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Muscarinic Receptor Stimulation Activates a Ca^{2+}-dependent Cl^- Conductance in Rat Distal Colon

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Abstract. Recently, it was observed that the acetylcholine analogue carbachol induces a transient stimulation of an apical Cl^- conductance in basolaterally depolarized rat distal colonic epithelium (Schultheiss et al., 2003). The further characterization of this conductance was the aim of the present study. All experiments were performed at basolaterally depolarized tissues (111.5 mmol·l^{-1} KCl buffer at the serosal side); in the absence of a K^+ gradient, a Cl^- current was driven across the apical membrane (107 mmol·l^{-1} K gluconate/4.5 mmol·l^{-1} KCl buffer on the mucosal side). Under these conditions, carbachol evoked an atropine-sensitive biphasic change in short-circuit current (I_{SC}), consisting of a transient increase followed by a long-lasting decrease, suggesting a stimulation of apical Cl^- conductance followed by an inhibition. This conductance was inhibited by SITS, but was resistant against glibenclamide, a blocker of CFTR. The carbachol-induced I_{SC} was dependent on the presence of mucosal Ca^{2+}. Ionomycin, a Ca^{2+} ionophore, mimicked the effect of carbachol. An antibody against bovine Ca^{2+}-activated Cl^- channel ClCa1 stained rat colonic epithelial cells both at the cell membrane as well as intracellularly, suggesting that the action of Ca^{2+} may be caused by a stimulation of a ClCa-type anion channel. The activation of apical Cl^- conductance by carbachol was resistant against any blockers of the phospholipase C/IP3/protein kinase C pathway tested (e.g., U-73122, 2-ABP, Li^+, staurosporine), but was inhibited by the NO-synthase blocker L-NNA. Vice versa, NO-donating compounds such as GEA 3162 or sodium nitroprusside evoked a transient increase of I_{SC}. Consequently, NO seems to be involved in the transient stimulation of apical Ca^{2+}-dependent Cl^- conductance after muscarinic receptor stimulation.

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Introduction

Epithelial Cl^- secretion can only occur if apical Cl^- channels are in an open state and an electrical and/or chemical gradient drives the extrusion of Cl^- ions. The dominant Cl^- channel in most epithelia seems to be the CFTR (cystic fibrosis transmembrane regulator) channel (Greger, 2000). However, depending on the type of epithelium, additional anion conductances may be present in the apical membrane. For example, in respiratory epithelia it is clearly established that a Ca^{2+}-activated Cl^- channel (ClCa) contributes to transepithelial Cl^- secretion (for review *see* Fuller & Benos, 2000; Fuller et al., 2001). The expression of different Cl^- exit pathways explains why CFTR knockout mice do not show the respiratory symptoms known from human patients suffering from cystic fibrosis (for review *see* e.g., Ko & Pederson, 2001).

In contrast to respiratory epithelia, at intestinal epithelia the existence of Ca^{2+}-dependent Cl^- channels is controversial. Studies by Anderson & Welsh (1991) demonstrate that colonic epithelial tumor cells only express a Ca^{2+}-activated Cl^- current when they are in an undifferentiated state, i.e., when they have not yet grown to confluency. In contrast, in the human tumor cell line T84 a DIDS-sensitive Ca^{2+}-dependent Cl^- current across the apical membrane was induced after permeabilization of the basolateral membrane (Merlin et al., 1998). In porcine ileum, RT-PCR and in-situ hybridization experiments revealed the presence of a transcript termed pClCa1 with high homology to the human isoform, hClCa1 (Gaspar et al., 2000), although its function as a Cl^- channel by its own has been questioned later on by the same group (Loewen et al., 2002; 2003) . A further

member of the ClCa family (mClCa6) has been isolated from mouse intestine, which, after expression in HEK 293 cells, leads to an ionomycin-stimulated whole-cell Cl^- current, which is sensitive to niflumic acid (Evans, Thoreson & Beck, 2004).

In rat colonic epithelium, the dominant response of the cells to an increase in the intracellular Ca^{2+} concentration is the stimulation of Ca^{2+}-dependent K^+ conductances (Böhme, Diener & Rummel, 1991; Strabel & Diener, 1995; Schultheiss & Diener, 1997); only after exposure to a carcinogen a stimulation of a Cl^- conductance by Ca^{2+}-dependent secretagogues was observed (Bleich et al., 1997). However, when the experimental conditions are selected in such a way that the predominant action of Ca^{2+} on K^+ channels is prevented, the transient stimulation of an apical Ca^{2+}-induced Cl^- current could be measured (Schultheiss et al., 2003). In this study, the basolateral membrane was depolarized by a high K^+ concentration (with KCl at the serosal side), which leads to the electrical elimination of this membrane (Fuchs, Larsen & Lindemann, 1977); in the absence of a K^+ gradient, a Cl^- current was driven across the apical membrane (i.e., with K gluconate at the mucosal side) in order to measure changes in apical Cl^- conductances.

The aim of the present study was to investigate in more detail this Ca^{2+}-activated Cl^- current. Our electrophysiological data in combination with immunohistochemical results demonstrate the presence of a stilbene-sensitive, Ca^{2+}-activated apical Cl^- conductance in rat distal colon, which seems to exhibit a quite unusual way of activation by the Ca^{2+}-dependent secretagogue, carbachol, involving the mediation by NO and the influx of Ca^{2+} across the apical membrane.

Materials and Methods

Solutions

All Ussing-chamber experiments were carried out in a serosal bathing solution containing (mmol·l^{-1}): KCl 111.5, $NaHCO_3$ 25, Na_2HPO_4 1.8, NaH_2PO_4 0.2, $CaCl_2$ 1.25, MgSO4 1 and glucose 12 to depolarize the basolateral membrane. In order to apply a serosal to mucosal Cl^- gradient, 107 mmol·l^{-1} Cl^- was replaced by the impermeable anion gluconate in the apical bathing solution, thus containing (mmol·l^{-1}): K gluconate 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. Both solutions were gassed with carbogen (5% CO_2/95% O_2; v/v) and kept at a temperature of 37°C; pH was 7.4.

For the experiments with the trivalent cation La^{3+}, a HEPES-buffered Tyrode solution was used to prevent binding of the lanthanide by CO_3^{2-}- and PO_4^{3-}-anions (Caldwell, Clemo & Baumgarten, 1998). This buffer on the serosal side contained (mmol·l^{-1}): KCl 145.4, $CaCl_2$ 1.25, $MgCl_2$ 1, HEPES (N-(2-hydroxyethyl) piperazine-N′-2-ethane-sulfonic acid) 10, glucose 12. The mucosal bathing solution for these experiments contained (mmol·l^{-1}): K gluconate 140, KCl 5.4, $CaCl_2$ 1.25, $MgCl_2$ 1, HEPES 10, glucose 12. Both solutions were kept at a temperature of 37°C, a pH of 7.4 and were gassed with O_2.

For the immunhistochemical experiments, a 100 mmol·l^{-1} phosphate buffer (80 mmol·l^{-1} Na_2HPO_4 and 20 mmol·l^{-1} NaH_2PO_4) was used.

Isolation of Tissues

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.

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}. I_{SC} is expressed as μEq·h^{-1}·cm^{-2}, i.e., the flux of a monovalent ion per time and area, with 1 μEq·h^{-1}·cm^{-2} = 26.9 μA·cm^{-2}. The baseline in electrical parameters was determined as mean over 3 min just before administration of a drug.

Immunohistochemistry

The tissue was rinsed in phosphate buffer and embedded in gelatin (gelatin type A from porcine skin; 100 g·l^{-1}). Then the tissue was cryofixed in N_2-cooled 2-methylbutane. Sections (16 μm thick) were cut and mounted on glass slides coated with gelatin containing chrome alum (chromium(III) potassium sulfate; 0.5 g·h^{-1}). Immunohistochemistry was performed using the avidin-biotin-horseradish peroxidase complex (ABC) technique (Vectastain ABC-Elite Kit; Vector Laboratories, Burlingame, CA). In short, after rehydration in phosphate buffer, the sections were incubated with peroxidase blocking reagent (Dako Cytomation, Carpinteria, CA) for 14 min at room temperature to decrease the endogenous peroxidase activity. After rinsing with phosphate buffer, the sections were incubated in phosphate buffer, containing 10% (v/v) donkey serum (Chemicon, Temecula, CA), 0.5% (v/v) triton X-100, 2% (w/v) bovine serum albumin (BSA) to block unspecific binding. Then the blocking solution was removed and the sections were incubated with α-p38 (generous gift from Catherine Fuller, Birmingham, AL), a rabbit polyclonal antibody against the 38 kD subunit of bovine ClCa1 (Ran & Benos, 1992) for 20 h at 4°C at a dilution of 1:50. The antibody was dissolved in phosphate buffer containing 0.5% (v/v) triton X-100, 0.5% (w/v) milk powder, 1% (w/v) BSA and 1% (v/v) donkey serum. After rinsing with phosphate buffer, the sections were incubated with biotinylated goat-anti-rabbit IgG (dilution of 1:200; Vector Laboratories) for 2 h at room temperature. After a further rinse with phosphate buffer, the sections were incubated for 1 h with Vectastain ABC reagent, followed by incubation with 3,3′-diaminobenzidine (DAB).

The sections were counterstained with Mayer's hematoxylin solution (10 min) followed by staining with eosin Y (0.5%, w/v;

both from Carl Roth, Karlsruhe, Germany) for 3 min. As negative control, some sections were incubated with a solution that did not contain anti-ClCa. The sections were examined on a light microscope (Olympus BX 50), photographed with a CCD camera (Olympus Camedia Digital Camera C3030 Zoom) and visualized by a computer program (Camedia Master 2.0, Olympus).

Drugs

2-APB (2-aminoethoxydiphenylborat; Calbiochem, Bad Soden, Germany), calmidazolium, dichlorobenzamil (Molecular Probes, Leiden, The Netherlands), DIDS (4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid disodium salt), glibenclamide, KN-62 (1-(N,O-bis(5-isoquinoline-sulfonyl)-N-methyl-L-tyrosyl)-4-phenyl-piperazine, Tocris Cookson, Bristol, UK), staurosporine, and U-73122 (1-[6((17ß-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione; Calbiochem, Bad Soden, Germany) were dissolved in dimethylsulfoxide (DMSO; final maximal concentration 2.5 $ml \cdot l^{-1}$). Indomethacin and ionomycin were dissolved in ethanol (final maximal concentration 2.5 $ml \cdot l^{-1}$). Atropine, carbachol, CPT-cGMP (8-(4-chlorophenylthio)-guanosine 3′,5′ cyclic monophosphate), GEA 3162 ([1,2,3,4-oxatriazolium, 5-amino-3-(3,4-dichlorophenyl)]-chloride, Alexis, Grünberg, Germany), $LaCl_3$, SITS (4-acetamido-4′-isothiocyanato-stilbene-2,2′-disulfonic acid sodium salt, Calbiochem), SNP (sodium nitroprusside), ruthenium red (Alfa Aesar, Karlsruhe, FRG), and $ZnCl_2$ were dissolved in aqueous stock solutions. Flufenamic acid was dissolved in a stock solution containing 0.5 $mol \cdot l^{-1}$ NaOH. L-NNA (N-Ω-nitro-L-arginine, Tocris Cookson) was dissolved in a stock solution containing 1 $mol \cdot l^{-1}$ HCl. If not indicated differently, drugs were from Sigma, Deisenhofen, Germany.

If drugs were dissolved in a solvent other than an aqueous solution, the same volume of solvent was administered to the control tissue.

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

Baseline Effect of Carbachol on the Apical Membrane

All experiments were carried out on basolaterally depolarized epithelia (i.e., with a 111.5 $mmol \cdot l^{-1}$ KCl buffer on the serosal side), and in the presence of a serosally to mucosally directed Cl^- gradient (i.e., with a 107 $mmol \cdot l^{-1}$ K gluconate/4.5 KCl $mmol \cdot l^{-1}$ buffer on the mucosal side). Under these conditions, the basolateral membrane is electrically eliminated (Fuchs et al., 1977; Schultheiss & Diener, 1997); therefore, all actions of intracellular Ca^{2+} on cellular K^+ conductances can no longer contribute to I_{SC} due to the missing driving force for K^+ movement. Since only a chemical driving force acts on Cl^- ions, changes in I_{SC} should therefore correspond to Cl^- currents (Schultheiss et al., 2003).

With this protocol, the cholinergic agonist carbachol (used at a concentration of $5 \cdot 10^{-5}$ $mol \cdot l^{-1}$ at the serosal side in all experiments) induced a biphasic change in I_{SC}. During the first phase, the current increased transiently by a maximal value of 0.43 ± 0.09 $\mu Eq \cdot h^{-1} \cdot cm^{-2}$, suggesting the activation of a Cl^- current by carbachol. In the second phase, the I_{SC} decayed again, reaching after 10 min a value of -0.55 ± 0.10 $\mu Eq \cdot h^{-1} \cdot cm^{-2}$ below the former baseline ($P < 0.05$ versus I_{SC} prior administration of carbachol, $n = 6$, *see*, e.g., Fig. 3). Both phases induced by carbachol were abolished when the tissues were pretreated with the muscarinic antagonist atropine (10^{-6} $mol \cdot l^{-1}$ at the serosal side; $n = 5$–7). In the absence of a concentration gradient for Cl^- ions, i.e, in the presence of an identical KCl buffer on both sides of the epithelium, carbachol did not induce any change in I_{SC} ($n = 7$, *data not shown*); consequently the transient increase in I_{SC} must represent the activation of a Cl^- current. The following results focus mainly on this first phase of the carbachol response.[1]

Carbachol Does not Activate the CFTR Cl^- Conductance

The carbachol-stimulated increase in I_{SC} had a different time course compared to the cAMP-stimulated Cl^- current across the apical membrane. Forskolin ($5 \cdot 10^{-6}$ $mol \cdot l^{-1}$ at the mucosal and serosal side), the activator of adenylate cyclases stimulating CFTR via protein kinase A-mediated phosphorylation, induced an increase in I_{SC} to a maximal value of 2.41 ± 0.32 $\mu Eq \cdot h^{-1} \cdot cm^{-2}$ above baseline, which after 10 min still amounted to 0.60 ± 0.09 $\mu Eq \cdot h^{-1} \cdot cm^{-2}$ above the former baseline ($P < 0.05$ versus baseline prior administration of forskolin, $n = 7$, Fig. 1). Subsequent administration of carbachol induced the same pattern of I_{SC} response as under control conditions: a fast transient increase of 0.93 ± 0.14 $\mu Eq \cdot h^{-1} \cdot cm^{-2}$ followed by a long-lasting decay by -1.06 ± 0.18 $\mu Eq \cdot h^{-1} \cdot cm^{-2}$ ($P < 0.05$ versus baseline prior administration of carbachol, $n = 7$).

In intact tissue, where the predominant action of carbachol on K^+ currents is not covered due to the high extracellular K^+ concentration as in the present experiments, a Cl^- secretion only results when the apical CFTR channels are kept open due to a release of prostaglandins from the subepithelial tissue, which stimulate the intracellular cAMP production (Strabel & Diener, 1995). However, pretreatment with indomethacin (10^{-6} $mol \cdot l^{-1}$ at the serosal side), a blocker of cyclooxygenases, did not prevent the I_{SC} response

[1]The obvious subsequent down-regulation of apical Cl^- conductance is most probably explained by the inhibition of apical Cl^- channels by carbachol (Barrett et al. 1998; Schultheiss, Ribeiro & Diener, 2001) via a mechanism that is not yet completely understood.

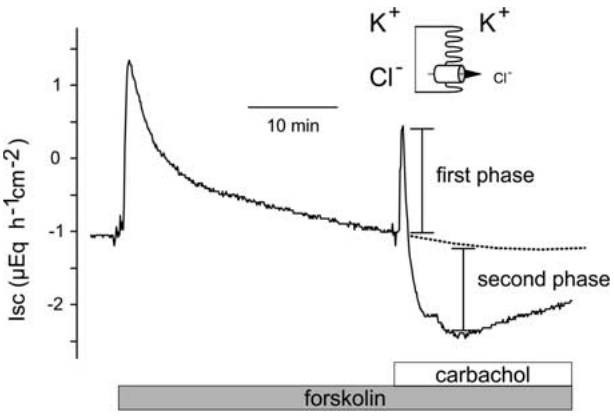

Fig. 1. I_{SC} response to forskolin ($5{\cdot}10^{-6}$ mol·l^{-1} at the mucosal and serosal side; *grey bar*) and to carbachol ($5{\cdot}10^{-5}$ mol·l^{-1} at the serosal side; *white bar*) under basolateral depolarized conditions (111.5 mmol·l^{-1} KCl at the serosal side) in the presence of a Cl^- gradient (107 mmol·l^{-1} K gluconate/4.5 mmol·l^{-1} KCl in the mucosal bathing solution, as indicated by the schematic drawing). Note the second phase of the carbachol-induced change in I_{SC} consisting of a long-lasting decrease, which was never observed with forskolin. Typical recording for $n = 7$; for statistics, *see* text.

induced by carbachol under basolaterally depolarized conditions (Table 1). Furthermore, glibenclamide ($5{\cdot}10^{-4}$ mol·l^{-1} at the mucosal side), a blocker of the CFTR Cl^- conductance (*see* e.g., Zhang et al., 2004), did not affect the stimulation of I_{SC} by carbachol (Table 1). Taken together, these data suggest that the carbachol-stimulated I_{SC} is not caused by the activation of the CFTR Cl^- conductance.

Carbachol Stimulates a Ca^+-Activated Cl^- Conductance in the Apical Membrane

Binding of carbachol at muscarinic receptors causes an increase of the intracellular concentration via a release of Ca^{2+} from intracellular stores and an influx from the extracellular space (Fischer et al., 1992; Frings, Schultheiss & Diener, 1999). However, in the absence of serosal Ca^{2+}, carbachol still activated an I_{SC} which amounted to 0.42 ± 0.09 µEq·h^{-1}·cm^{-2} ($P < 0.05$ versus I_{SC} prior to administration of carbachol, $n = 6$; Fig. 2), not different from the response observed in the serosal presence of the divalent cation. In contrast, when Ca^{2+} was omitted from the buffer on the mucosal side, the carbachol response was completely suppressed (maximal increase in I_{SC} induced by carbachol: 0.09 ± 0.06 µEq·h^{-1}·cm^{-2}, not significantly different from baseline; $n = 5$; Fig. 2).

The action of carbachol was mimicked by ionomycin (10^{-7} mol·l^{-1} at the mucosal side), a Ca^{2+} ionophore, which induced a small, but consistent increase in I_{SC} of 0.22 ± 0.04 µEq·h^{-1}·cm^{-2} ($P < 0.05$ versus baseline; $n = 7$). Like in the case of carbachol, the ionomycin-induced stimulation of I_{SC} across the apical membrane was followed by a long-lasting decrease (*data not shown*). These results show that an increase of the cytoplasmic Ca^{2+} concentration is required for the activation of the current across the apical membrane driven by the applied chemical Cl^- gradient, suggesting the presence of a Ca^{2+}-activated Cl^- conductance (ClCa), which might be the target for the action of carbachol.

Stilbenes are known to inhibit ClCa channels (Nilius et al., 1997, Nilius & Droogmans, 2003). Indeed, pretreatment with SITS (10^{-3} mol·l^{-1} at the mucosal side) significantly inhibited the carbachol-stimulated I_{SC} by about 2/3 (Fig. 3, Table 1). A similar inhibition was observed for the ionomycin-evoked increase in I_{SC}, which was reduced by more than 50% in the presence of DIDS (0.10 ± 0.01 µEq·h^{-1}·cm^{-2}, $n = 9$ in the presence of DIDS compared to 0.22 ± 0.04 µEq·h^{-1}·cm^{-2}, $n = 7$ in the absence of DIDS; $P < 0.05$). Another stilbene, DIDS (10^{-4} mol·l^{-1} at the mucosal side), which blocks ClCa from bovine respiratory epithelia (Ran et al., 1992), was ineffective (Table 1).

This electrophysiological evidence for the presence of a Ca^{2+}-activated Cl^- conductance was supported by immunohistochemical experiments using an antibody against bovine ClCa1. Similar to its application to another epithelium from the rat, i.e., exocrine pancreas (Thévenod et al., 2003), an immunoperoxidase reaction (avidin-biotin-horseradish peroxidase technique) was used to investigate the binding of this antibody. With this technique, the peroxidase activity, visualized by the precipitation of the oxidized form of the dye 3,3′-diaminobenzidine (DAB), was found near cellular membranes and diffusely within the cytoplasm of crypt epithelium (Fig. 4). When the primary antibody was omitted, only a faint background signal was measured. Consequently, it seems reasonable to conclude that the carbachol-stimulated I_{SC} across the apical membrane, which is driven by a chemical Cl^- gradient and inhibited by SITS, may be mediated by a ClCa-like anion channel.

Attempts to Characterize the Mucosal Ca^{2+}-Entry Pathway

The next set of experiments focused on the question, which influx pathway was used by Ca^{2+} for entering the cell from the mucosal side after stimulation by carbachol. In the basolateral membrane of rat colonic crypts, non-selective La^{3+}-sensitive cation channels, which are activated by depletion of intracellular Ca^{2+} stores, play a prominent role in Ca^{2+} influx (Frings et al., 1999). However, when lanthanum (10^{-3} mol·l^{-1}) was applied at the mucosal side, the stimulation of I_{SC} by carbachol was unaffected (Table 2). In *Xenopus* oocytes, endogenous Ca^{2+} channels activated by store depletion are inhibited by Zn^{2+} (Yao & Tsien, 1997). However, pretreatment with $ZnCl_2$ (10^{-4} mol·l^{-1} at the mucosal side) did not affect the stimulation of I_{SC} by

Table 1. Effect of drugs directly or indirectly interacting with apical Cl^- channels on carbachol-stimulated of I_{SC}

Inhibitor	Carbachol-stimulated current (ΔI_{SC}; $\mu Eq \cdot h^{-1} \cdot cm^{-2}$)			
	+ Inhibitor	*n*	− Inhibitor	*n*
Indomethacin	0.20 ± 0.06*	6	0.29 ± 0.07*	7
Glibenclamide	0.32 ± 0.04*	6	0.23 ± 0.03*	5
SITS	0.10 ± 0.02*#	8	0.30 ± 0.07*	11
DIDS	0.25 ± 0.08*	6	0.29 ± 0.06*	7

I_{SC}, stimulated by carbachol ($5 \cdot 10^{-5}$ mol·l^{-1} at the serosal side), across the apical membrane in the absence or presence of inhibitors interfering with apical Cl^- channels. Tissues were basolaterally depolarized (111.5 mmol·l^{-1} KCl at the mucosal side) in the presence of a serosally to mucosally directed Cl^- gradient (107 mmol·l^{-1} K gluconate/4.5 KCl mucosal bathing solution). Concentration of inhibitors were: DIDS (10^{-4} mol·l^{-1} at the mucosal side), glibenclamide ($5 \cdot 10^{-4}$ mol·l^{-1} at the mucosal side), indomethacin (10^{-6} mol·l^{-1} at the serosal side), SITS (10^{-3} mol·l^{-1} at the mucosal side). The values represent the maximal carbachol- induced I_{SC} given as difference to the baseline just prior to administration of the cholinergic agonist (ΔI_{SC}) and are means ± SEM. *$P < 0.05$ versus I_{SC} prior to administration of carbachol; #$P < 0.05$ versus carbachol-response in the absence of the respective inhibitor.

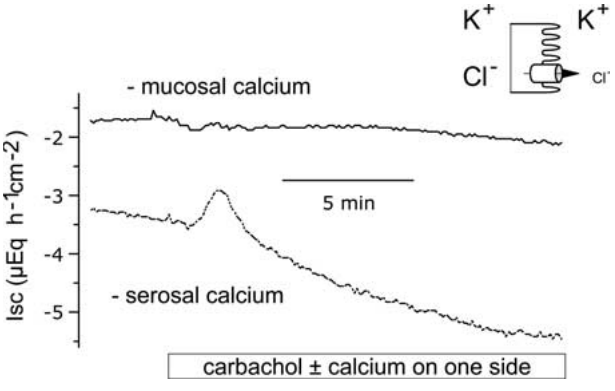

Fig. 2. The response evoked by carbachol ($5 \cdot 10^{-5}$ mol·l^{-1} at the serosal side; *white bar*) was suppressed in the absence of Ca^{2+} in mucosal bathing solution (− mucosal calcium, *solid line*), whereas in the absence of serosal Ca^{2+} (− serosal calcium, *dashed line*) a current across the apical membrane could still be induced. Tissues were basolaterally depolarized (111.5 mmol·l^{-1} KCl at the serosal side) in the presence of a Cl^- gradient (107 mmol·l^{-1} K^+ gluconate/4.5 mmol·l^{-1} KCl in the mucosal bathing solution, as indicated by the schematic drawing). Typical recording for $n = 5$–6; for statistics, *see* text.

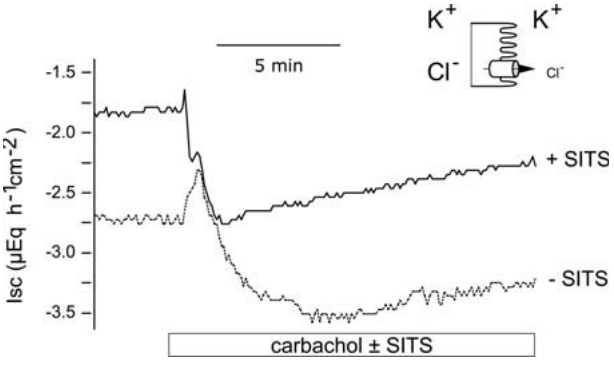

Fig. 3. The stimulation of I_{SC} by carbachol ($5–10^{-5}$ mol·l^{-1} at the serosal side; *white bar*) was inhibited in the presence of SITS (+ SITS, *solid line*), whereas in the absence of SITS (− SITS, *dashed line*) a significant increase in I_{SC} was observed. Tissues were basolaterally depolarized (111.5 mmol·l^{-1} KCl at the serosal side) in the presence of a Cl^- gradient (107 mmol·l^{-1} K^+ gluconate/4.5 mmol·l^{-1} KCl in the mucosal bathing solution, as indicated by the schematic drawing). Typical recording for $n = 8$–11; for statistics, *see* Table 1.

carbachol (Table 2). Also flufenamic acid ($5 \cdot 10^{-6}$ mol·l^{-1} at the mucosal side), another blocker of non-selective cation conductances (Siemer & Gögelein, 1992), did not inhibit the carbachol-stimulated current[2] (Table 2).

An alternative pathway for Ca^{2+} uptake across intestinal epithelia is represented by the Ca^{2+}-selective channels, $ECaC_1$ or $ECaC_2$ (now termed TRPV5 and TRPV6, respectively; Vennekens et al., 2002). However, ruthenium red, a potent blocker of both types of ECaC (Nilius et al., 2002), did not affect the carbachol-stimulated I_{SC} (Table 2).

Recently, a Na^+-Ca^{2+}-exchanger (NCX) was identified in the apical membrane of rat distal colonic epithelium (Schultheiss et al., 2003). Such a transport mechanism, when working in the reversed mode, can also mediate Ca^{2+} uptake (Blaustein & Lederer, 1999). Therefore, tissues were pretreated with dichlorobenzamil, a selective blocker of NCX (Kaczorowski et al., 1985). Again, the carbachol-induced increase in I_{SC} was unaffected (Table 2). Consequently, none of these inhibitor experiments gave a hint for the identification of the mucosal Ca^{2+} influx pathway necessary for the activation of the apical Cl^- conductance by carbachol.

Signalling Pathways Mediating the Carbachol Response

The following experiments served to elucidate the signal transduction mechanism involved in the

[2]However, in contrast to $LaCl_3$ or $ZnCl_2$, flufenamic acid suppressed the secondary long-lasting decrease in I_{SC} induced by carbachol (*data not shown*).

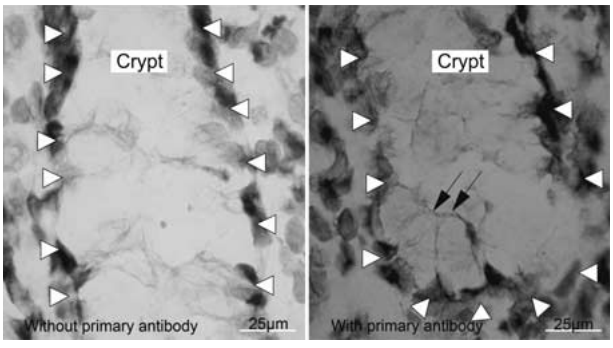

Fig. 4. Immunhistochemical staining with the avidin-biotin-horseradish peroxidase (ABC) technique led to the precipitation of the oxidized form of the dye 3,3′-diaminobenzidine (DAB) near cellular membranes (*black arrows*) and diffusely within the cytoplasm of crypt epithelium (*right picture*). When the primary antibody against bC1Ca1 was omitted, only a faint background signal of DAB was measured (*left picture*). White triangles mark the nuclei of the crypt cells located in the basal part of the enterocytes.

activation of the apical Cl^- conductance. Since ClCa in respiratory epithelia is stimulated by multifunctional calmodulin-dependent kinase II (CaMK-II; Fuller & Benos, 2000), calmidazolium (10^{-7} mol·l^{-1} at the serosal side), an inhibitor of the Ca^{2+}-calmodulin complex (*see*, e.g., Worrell & Frizzell, 1991), and KN-62 ($4{\cdot}10^{-6}$ mol·l^{-1} at the mucosal and serosal side), a blocker of CaMK-II (Casnellie, 1991), were used. However, neither of these inhibitors did significantly affect the carbachol-stimulated I_{SC} (Table 3).

Inhibition by atropine (*see* above) demonstrates the involvement of muscarinic receptors in the carbachol effect. Binding of carbachol at such receptors stimulates the phospholipase C pathway (PLC), leading to the production of inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol, resulting in an increase in the intracellular Ca^{2+} concentration and/or an activation of protein kinase C, respectively. Several blockers of this pathway were tested for their ability to interfere with the carbachol-stimulated I_{SC}. In the first set of experiments U-73122 (10^{-5} mol·l^{-1} at the serosal side), a blocker of PLC (Taylor & Broad, 1998), was administered, but the carbachol-induced increase of I_{SC} was not affected (Table 3). Inhibiting IP_3 turnover with LiCl (10^{-2} mol·l^{-1} at the mucosal and serosal side), blocking the myo-inositol-1-phosphatase (Berridge & Irvine, 1989; Jenkinson, Nahorski & Challiss, 1994; Wolfson et al., 1998), was also ineffective in inhibiting the carbachol-stimulated rise in I_{SC} (Table 3). However, in the case of LiCl, the carbachol-induced secondary decrease in I_{SC} was completely suppressed, i.e., 10 min after administration of carbachol in the presence of LiCl the current had fallen by 0.01 ± 0.11 µEq·h^{-1}·cm^{-2} (n = 7) below the baseline prior to administration of carbachol compared to −1.77 ± 0.22 µEq·h^{-1}·cm^{-2} in the absence of LiCl ($P < 0.05$ versus response in the presence of Li^+; n = 6). Inhibition of the PLC/IP_3 signalling pathway more downstream, i.e., block of IP_3 receptors with 2-APB (10^{-4} mol·l^{-1} at the mucosal and serosal side; for reference of this inhibitor, *see* Bakowski, Glitsch & Parekh, 2001), or protein kinases with staurosporine (10^{-6} mol·l^{-1} at the serosal side; for reference of this inhibitor, *see* Tamaoki et al., 1986), proved to be ineffective, too (Table 3).

Involvement of NO

Recently we observed that GEA 3162, a lipophilic compound liberating NO in aqueous solutions (Kankaanranta et al., 1996; Holm et al., 1998), evokes a Ca^{2+}-dependent transepithelial Cl^- secretion in rat distal colon (Schultheiss et al., 2002). Therefore, in a final set of experiments, the role of NO for stimulating apical Cl^- conductance was investigated. GEA 3162 ($5{\cdot}10^{-4}$ mol·l^{-1} at the serosal side) induced a transient activation of I_{SC} of 1.00 ± 0.26 µEq·h^{-1}·cm^{-2} ($P < 0.05$ versus baseline prior to administration of the drug; n = 8; Fig. 5), when tested under the same conditions as carbachol, i.e., with basolateral depolarization and with an applied Cl^- gradient. When tissues were pretreated with GEA, carbachol no longer induced any increase in I_{SC}, suggesting that both might activate the same conductance (Fig. 5). Another NO-liberating compound, sodium nitroprusside (SNP, $5{\cdot}10^{-4}$ mol·l^{-1} at the serosal side) had the same action: it stimulated a transient increase in I_{SC}, which amounted to 0.18 ± 0.04 µEq·h^{-1}·cm^{-2} ($P < 0.05$ versus baseline prior to administration of SNP, n = 6) and carbachol in the presence of SNP no longer induced any increase in Cl^- current (*data not shown*; n = 7). As it was observed with carbachol, the SNP-stimulated I_{SC} was dependent on the presence of mucosal Ca^{2+}: in the absence of mucosal Ca^{2+}, SNP ($5{\cdot}10^{-4}$ mol·l^{-1} at the serosal side) no longer stimulated I_{SC} (0.04 ± 0.02 µEq·h^{-1}·cm^{-2} in the absence and 0.24 ± 0.05 µEq·h^{-1}·cm^{-2} in the presence of mucosal Ca^{2+}, respectively; $P < 0.05$ versus absence of Ca^{2+}; n = 6). The effect of NO was not mimicked by CPT-cGMP, a cell-permeable analogue of cGMP. In the presence or absence of CPT-cGMP (10^{-4} mol·l^{-1} at the serosal side) the carbachol-induced increase in I_{SC} was identical (*data not shown*, n = 7–8). CPT-cGMP alone induced an increase in I_{SC}, which amounted to 0.41 ± 0.05 µEq·h^{-1}·cm^{-2} ($P < 0.05$ versus baseline prior to administration of the drug; n = 8), an action that is in accordance with the known ability of cGMP to induce CFTR activation via phosphorylation (for review *see*, e.g., Jentsch et al., 2002).

Vice versa, blocking NO-synthases with the non-selective blocker N-Ω-nitro-L-arginine (L-NNA, 10^{-2} M at the mucosal and serosal side) significantly inhibited the carbachol-stimulated I_{SC}. In the presence of L-NNA, the carbachol-induced increase in I_{SC} only amounted to 0.09 ± 0.03 µEq·h^{-1}·cm^{-2} (n = 6)

Table 2. Effect of putative inhibitors of Ca^{2+} entry pathways on carbachol-induced stimulation of I_{SC}

Inhibitor	Carbachol-stimulated current (ΔI_{SC}; $\mu Eq \cdot h^{-1} \cdot cm^{-2}$)			
	+ Inhibitor	*n*	− Inhibitor	*n*
$LaCl_3$	0.59 ± 0.32	10	0.44 ± 0.10	10
Flufenamic acid	0.22 ± 0.03*	8	0.38 ± 0.11*	9
Ruthenium red	0.59 ± 0.06*	6	0.33 ± 0.11*	6
$ZnCl_2$	0.29 ± 0.09*	7	0.25 ± 0.05*	8
Dichlorobenzamil	0.30 ± 0.14*	7	0.34 ± 0.14*	7

I_{SC}, stimulated by carbachol ($5 \cdot 10^{-5}$ mol·l^{-1} at the serosal side), across the apical membrane in the absence or presence of putative inhibitors of Ca^{2+}-entry pathways. Tissues were basolaterally depolarized (111.5 mmol·l^{-1} KCl at the serosal side) in the presence of a serosally to mucosally directed Cl^- gradient (107 mmol·l^{-1} gluconate/4.5 KCl mucosal bathing solution). Concentration of inhibitors were: dichlorobenzamil (10^{-4} mol·l^{-1} at the mucosal side), flufenamic acid ($5 \cdot 10^{-6}$ mol·l^{-1} at the mucosal side), LaCl3 (10^{-3} mol·l^{-1} at the mucosal side), ruthenium red ($5 \cdot 10^{-4}$ mol·l^{-1} at the mucosal and serosal side), $ZnCl_2$ (10^{-4} mol·l^{-1} at the mucosal side). The experiments with $LaCl_3$ were performed in a HEPES-buffered Tyrode solution in order to prevent binding of this trivalent cation to HCO_3^- or PO_4^{3-} anions. The values represent the maximal carbachol-induced I_{SC} given as difference to to the baseline just prior to administration of the cholinergic agonist (ΔI_{SC}) and are means ± SEM. *$P < 0.05$ versus I_{SC} prior to administration of carbachol.

Table 3. Effect of inhibitors of calmodulin- and phospholipase C pathway on carbachol-induced stimulation of I_{SC}

Inhibitor	Carbachol-stimulated current (ΔI_{SC}; $\mu Eq \cdot h^{-1} \cdot cm^{-2}$)			
	+ Inhibitor	*n*	− Inhibitor	*n*
2-APB	0.12 ± 0.04*	6	0.22 ± 0.05*	8
Calmidazolium	0.21 ± 0.06*	10	0.24 ± 0.10*	7
KN−62	0.59 ± 0.20*	7	0.58 ± 0.16*	6
LiCl	0.29 ± 0.11*	7	0.50 ± 0.24*	6
Staurosporine	0.81 ± 0.15*#	8	0.42 ± 0.16*	9
U-73122	0.57 ± 0.20*	7	0.33 ± 0.12*	10

I_{SC}, stimulated by carbachol ($5 \cdot 10^{-5}$ mol·l^{-1} at the serosal side), across the apical membrane in the absence or presence of inhibitors of calmodulin and phospholipase C pathways. Tissues were basolaterally depolarized (111.5 mmol·l^{-1} KCl at the serosal side) in the presence of a serosally to mucosally directed Cl^- gradient (107 mmol·l^{-1} K gluconate/4.5 KCl mucosal bathing solution). Concentration of inhibitors were: 2-APB (10^{-4} mol·l^{-1} at the mucosal and serosal side), calmidazolium (10^{-7} mol·l^{-1} at the serosal side), KN-62 ($4 \cdot 10^{-6}$ mol·l^{-1} at the mucosal and serosal side), LiCl (10^{-2} mol·l^{-1} at the mucosal and serosal side), staurosporine (10^{-6} mol·l^{-1} at the serosal side), U-73122 (10^{-5} mol·l^{-1} at the serosal side). The values represent the maximal carbachol-induced I_{SC} given as difference to the baseline just prior to administration of the cholinergic agonist (ΔI_{SC}) and are means ± SEM. *$P < 0.05$ versus I_{SC} prior administration of carbachol; # $P < 0.05$ versus carbachol-response in the absence of the respective inhibitor.

compared to 0.40 ± 0.12 $\mu Eq \cdot h^{-1} \cdot cm^{-2}$ in the absence of the inhibitor ($P < 0.05$ versus carbachol-response in the absence of the inhibitor; $n = 7$).

Several action sites can be thought of for the interaction between NO and the carbachol response. NO might be responsible for the activation of the yet unidentified Ca^{2+} influx pathway across the apical membrane or — vice versa — as constitutive NO-synthases are dependent on Ca^{2+} (*see*, e.g., Alican & Kubes, 1996), the Ca^{2+} influx across this membrane might stimulate the production of NO being involved in the activation of the presumed apical Ca^{2+}-dependent Cl^- channel. In order to differentiate between these two possibilities, the effect of the Ca ionophore ionomycin (10^{-7} mol·l^{-1} at the mucosal and serosal side) was tested in the absence and in the presence of the NO-synthase(s) blocker L-NNA (10^{-2} mol·l^{-1} at the mucosal and serosal side). L-NNA did not inhibit the increase in I_{SC} evoked by ionomycin (0.13 ± 0.03 $\mu Eq \cdot h^{-1} \cdot cm^{-2}$, $n = 6$ in the absence and 0.17 ± 0.04 $\mu Eq \cdot h^{-1} \cdot cm^{-2}$ in the presence of L-NNA, $n = 8$; difference not significant). Consequently, it seems reasonable to assume that the involvement of NO in the mediation of the carbachol response at the apical membrane is localized upstream from the apical Ca^{2+} influx.

Discussion

The results of the present study show that carbachol induces a biphasic change of I_{SC} across the apical membrane, consisting of a fast transient increase followed by a long-lasting decay (*see* Fig. 1). After basolateral depolarization, the basolateral membrane is electrically eliminated (Fuchs et al., 1977; Schultheiss & Diener, 1997), so that changes in currents induced by drugs should reflect changes in the

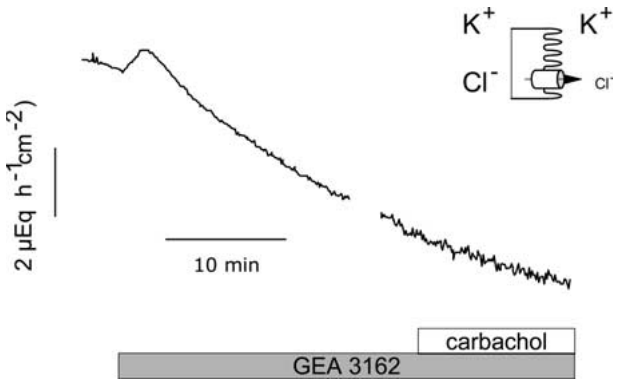

Fig. 5. The NO-donor GEA 3162 ($5{\cdot}10^{-4}$ mol·l^{-1} at the serosal side; *grey bar*) itself stimulates a transient increase in I_{SC}, and prevents the action of a subsequent administration of carbachol ($5{\cdot}10^{-4}$ mol·l^{-1} at the serosal side; *white bar*). The line interruption is caused by omission of a time period of 2.5 min. Tissues were basolaterally depolarized (111.5 mmol·l^{-1} KCl at the serosal side) in the presence of a Cl^- gradient (107 mmol·l^{-1} K gluconate/4.5 mmol·l^{-1} KCl in the mucosal bathing solution, as indicated by the schematic drawing). Typical recording for $n = 8$; for statistics, *see* text. In a time-dependent control, performed in the absence of GEA 3162, carbachol evoked a transient increase in I_{SC} of 0.4 ± 0.1 µEq·h^{-1}·cm^{-2} ($P < 0.05$ versus baseline prior to administration of carbachol, $n = 8$), whereas in all 8 tissues pretreated with GEA 3162 no increase in I_{SC} was observed.

conductance in the apical membrane. In order to measure ionic currents across this membrane, a driving force must exist, i.e., in our case, a chemical Cl^- gradient. Sensitivity of the carbachol-induced I_{SC} to SITS demonstrates that the changes in I_{SC} reflect indeed transcellular Cl^- currents and not Cl^- movement across the paracellular pathway driven by the applied concentration gradient.

The carbachol-activated Cl^- current does not pass through the CFTR channel. Several observations argue against a contribution of CFTR to the inital and transient carbachol-stimulated increase in I_{SC}. The carbachol-activated Cl^- current was resistant against block of cyclooxygenase(s) by indomethacin (*see* Table 1), indicating that prostaglandins, which in rat colonic epithelium via stimulation of the tonic production of cAMP are necessary to keep the CFTR open (Strabel & Diener, 1995), are not involved here. Vice versa, stimulation of the cAMP- or the cGMP-pathway, known to lead to CFTR activation via phosphorylation, stimulated an I_{SC} with a completely different time course (Fig. 1). Glibenclamide, a well known blocker of CFTR, did not inhibit the carbachol-induced activation of Cl^- current at all (Table 1), suggesting that carbachol exerts its action at a different type of anion conductance. The stimulation of I_{SC} by carbachol was dependent on the presence of mucosal Ca^{2+} (Fig. 2). Finally SITS, a non-specific but potent blocker of Ca^{2+}-activated Cl^- conductances (Nilius & Droogmans, 2003), significantly inhibited the carbachol effect (Fig. 3), suggesting the activation of a Ca^{2+}-dependent Cl^- conductance by carbachol.

These functional data were supported by immunoperoxidase staining using an antibody against bClCa1, which revealed the presence of this antigen within the colonic epithelial cells (Fig. 4). These experiments showed the localization of the product of the immunohistochemical reaction not only near the apical membrane, but also near the lateral border of the cell and diffusively within the cytoplasm. As in rat pancreatic epithelial cells, ClCa protein has been identified also in zymogen granules (Thévenod et al., 2003), this protein might exert additional functions in the task, such as serving as a plasma membrane exit pathway for Cl^-.

The transduction mechanism mediating the signalling cascade between carbachol binding to the muscarinic receptor to the activation of an apical anion conductance is unclear. The classical pathway initiated by carbachol, i.e., activation of the phospholipase C (PLC) with the consecutive production of IP_3, is very likely not involved in this process, since neither blockade of PLC nor inhibition of IP_3 production nor inhibition of IP_3 receptors had any effect on carbachol-stimulated I_{SC} (*see* Table 3). Ryanodine receptors, which have been shown to play a role in mediating transepithelial Ca^{2+}-dependent Cl^- secretion (Kocks, Schultheiss & Diener, 2002), seem also not to be involved, because ruthenium red, a potent blocker of ryanodine receptors, did not inhibit the carbachol-stimulated Cl^- current (*see* Table 2). Besides the activation of PLC, binding of, e.g., acetylcholine to muscarinic receptors can lead to the activation of phospholipase D (PLD; Mamoon et al., 1999). However, stimulation of one of these pathways, i.e., the PLC or the PLD pathway, results finally in the activation of protein kinases. Since staurosporine, which unspecifically blocks protein kinases (Tamaoki et al., 1986), did not prevent the stimulation of I_{SC} by carbachol (Table 3), a contribution of the PLD pathway seems also not very likely. ClCa activation in respiratory epithelia is primarily mediated by Ca^{2+}-dependent kinases such as CaMK II (Fuller & Benos, 2000). However, both calmidazolium, an inhibitor of the Ca^{2+}-calmodulin complex, and KN-62, a specific blocker of CaMK-II, did not inhibit the carbachol-stimulated I_{SC} (Table 3).

In contrast, there seems to be an interaction between NO and the carbachol-induced Cl^- current. GEA 3162 and SNP, two drugs liberating NO, evoked themselves a Ca^{2+}-dependent increase in I_{SC} across the apical membrane. After stimulation of Ca^{2+}-activated Cl^- conductance by these NO-donors, the subsequent administration of carbachol was ineffective. Furthermore, blockade of NO-synthases by L-NNA prevented the carbachol action.

At present, we cannot distinguish which type of NO-synthase is affected by carbachol. In most tissues,

the inducible form of NO-synthase (iNOS) is not expressed under basal conditions; however, in mouse small intestine, iNOS has been observed already under basal conditions near the apical pole of the enterocytes (Rumbo et al., 2005). A plausible explanation for the interaction of carbachol with the NO-pathway may represent the known Ca^{2+} dependence of the constitutive forms of NO-synthase (*see*, e.g., Alican & Kubes, 1996). However, if the assumed Ca^{2+} influx across the apical membrane led to the activation of an NO-synthase, the increase in I_{SC} evoked by ionomycin should be sensitive to inhibition of NO-synthases with L-NNA, which was clearly not the case. Consequently, it is more reasonable to assume that NO may play a role more upstream from the activation of the apical Ca^{2+} influx pathway, finally leading to the activation of the Ca^{2+}-dependent Cl^- conductance. The classical action of NO consists of the stimulation of a soluble guanylylcyclase, leading to an increase of the intracellular cGMP concentration (*see*, e.g., Pfeilschifter, Eberhardt & Beck, 2001). However, the membrane-permeable cGMP analogue, CPT-cGMP, did not mimic the action of the NO-donors, suggesting an alternative mechanism of action, such as, e.g., N-nitrosylation.

The influx pathway for Ca^{2+} across the apical membrane involved in the activation of I_{SC} by carbachol remains obscure. Neither blockers of non-selective channels such as $LaCl_3$, flufenamic acid or $ZnCl_2$, nor inhibitors of epithelial apical Ca^{2+} conductances ($ECaC_1$ or $ECaC_2$), inhibited the carbachol-induced stimulation of Cl^- conductance (*see* Table 2). The same was observed with the Na^+-Ca^{2+}-exchanger (NCX), which has recently been shown to exist in the apical membrane of rat distal colonic epithelium (Schultheiss et al., 2003). Consequently, the nature of the apical Ca^{2+} influx mechanism remains to be resolved.

What is the physiological significance of this additional apical Cl^- conductance? When compared to the effect of a typical agonist of the cAMP pathway, such as forskolin, the Cl^- current stimulated by carbachol is small and, even more importantly, extremely transient and followed by a down-regulation, i.e., an inhibition of apical Cl^- channels via a mechanism that is not yet completely understood (Barrett et al., 1998; Schultheiss, Ribeiro & Diener 2001). Consequently, the opening of apical Cl^- channels induced by carbachol can only transiently contribute to stimulation of transepithelial Cl^- channels compared, e.g., to the action of a typical cAMP-dependent secretagogue such as forskolin, which stimulates a Cl^- secretion stable over 5 h and more (Mestres, Diener & Rummel, 1990). Furthermore, this action of carbachol can only be observed under conditions where the dominant action of the cholinergic agonist on basolateral and apical K^+ channels is suppressed by the high K^+ concentration on both sides of the tissue so that changes in K^+ conductances no longer contribute to I_{SC}. If only the basolateral membrane is depolarized without an applied Cl^- gradient (i.e., with NaCl at the mucosal side), carbachol stimulates a pronounced negative I_{SC} due to opening of Ca^{2+}-dependent apical K^+ channels (Schultheiss et al., 2003). Consequently, under physiological conditions, the contribution of the Ca^{2+}-dependent Cl^- conductance to transepithelial secretion will be small and only transient; this is probably the reason why it was not observed in previous experiments, having been hidden by the strong activation of K^+ channels by carbachol (Böhme et al., 1991; Strabel & Diener, 1995).

Taken together these data demonstrate the presence of a Ca^{2+}-activated Cl^- conductance in the apical membrane of rat colonic epithelia. Its activation after muscarinic receptor stimulation involves NO-synthase activity and an influx of Ca^{2+} across the apical membrane.

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