3^--free solution (*see* Materials and Methods). Values are given as difference to the baseline just prior administration of the inhibitor (ΔI_{sc} or ΔG_t, respectively) and are means ± SEM, $*P < 0.05$ versus I_{sc}/G_t prior to administration of the drug. Concentrations of the inhibitors were charybdotoxin ($2 \cdot 10^{-7}$ mol $\cdot$ l^{-1} at the mucosal side), dichlorobenzamil (10^{-4} mol $\cdot$ l^{-1} at the mucosal side), gadolinium (Gd^{3+}, 10^{-3} mol $\cdot$ l^{-1} at the mucosal side), indomethacin (10^{-6} mol $\cdot$ l^{-1} at the serosal side), lanthanum (La^{3+}, 10^{-3} mol $\cdot$ l^{-1} at the mucosal side), LiCl (10^{-2} mol $\cdot$ l^{-1} at the mucosal and serosal side), N-Ω-Nitro-L-Arginine (L-NNA, 10^{-3} mol $\cdot$ l^{-1} at the mucosal and serosal side), propranolol (10^{-4} mol $\cdot$ l^{-1} at the mucosal and serosal side), ruthenium red ($5 \cdot 10^{-4}$ mol $\cdot$ l^{-1} at the mucosal and the serosal side), ryanodine (10^{-3} mol $\cdot$ l^{-1} at the mucosal and serosal side), staurosporine (10^{-6} mol $\cdot$ l^{-1} at the serosal side), tetraethylammonium (TEA, $5 \cdot 10^{-3}$ mol $\cdot$ l^{-1} at the mucosal side), U-73122A (10^{-5} mol $\cdot$ l^{-1} at the serosal side).

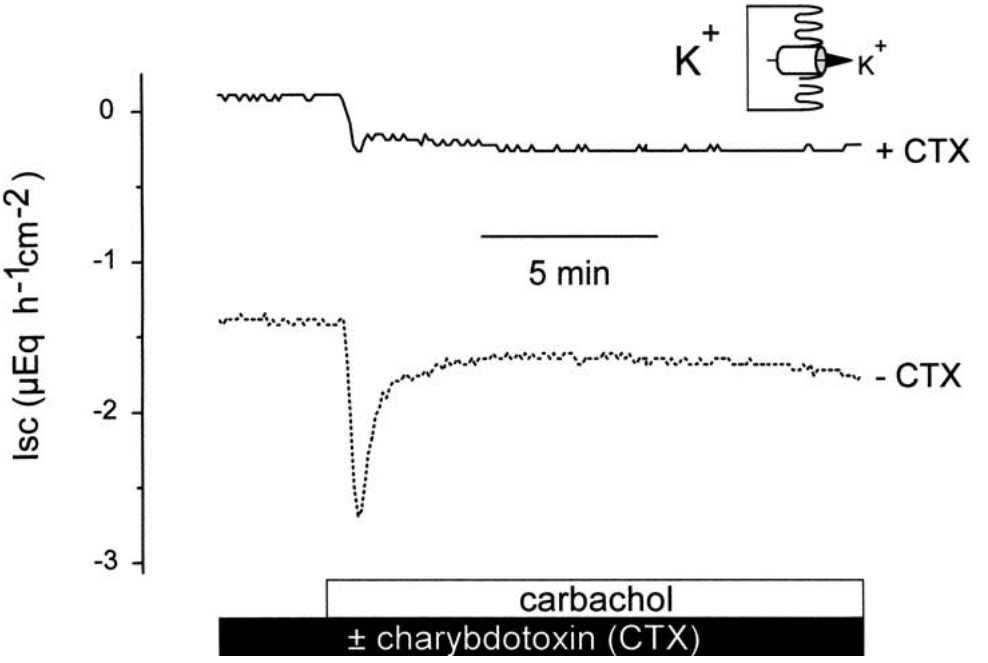

Fig. 4. Effect of carbachol ($5 \cdot 10^{-5}$ mol $\cdot$ l^{-1} at the serosal side; *white bar*) on I_{sc} across the apical membrane in the absence (−CTX; *dashed line*) and presence (+CTX; *solid line*) of charybdotoxin ($2 \cdot 10^{-7}$ mol $\cdot$ l^{-1} at the mucosal side; *black bar*). The basolateral membrane was depolarized by a serosal bathing solution containing a high K^+ concentration (111.5 mmol $\cdot$ l^{-1} KCl, as indicated by the schematic inset). Typical recording for $n = 6$; for statistics, *see* Table 4.

of phospholipase C by U-73122 (10^{-5} mol $\cdot$ l^{-1} at the serosal side) (Taylor & Broad, 1998) did not affect the 1st phase of carbachol-induced apical K^+ current, whereas the long-lasting second phase was completely suppressed, as one should expect (Table 4).

Binding of carbachol at muscarinic receptors has been reported to activate a phospholipase D in some cell types (Mamoon et al., 1999), which, via the subsequent involvement of a phosphatidic acid phosphatase, leads to the production of diacylgly-

Table 4. Effect of putative inhibitors on carbachol-induced K^+-currents across the apical membrane

Inhibitor	ΔI_{sc} ($\mu Eq \cdot h^{-1} \cdot cm^{-2}$				
	Carbachol without inhibitor		Carbachol with inhibitor		
	1st phase	2nd phase	1st phase	2nd phase	
Charybdotoxin	−0.9 ± 0.2*	−0.3 ± 0.1*	−0.4 ± 0.1*#	−0.3 ± 0.1*	7–8
Dichlorobenzamil	−1.1 ± 0.2*	−0.3 ± 0.1	−0.6 ± 0.1#	−0.2 ± 0.1	7–8
Gd^{3+}	−1.2 ± 0.1*	0.2 ± 0.1	−1.3 ± 0.2*	−0.1 ± 0.1	6–7
Indometacin	−1.6 ± 0.2*	−0.3 ± 0.1*	−1.6 ± 0.1*	−0.3 ± 0.1*	6
La^{3+}	−0.8 ± 0.1*	0.0 ± 0.2	−0.5 ± 0.2*	−0.1 ± 0.1	6
L-NNA	−1.3 ± 0.1*	−0.6 ± 0.1*	−1.2 ± 0.1*	−0.6 ± 0.1*	8
Propranolol	−0.8 ± 0.1*	−0.4 ± 0.1*	−1.3 ± 0.1*#	−0.7 ± 0.1*#	6
Ruthenium red	−1.0 ± 0.2*	−0.1 ± 0.1	−1.3 ± 0.1*	0.0 ± 0.2	5–6
Ryanodine	−1.0 ± 0.2*	−0.5 ± 0.1*	−1.5 ± 0.2*#	−0.5 ± 0.1*	7
Staurosporine	−1.2 ± 0.2*	−0.4 ± 0.1*	−1.4 ± 0.2*	−0.2 ± 0.0*#	5–6
TEA	−1.4 ± 0.3*	−0.3 ± 0.2	−0.6 ± 0.3*#	−0.3 ± 0.2	6–8
U-73122	−0.8 ± 0.2*	−0.2 ± 0.2	−1.2 ± 0.2*	−0.0 ± 0.2	6–8

Effect of putative inhibitors on the I_{sc} induced by carbachol ($5 \cdot 10^{-5}$ mol $\cdot$ l^{-1} at the serosal side) in basolaterally depolarized tissues (serosal bathing solution containing 111.5 mmol $\cdot$ l^{-1} KCl). Experiments with lanthanides were performed in a HEPES-buffered, HCO_3^--free solution (*see* Materials and Methods). In the control experiments, only the solvent of the inhibitor to be tested was administered. The 1st phase of the carbachol response was measured at the early maximum of the negative I_{sc} induced by the cholinergic agonist; the 2nd phase was measured 10 min after administration (I_{10}) of carbachol. For concentrations of the inhibitors, *see* Table 3. Values are given as difference to the baseline just prior administration of carbachol (ΔI_{sc}) and are means ± SEM; *$P < 0.05$ versus baseline I_{sc} prior to administration of carbachol, #$P < 0.05$ versus same phase in the absence of the putative inhibitor.

cerol, a well-known activator of conventional and novel types of protein kinase C. However, propranolol (10^{-4} mol $\cdot$ l^{-1} at the mucosal and the serosal side), which in high concentrations is known to inhibit phosphatidic acid phosphatase (Oprins et al., 2001), failed to inhibit the carbachol-induced I_{sc} across the apical membrane and even enhanced this current (Table 4). Both the phospholipase C as well as the phospholipase D pathway finally lead to a stimulation of protein kinase C via production of diacylglycerol. In order to block this kinase, staurosporine (10^{-6} mol $\cdot$ l^{-1} at the serosal side), a non-selective protein kinase inhibitor (Tamaoki et al., 1986), was used. In accordance with the results obtained with U-73122, this blocker inhibited the 2nd phase of the carbachol-induced current significantly, but left the 1st phase unaffected (Table 4). Taken together, these data suggest that neither phospholipases nor protein kinases are involved in the fast transient activation of apical K^+ conductances by carbachol, whereas the long-lasting activation is mediated by the classical IP_3/diacylglycerol pathway.

Several other signal transduction mechanisms have been reported in muscarinic receptor-evoked intestinal secretion, such as ryanodine receptors (Kocks, Schultheiss & Diener, 2002), NO produced by NO-synthases (Bischof et al., 1995), or prostaglandins, whose synthesis is stimulated by carbachol (Craven & DeRubertis, 1981). However, neither inhibitors of ryanodine receptors, such as ruthenium red ($5 \cdot 10^{-4}$ mol $\cdot$ l^{-1} at the mucosal and the serosal side; Franzini-Armstrong & Protrasi, 1997) or ryanodine in a high concentration (Kocks, Schultheiss & Diener, 2002), nor an inhibitor of NO-synthases, N-Ω-nitro-L-arginine (L-NNA; 10^{-3} mol $\cdot$ l^{-1} at the mucosal and the serosal side), nor the inhibitor of cyclooxygenases, indomethacin (10^{-6} mol $\cdot$ l^{-1} at the serosal side), had any inhibitory effect on the carbachol-induced apical K^+ current (Table 4).

Origin of Ca^{2+} Activating the Transient Ca^{2+}-dependent Apical K^+ Conductances

All data presented above suggest that none of the common regulatory pathways is capable of stimulating the fast transient charybdotoxin-sensitive K^+ conductance in the apical membrane. To confirm the significance of Ca^{2+} for activation of the apical K^+ conductances, studies with Ca^{2+}-free serosal bathing solutions were performed. Depolarization of the basolateral membrane in the absence of serosal Ca^{2+} resulted in a decrease in I_{sc} from 1.6 ± 0.4 to −0.6 ± 0.2 $\mu Eq \cdot h^{-1} \cdot cm^{-2}$ ($P < 0.05$, $n = 7$). Unexpectedly, this response was concomitant with a slight decrease in G_t from 18.5 ± 3.2 to 16.4 ± 2.0 mS $\cdot$ cm^{-2} ($P > 0.05$). In the absence of serosal Ca^{2+}, the 2nd phase of the negative I_{sc} induced by carbachol ($5 \cdot 10^{-5}$ mol $\cdot$ l^{-1} at the serosal side) was abolished, whereas the 1st phase was unaffected (for I_{sc} and G_t data, *see* Table 5). Subsequent administration of $CaCl_2$ (10^{-3} mol $\cdot$ l^{-1} at the serosal side) induced a further negative I_{sc} of −0.2 ± 0.0 $\mu Eq \cdot h^{-1} \cdot cm^{-2}$ ($P < 0.05$ versus current prior administration of $CaCl_2$). The G_t

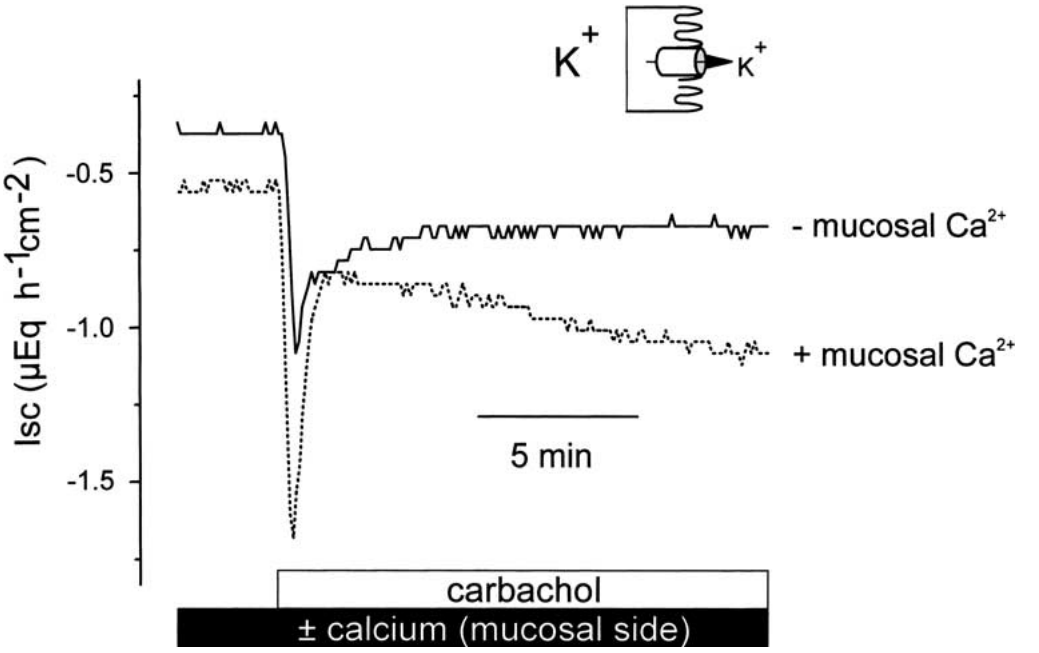

Fig. 5. Effect of carbachol ($5 \cdot 10^{-5}$ mol $\cdot$ l^{-1} at the serosal side; *white bar*) on I_{sc} across the apical membrane in the presence (*dashed line*) and absence (*solid line*) of mucosal Ca^{2+} ($1.25 \cdot 10^{-3}$ mol $\cdot$ l^{-1} at the mucosal side; *black bar*). The basolateral membrane was depolarized by a serosal bathing solution containing a high K^+ concentration ($111.5 \cdot$ mmol $\cdot$ l^{-1} KCl, as indicated by the schematic inset). Typical recording for $n = 6$–7; for statistics, *see* Table 5.

changed at the same time by 0.1 ± 0.1 mS $\cdot$ cm^{-2} ($P > 0.05$ versus conductance prior administration of $CaCl_2$).

The same experiment was repeated in the absence of mucosal Ca^{2+}. Depolarizing the basolateral membrane in the absence of mucosal Ca^{2+} yielded a decrease in I_{sc} by -2.0 ± 0.4 µEq $\cdot$ h^{-1} $\cdot$ cm^{-2}; simultaneously G_t increased from 12.7 ± 1.4 mS $\cdot$ cm^{-2} to 16.6 ± 1.5 mS $\cdot$ cm^{-2} ($P < 0.05$, $n = 6$). This increase in G_t by 4.0 ± 1.2 mS $\cdot$ cm^{-2} was not statistically different from the increase in G_t evoked by basolateral depolarization in a series of control experiments performed in parallel, in which G_t increased by 3.4 ± 1.7 mS $\cdot$ cm^{-2} ($n = 6$).

In the absence of mucosal Ca^{2+} the response to the muscarinic agonist ($5 \cdot 10^{-5}$ mol $\cdot$ l^{-1} at the serosal side) during the 1st phase was reduced significantly, whereas the second, long-lasting phase remained unaffected (Fig. 5; for I_{sc} and G_t *see* Table 5). These results suggest a role of mucosal Ca^{2+} for stimulating the apical K^+ conductance.

Involvement of an Apical Na^+/Ca^{2+} Exchanger in Carbachol Stimulation of Apical K^+ Conductance

The next set of experiments served to characterize the pathway by which Ca^{2+} enters the cell from the mucosal side. Since the mRNA for an apical Ca^{2+} channel (CaT1 or ECaC2, renamed now as TRPV5) was found in the rat colonic epithelium (Peng et al., 1999), inhibitors of this channel, such as ruthenium red (Nilius et al., 2001) or the lanthanides La^{3+} and Gd^{3+} (Peng et al., 1999) were used. Experiments with lanthanides were performed in a HEPES-buffered, HCO_3^--free solution in order to avoid precipitation as CO_3^{2-} salts (*see* Materials and Methods). However, none of these inhibitors affected the activation of carbachol-induced K^+ currents across the apical membrane (Table 4).

An alternative pathway for Ca^{2+} entering the cell could be a Na^+/Ca^{2+} exchanger working under Ca^{2+}-influx conditions, as described for the heart during the contraction period (Blaustein & Lederer, 1999). Dichlorobenzamil (10^{-4} mol $\cdot$ l^{-1} at the mucosal side), a blocker of Na^+/Ca^{2+} exchangers (Kaczorowski et al., 1985), induced an increase in I_{sc}, which, however, did not reach statistical significance (for effects of the inhibitor on baseline I_{sc} and G_t *see* Table 3). In the presence of this inhibitor, the 1st phase of the carbachol-induced I_{sc} across the apical membrane was reduced to nearly half the value of an untreated control, whereas the 2nd phase remained unaffected (Table 4).

These functional data were confirmed by immunostaining experiments, in which a polyclonal antibody against the cardiac form of the exchanger (NCX1) was used (Fig. 6). These experiments revealed the presence of the Na^+/Ca^{2+} exchanger within the apical region of the colonic enterocytes, since the immunoreaction was concentrated around the lumen of the crypts.

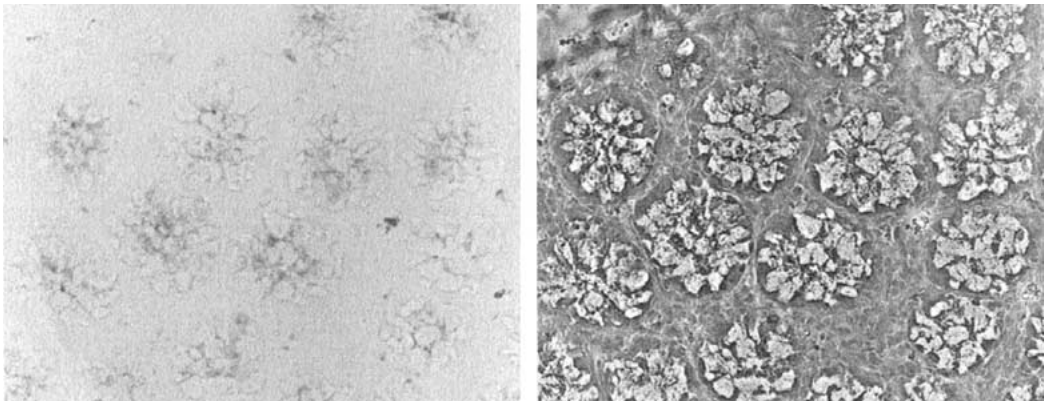

Fig. 6. Immunohistochemical visualization of the sodium-calcium exchanger within the apical region of the colonic enterocytes (*left*). The immunoreaction is concentrated around the lumen of the crypts, which is demonstrated by phase-contrast microscopy (*right*). 300×

Is Depolarization of the Apical Membrane the Cause for the Reversal of the Apical Na^+/Ca^{2+} Exchanger?

If an apical Na^+/Ca^{2+} exchanger is responsible for the transient influx of Ca^{2+} across the apical membrane, the driving force for this transporter, which usually works in the Ca^{2+}-efflux mode, has to be altered (Blaustein & Lederer, 1999). One possibility for this might be a depolarization of the membrane, e.g., via stimulation of Ca^{2+}-dependent Cl^- channels, as they have been observed in bronchial epithelium or intestine of the pig (Gaspar et al., 2000). In order to identify a Ca^{2+}-dependent apical Cl^- conductance, the basolateral membrane was depolarized (107 mmol $\cdot$ l^{-1} KCl solution at the serosal side) and the solution facing the apical membrane was exchanged against a K-gluconate-rich solution (107 mmol $\cdot$ l^{-1} KGluc buffer at the mucosal side). Thus, the basolateral membrane was bypassed due to the depolarization; simultaneously, a chemical K^+ gradient at the apical membrane was abolished by the high K^+ concentration on the apical side. Therefore, the only ion gradient present at the apical membrane was a Cl^- gradient, and all changes in I_{sc} induced by carbachol under these conditions should reflect changes in the apical Cl^{-1} conductance. Indeed, under these conditions carbachol ($5 \cdot 10^{-5}$ mol $\cdot$ l^{-1} at the serosal side) induced a transient increase in I_{sc} of 0.4 $\pm$ 0.1 μEq $\cdot$ h^{-1} $\cdot$ cm^{-2}, paralleled by an increase in G_t by 0.6 $\pm$ 0.2 mS $\cdot$ cm^{-2} ($P < 0.05$ for both versus the corresponding value prior administration of carbachol, $n = 10$, *see* Fig. 7). This increase was followed by a strong, long-lasting decrease of I_{sc} by -1.0 ± 0.1 μEq $\cdot$ h^{-1} $\cdot$ cm^{-2} below the baseline prior to the administration of the agonist ($P < 0.05$ versus current prior to administration) and of G_t by -0.7 ± 0.5 mS $\cdot$ cm^{-2} ($P > 0.05$). Thus, carbachol is able to transiently activate an apical Cl^- channel, a response that is followed by a long-lasting inhibition of the dominant apical Cl^- conductance, i.e., of the CFTR channels, as described earlier (Schultheiss, Ribeiro & Diener, 2001)[2].

Discussion

The results of this study show that carbachol induces a biphasic increase in the K^+-conductance of both the apical and the basolateral membrane. Preincubation with LiCl strongly inhibits the activation of K^+ conductances during the late phase of the carbachol response in both membranes. However, the sensitivity against preincubation with LiCl of the fast K^+ conductance activated by carbachol differs in both membranes: LiCl inhibits this K^+ current across the basolateral membrane by more than 60% (Fig. 2, Table 1), but leaves the carbachol-stimulated K^+ current across the apical membrane unaffected (Fig. 3, Table 2). Inhibition of the carbachol response by LiCl is in accordance with the general model, which states that carbachol, after binding to muscarinic receptors, activates a phospholipase C. The enhanced production of IP_3 induces an increase of the cytoplasmic Ca^{2+} concentration. LiCl is a blocker of the enzyme myo-inositol-1-phosphatase (Berridge & Irvine, 1989; Jenkinson, Nahorski & Challiss, 1994; Wolfson et al. 1998) and thereby causes an inhibition of the regeneration of myo-inositol, a precursor for the production of phosphatidylinositols (Shears, 1998). Consequently, it is not astonishing that preincubation with LiCl nearly suppressed the stimulation of the basolateral K^+ conductances (Fig. 2) and the increase in the intracellular Ca^{2+} concentration induced by carbachol. This implies, however, that the spontaneous turnover of IP_3 at the rat distal colon must be quite high (so that the 20-min preincubation

[2]The inhibition of the apical Cl^- channels is in part due to the liberation of fatty acids after activation of Ca^{2+}-dependent phospholipase(s) A_2 by carbachol.

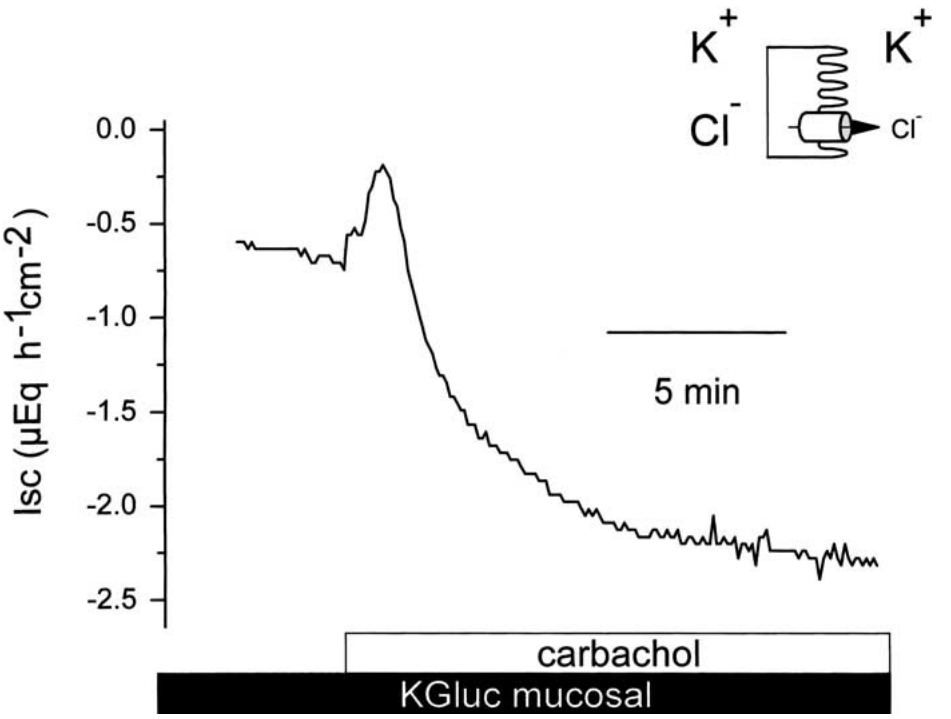

Fig. 7. Effect of carbachol ($5 \cdot 10^{-5}$ mol $\cdot$ l^{-1} at the serosal side; *white bar*) on Cl^- current across the apical membrane. The tissue was incubated with a KCl buffer (111.5 mmol $\cdot$ l^{-1} KCl) at the serosal and a K gluconate buffer (111.5 mmol $\cdot$ l^{-1} KGluc; *black bar*) at the mucosal side in order to drive a Cl^- current across the apical membrane (as indicated by the schematic inset). Typical recording for $n = 10$; for statistics, *see* text.

with LiCl can already reduce the available precursors for IP_3 production).

To our surprise, the carbachol effect at the apical membrane showed a different behavior. Under conditions in which the basolateral membrane was electrically eliminated by a high concentration of KCl in the bathing solution (Fuchs, Larsen & Lindemann, 1977), carbachol stimulates K^+ currents across the apical membrane, which can be inhibited by mucosally applied K^+-channel blockers (Schultheiss & Diener, 1997), leading to an enhanced K^+efflux, as demonstrated by experiments with $^{86}Rb^+$ (Heinke, Hörger & Diener, 1998). As it was observed for the basolateral membrane, there are (at least) 2 different K^+ conductances present on the apical side: one with a fast activating kinetics, which is LiCl resistant, and the second with a slower kinetics being LiCl-sensitive.

The slow apical carbachol-activated K^+ conductance seems to be activated via the 'classical' Ca^{2+}-signaling pathway. Its activation was prevented by U-73122 (Table 4), an inhibitor of phospholipase C (Taylor & Broad, 1998), staurosporine (Table 4), a nonspecific inhibitor of protein kinases (Tamaoki et al., 1986), or Ca^{2+} removal from the serosal solution (Table 5).

In contrast, the activation of the fast carbachol-induced apical K^+ current proved to be resistant against the blockade of inositol-1,4,5-trisphosphate turnover by LiCl (Fig. 3), inhibition of phospholipase C, or blockade of protein kinases (Table 4), suggesting that this response is not mediated by the 'classical' phospholipase C/IP_3 pathway. These observations confirm earlier efflux experiments, in which the protein kinase inhibitor staurosporine was unable to inhibit the early response in carbachol-induced apical Rb^+ efflux, but suppressed the late phase of the response completely (Heinke, Ribeiro & Diener, 1999). Also the alternative production of diacylglycerol by phospholipase D, which has been shown to lead to the production of the protein kinase C activator diacylglycerol in some epithelial cells (Oprins et al., 2001), is obviously not involved, as shown by the resistance against a high concentration of propranolol (Table 4).

The data obtained with K^+-channel blockers suggested, however, that also during the early phase of carbachol stimulation of apical K^+ conductance Ca^{2+}-dependent K^+ channels are involved (Fig. 4). Charybdotoxin, a specific inhibitor of Ca^{2+}-dependent K^+ channels (Cook & Quast, 1990), suppressed this conductance nearly completely (Table 4). Where do the Ca^{2+} ions necessary for the activation of these channels come from? Obviously, the presence of serosal Ca^{2+} is not a prerequisite for the early effect of carbachol on the apical membrane, although in the absence of this cation the late carbachol-induced apical K^+ current is abolished (Table 5). In contrast, this early K^+ current proved to be sensitive to

Table 5. Dependence of the carbachol-induced K^+ currents currents across the apical membrane on the presence of Ca^{2+} in the bathing solution

	Carbachol with Ca^{2+} ΔI_{sc} ($\mu Eq \cdot h^{-1} \cdot cm^{-2}$)		Carbachol with Ca^{2+} ΔI_{sc} ($\mu Eq \cdot h^{-1} \cdot cm^{-2}$)		n
	1st phase	2nd phase	1st phase	2nd phase	
Serosal Ca^{2+}-free	−1.0 ± 0.2*	−0.5 ± 0.1*	−1.3 ± 0.2*	−0.1 ± 0.1#	1
Mucosal Ca^{2+}-free	−1.1 ± 0.1*	−0.5 ± 0.2*	−0.8 ± 0.1*#	−0.5 ± 0.1*	6–7
ΔG_t ($mS \cdot cm^{-2}$)					
	1st phase	2nd phase	1st phase	2nd phase	
Serosal Ca^{2+}-free	−0.1 ± 0.2	−3.1 ± 0.8*	−0.2 ± 0.2	0.1 ± 0.7#	7
Mucosal Ca^{2+}-free	0.8 ± 0.6	2.2 ± 0.7*	1.9 ± 1.0	0.4 ± 0.5	6–7

Effect of Ca^{2+} omission on changes of I_{sc} and G_t induced by carbachol ($5 \cdot 10^{-5}$ mol $\cdot$ l^{-1} at the serosal side) in basolaterally depolarized tissues (serosal bathing solution containing 111.5 mmol $\cdot$ l^{-1} KCl, effect indicated at the first columne). The first phase of the carbachol response was measured at the early maximal decrease in I_{sc}, the second phase, 10 min after administration (I_{10} or G_{10}, respectively) of carbachol. Ca^{2+} was omitted either from the serosal or the mucosal bathing solution; the response was compared with a group of tissues, which were bathed with a solution containing Ca^{2+} ($1.25 \cdot 10^{-3}$ mol $\cdot$ l^{-1}) at the mucosal and the serosal side of the tissue. Values are given as difference to the baseline just prior to administration of carbachol (ΔI_{sc} or ΔG_{10}, respectively) and are means ± SEM; *$P < 0.05$ versus I_{sc} or G_{10} prior to administration of carbachol, #$P < 0.05$ versus same phase in the presence of Ca^{2+} ($1.25 \cdot 10^{-3}$ mol $\cdot$ l^{-1}). n = 6–7.

removal of mucosal Ca^{2+} (Fig. 5, Table 5). The modest increase in tissue conductance under these conditions (*see* Results) confirms the integrity of the tight junctions under these experimental conditions. Consequently, it seems reasonable to conclude that the charybdotoxin-sensitive apical K^+ channels are stimulated by carbachol due to an influx of Ca^{2+} ions across the apical membrane, which probably leads to a local increase in the intracellular Ca^{2+} concentration at this membrane, probably too small to be recognized in our conventional fura-2 experiments.

Several transporters might be responsible for mediation of this Ca^{2+} influx. One possibility is Ca^{2+}-permeable channels of the TRPV family, i.e., TRPV5 (formerly named ECaC1 or CaT2) or TRPV6 (formerly named ECaC2 or CaT1; for review *see* Vennekes et al., 2002). However, neither lanthanides, such as La^{3+} or Gd^{3+}, nor ruthenium red, which all act as potent blockers of these channels (Peng et al., 1999; Nilius et al., 2001), had any significant effect on the carbachol-induced K^+ current across the apical membrane (Table 4). As the lanthanides are also effective inhibitors of the non-selective cation channel activated after store depletion in rat colon (Frings, Schultheiss & Diener, 1999), these results exclude the involvement of such a non-selective cation conductance in the mediation of the carbachol response. In contrast, inhibitor experiments with dichlorobenzamil, an inhibitor of the Na^+/Ca^{2+} exchanger (Kaczorowski et al., 1985; Seip et al., 2001), suggest a role of this antiporter for Ca^{2+} loading across the apical membrane (Table 5). This exchanger contributes to the increase in the intracellular Ca^{2+} concentration after store depletion, because the non-selective cation channels activated by store depletion mediate an influx of Na^+, which reduces the driving force for Ca^{2+} exit via the Na^+/Ca^{2+} exchanger (Seip et al., 2001). A similar situation is known from the heart (Blaustein & Lederer, 1999). Immunohistochemical staining confirmed the presence of this exchanger in the apical membrane of rat colonic epithelial cells (Fig. 6).

Under normal conditions, this exchanger is thought to work in the Ca^{2+}-extruding mode (Blaustein & Lederer, 1999). A reversal into the Ca^{2+}-loading mode is only possible after changing the driving forces, e.g., removal of extracellular Na^+ in order to revert the Na^+ gradient at the membrane (*see*, e.g., Seip et al., 2001). However, a significant Na^+ influx across the apical membrane is quite improbable, because the rat colon does not express epithelial Na^+ channels, when the rats are kept under standard diet conditions (Binder & Sandle, 1994). An influx of Na^+ across the basolateral membrane after basolateral depolarization can also probably be excluded, because the intracellular Na^+ concentration is very likely higher than the Na^+ concentration in the serosal depolarization solution (*see* also Materials and Methods). An alternative way to revert the mode of this electrogenic Na^+/Ca^{2+} exchanger, which exchanges 3 Na^+ ions against 1 Ca^{2+} ion, might be a depolarization of the apical membrane induced by carbachol. In airway epithelium, a Ca^{2+}-dependent apical Cl^- conductance has been identified (*see*, e.g., Anderson & Welsh, 1991); the underlying ClCa channel has also been found in porcine small intestine (Gaspar et al., 2000), although its functional employment in transepithelial Cl^--secretion is questionable (Strabel & Diener, 1995). In order to find out whether the activation of such a Ca^{2+}-dependent apical Cl^- conductance could be responsible for a transient depolarization of the apical membrane, which then might revert the driving force for the apical Na^+/Ca^{2+} exchanger, tissues were basolaterally depolarized under conditions in which only a Cl^- gradient was present at the apical membrane. To this

purpose, Na^+ in the apical bathing solution was equimolarly replaced by K^+ for eliminating the K^+ gradient, and Cl^- in the same solution was equimolarly substituted by gluconate to obtain a chemical Cl^--gradient from the serosal to the mucosal side. Indeed, in these experiments carbachol induced a transient increase in (Cl^-) current across the apical membrane (Fig. 7). The transient nature of this response is in accordance with the typical behavior of the ClCa channels in airway epithelium, where these channels are rapidly downregulated by a metabolite of IP_3, the inositol-3,4,5,6-tetrakisphosphate (Vajanaphanich et al., 1994). This rapid downregulation is most probably also responsible for the fact that this Ca^{2+}-dependent Cl^- conductance has been overlooked previously, because it will be rapidly covered by the dominant increase in the Ca^{2+}-dependent K^+ conductance stimulated by carbachol (Böhme, Diener & Rummel, 1989; Strabel & Diener, 1995). The transient activation of an apical Cl^- conductance by carbachol is followed by a long-lasting inhibition (as indicated by a fall of the I_{sc} below the value prior to administration of carbachol; Fig. 7). This inhibition of the spontaneously open, cAMP-dependent Cl^- conductance, which is mediated by CFTR channels, has already been described earlier (Schultheiss, Ribeiro & Diener, 2001).

Taken together these data demonstrate that carbachol activates several types of Ca^{2+}-dependent K^+ conductances in the apical and the basolateral membrane. The stimulation of the basolateral K^+ channels can be inhibited by blockade of IP_3 turnover with LiCl; this conductance maintains the major driving force for transepithelial Cl^- secretion. On the apical side a transient activation of a Ca^{2+}-dependent K^+ conductance seems to be mediated by the reversal of the action mode of an apically localized Na^+/Ca^{2+} exchanger. The reason for this seems to be the short-lasting depolarization of the apical membrane after opening of Ca^{2+}-dependent Cl^- channels. The mechanisms by which the signal from the muscarinic receptor is translocated to these apical channels, has still to be elucidated.

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