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Ca^{2+}-dependent and -independent Cl^- secretion stimulated by the nitric oxide donor, GEA 3162, in rat colonic epithelium

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Abstract

The lipophilic nitric oxide-liberating drug, 1,2,3,4-oxatriazolium,5-amino-3-(3,4-dichlorophenyl)-chloride (GEA 3162), concentration-dependently induced a Cl^- secretion in rat colon. At a low concentration (5×10^{-5} M), the action was Ca^{2+}-dependent, whereas at a high concentration (5×10^{-4} M), the response was independent from extracellular Ca^{2+}. Fura-2 experiments at isolated colonic crypts revealed that GEA 3162 induced an increase of the cytoplasmic Ca^{2+} concentration due to an influx of extracellular Ca^{2+}, probably mediated by an activation of a nonselective cation conductance as demonstrated by whole-cell patch-clamp studies. After depolarization of the basolateral membrane, GEA 3162 (5×10^{-4} M) stimulated a current, which was suppressed by glibenclamide but was resistant against blockade of protein kinases by staurosporine, suggesting an activation of apical Cl^- channels directly by the nitric oxide (NO) donor. After permeabilizing the apical membrane with the ionophore, nystatin, GEA 3162 (5×10^{-4} M) activated basolateral K^+ conductances and the Na^+–K^+-ATPase. Thus, the lipophilic NO donor GEA 3162 stimulates a Cl^- secretion in a Ca^{2+}-dependent and -independent manner.

Keywords: Cl^- channel; Ca^{2+}, intracellular; K^+ channel; Na^+–K^+-ATPase; NO; Colon, rat

1. Introduction

In epithelia, as well as in other nonexcitable cells, a depletion of intracellular Ca^{2+} stores triggers a Ca^{2+} influx. The signals, by which the Ca^{2+}-storing organelles activate the so-called store-operated Ca^{2+} channels, remain obscure (Parekh and Penner, 1997). There are data arguing both for a direct mechanical interaction (see, e.g., Yao et al., 1989), as well as for the involvement of a soluble messenger substance (see, e.g., Trepakova et al., 2000).

We previously demonstrated that carbachol, as well as depletion of the intracellular Ca^{2+} stores with chelators, activate a store-operated nonselective cation conductance in the rat colonic epithelium (Frings et al., 1999). This conductance is responsible for the influx of extracellular Ca^{2+} and is involved in the Ca^{2+}-dependent Cl^- secretion induced by stimulation of muscarinic receptors with carbachol (Böhme et al., 1991; Strabel and Diener, 1995; Bleich et al., 1996; Seip et al., 2001). Nitric oxide (NO) has been debated to be one of the intracellular signals involved in the regulation of store-operated Ca^{2+} influx. In the human colonic tumor cell line HT29/B6, sodium nitroprusside, a NO-liberating drug, evoked an increase of the cytoplasmic Ca^{2+} concentration in resting cells (Bischof et al., 1995). The effect of sodium nitroprusside was more pronounced after prestimulation of the cells with the stable acetylcholine analogue, carbachol. Similar results obtained at mouse parotid acini support a role of NO in agonist-mediated Ca^{2+} release and entry (Watson et al., 1999). Other data show that NO donors such as sodium nitroprusside induce a Cl^- secretion in rat colon (Tamai and Gaginella, 1993; Wilson et al., 1993). The colonic epithelium possesses the ability to produce NO as these cells express the constitutive form of NO synthase, producing NO in a Ca^{2+}-dependent manner (Wilson et al., 1996). Therefore, we asked whether NO donors might act by stimulating the influx of extracellular Ca^{2+} via the store-operated nonselective cation conductance. To this purpose, Ussing chamber, patch-clamp and fura-2 experiments were performed, revealing indeed the activation of a Ca^{2+} entry pathway by NO. However,

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carbachol-induced Cl^- secretion was not prevented by inhibition of NO synthases, indicating that NO is not the physiological signal involved in the communication between the intracellular stores and the channels in the plasma membrane. Our data furthermore demonstrate additional action sites of the NO donor 1,2,3,4-oxatriazolium,5-amino-3-(3,4-dichlorophenyl)-chloride (GEA 3162), i.e., the apical Cl^- conductance, the basolateral K^+ conductance and the Na^+–K^+-ATPase.

2. Materials and methods

2.1. Solutions

The Ussing chamber experiments were carried out in a bathing solution containing (in mM): 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 a gas mixture of 5% CO_2 and 95% O_2; the pH was 7.4. For the depolarization of the basolateral membrane, a 111.5-mM KCl solution was used, in which NaCl was equimolarly replaced by KCl. In order to apply a K^+ gradient from the mucosal to the serosal side, the KCl concentration in the standard HCO_3^--buffered solution was increased to 13.5 mM, while reducing the NaCl concentration to 98 mM in order to maintain isoosmolarity. For the Na^+-free solution, NaCl was replaced by *N*-methyl-D-glucamine ($NMDG^+$) Cl^-. In the Cl^--free buffer, NaCl was substituted by Na gluconate. In experiments in which $CaCl_2$ was omitted, the concentration of $MgSO_4$ was raised to 5 mM.

For the experiments with isolated crypts, the following buffers were used. The ethylene diamino tetraacetic acid (EDTA) solution for the crypt isolation contained (in mM): 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 bovine serum albumin. The pH was adjusted to 7.4 by Tris. The high K^+ Tyrode for the storage of the crypts consisted of (in mM): K gluconate 100, KCl 30, NaCl 20, $CaCl_2$ 1.25, $MgCl_2$ 1, HEPES 10, glucose 12, Na pyruvate 5 and 1 g/l bovine serum albumin. The solution was adjusted with KOH to a pH of 7.4. The medium for the superfusion of the crypts was a Tyrode solution containing (in mM): NaCl 140, KCl 5.4, $CaCl_2$ 1.25, $MgCl_2$ 1, glucose 12, HEPES 10. The pipette solution was a K gluconate/KCl solution, which contained (in mM): K gluconate 100, KCl 30, NaCl 10, $MgCl_2$ 2, EGTA 0.1, Tris 10, ATP 5; pH was 7.2. For measurements with the perforated patch method, the ionophore nystatin was used. From a stock solution containing 50 g/l nystatin dissolved in dimethylsulfoxide (DMSO), 6 μl were added to 1-ml pipette solution just prior the beginning of the experiment.

2.2. Tissue preparation

Wistar rats were used with a weight of 180–220 g. The animals had free access to water and food until the day of the experiment. Animals were stunned by a blow on the head and killed by exsanguination (approved by Regierungspräsidium Gießen, Gießen, Germany). The serosa and muscularis propria were stripped away by hand to obtain the mucosa–submucosa preparation of the colon descendens. Two segments of the distal and the proximal colon of each rat were prepared.

2.3. Short-circuit current measurement

The tissue was mounted in a modified Ussing chamber, bathed with a volume of 3.5 ml on each side of the mucosa and short-circuited by a voltage clamp (Ing. Buero Mußler, Aachen, Germany) with correction for solution resistance. The exposed surface of the tissue was 1 cm^2. Short-circuit current (Isc) was continuously recorded and tissue conductance (Gt) was measured every min. Isc is expressed as $\mu Eq \cdot h^{-1} \cdot cm^{-2}$, i.e., the flux of a monovalent ion per time and area with $1\ \mu Eq \cdot h^{-1} \cdot cm^{-2} = 26.9\ \mu A \cdot cm^{-2}$. Tissues were left for 1 h to stabilize Isc before the effect of drugs was studied. The baseline in electrical parameters was determined as mean over 3 min just before administration of a drug. In those experiments, in which the Isc did not stabilize, i.e., the administration of drugs during the decaying phase of the nystatin-induced Isc or under basolateraly depolarized conditions, the theoretical course of Isc was calculated by linear regression analysis. To do this, the Isc 3 min prior administration of the drug (30 data points, as Isc was registered every 6 s) was used to calculate the regression line. Only regressions exceeding with $r^2 > 0.8$ were used. This regression served to extrapolate the decay of Isc in the absence of GEA 3162, which was subtracted from the maximal increase in Isc evoked by GEA 3162 as depicted in the inset of Fig. 7A.

2.4. Crypt isolation

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 isolate intact crypts. They were collected in an intracellular-like high K^+ Tyrode buffer (Böhme et al., 1991). The mucosa was kept at 37 °C during the isolation procedure. All further steps including the patch-clamp experiments were carried out at room temperature.

2.5. Patch-clamp experiments

The crypts were pipetted into the experimental chamber (volume of the chamber 0.5 ml). The crypts were fixed to the glass bottom of the chamber with the aid of poly-L-lysine (0.1 g/l). The preparation was superfused hydrostatically throughout the experiment (perfusion rate about 1 ml/min). The chamber was mounted on the stage of an inverted microscope (Olympus IX-70).

Patch pipettes were pulled from thick-walled borosilicate glass capillaries (Jencons Scientific, Bedfordshire, UK;

outer diameter 2 mm, inner diameter 1–1.25 mm) on a two-stage puller (H. Ochotzki, Homburg/Saar, Germany). The pipette tip was prefilled with the normal pipette solution by dipping it into this solution for 8–10 s. The pipette was then backfilled with a solution containing nystatin using a syringe. Opening of the patch was indicated by an increase of the capacitance, a decrease of the resistance, and a stable membrane potential under current-clamp conditions. Membrane capacitance was corrected for by cancellation of the capacitance transient (subtraction) using a 50 mV pulse.

Patch-clamp currents were recorded on a RK-400 amplifier (Biologics, Meylan, France). Current and voltage signals were digitized at 48 kHz and stored on a modified digital audio recorder (DTR-1200, Biologics, Meylan, France). The reference point for the patch potentials was the extracellular side of the membrane assumed to have zero potential. Current–voltage (I–V) curves were obtained by clamping the cell to a holding potential of −80 mV and stepwise depolarization for 30 ms. After each depolarization, the cell was clamped again to the holding potential for 1 s before the following voltage step (incremented by 10 mV) was applied. For statistical comparison of membrane currents, outward current was measured at the end of a pulse depolarizing the cell for 30 ms from −80 to +60 mV, and inward current was measured at the holding potential of −80 mV.

2.6. Fura-2 measurements

Relative changes in intracellular Ca^{2+} concentration were measured using the Ca^{2+}-sensitive fluorescent dye, fura-2 (Grynkiewicz et al., 1985), as described previously (Diener et al., 1991). The crypts were pipetted into the experimental chamber with a volume of about 3 ml. The crypts were fixed to the glass bottom of the chamber with the aid of poly-L-lysine. The crypts were loaded for 60 min with 2.5×10^{-6} M fura-2/AM (fura-2-acetoxymethylester) in the presence of 0.05% Pluronic® (Molecular Probes, Leiden, Netherlands). Then the fura-2 was washed away. The preparation was superfused hydrostatically throughout the experiment with NaCl Ringer. Perfusion rate was about 1 ml/min.

Experiments were carried out on an inverted microscope (Olympus IX-50) equipped with an epifluorescence set-up 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.

2.7. Drugs

Fura-2/AM (Molecular Probes, Leiden, Netherlands), (9S,10R,12R)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1-*H*-diindolol[1,2,3-fg:3′,2′,1′-k] pyrrolol [3,4-i][1,6]benzodiazocine-10-carboxylic acid methyl ester (KT 5823, Calbiochem, Bad Soden, Germany), 1*H*-[1,2,4]Oxodiazolo[4,3-a]quinoxalin-1-one (ODQ), staurosporine and *S*-nitroso-*N*-acetylpenicillamine (SNAP) were dissolved in dimethylsulfoxide (DMSO; final maximal concentration 2.5 ml/l). Pluronic® was dissolved in DMSO as a 200 g/l stock solution (final DMSO concentration 2.5 ml/l). Nystatin was dissolved in dimethylsulphoxide (DMSO; final concentration 2 ml/l). *N*-Ω-nitro-L-arginine (L-NNA) was dissolved as a stock solution in 1 M HCl, tetrodotoxin (TTX) was dissolved as a stock solution in citrate buffer (20 mM). 6-Anilino-5,8-quinolinequinone (LY 83583), bumetanide and indomethacin were added from ethanolic stock solutions (final maximal concentration 2.5 μl/ml); carbachol, 1,2,3,4-oxatriazolium,5-amino-3-(3,4-dichlorophenyl)-chloride (GEA 3162, Alexis, Grünberg, Germany), *N*(g)-nitro-L-arginine methylester hydrochloride (L-NAME, Tocris Cookson, Bristol, UK), sodium nitroprusside were dissolved in aqueous stock solution and diluted in salt buffer just before use. If not indicated differently, drugs were from Sigma, Deisenhofen, Germany.

2.8. Statistics

Results are given as means ± 1 S.E.M. When the means of several groups had to be compared first, an analysis of variances was performed. If the analysis of variances indicated significant differences between the groups investigated, further comparison was carried out by a Student's *t*-test (paired or unpaired as appropriate) or by the Mann Whitney *U*-test. An *F*-test was applied to decide which test method was to be used. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Comparison of different nitric oxide-liberating drugs

Before testing the possible involvement of nitric oxide (NO) in the regulation of store-operated cation conductances, we first compared the efficiency of structurally different NO-liberating drugs, i.e., *S*-nitroso-*N*-acetylpenicillamine (SNAP), sodium nitroprusside and the lipophilic compound 1,2,3,4-oxatriazolium,5-amino-3-(3,4-dichlorophenyl)-chloride (GEA 3162), to induce a change in short-circuit current (Isc). The most efficient one proved to be GEA 3162, which concentration-dependently increased Isc (Fig. 1). In the distal colon, the maximally tested concentration of 5×10^{-4} M (at the serosal side) induced an increase in Isc of $4.1 \pm 0.4\ \mu Eq \cdot h^{-1} \cdot cm^{-2}$ ($n = 6, P < 0.05$). At this concentration, Gt increased by $2.2 \pm 0.5\ mS \cdot cm^{-2}$ ($n = 6, P < 0.05$). In contrast, the two other NO-liberating drugs, i.e., SNAP and sodium nitroprusside, were less effective. At a concentration of 5×10^{-5} M (at the serosal side), SNAP induced only a

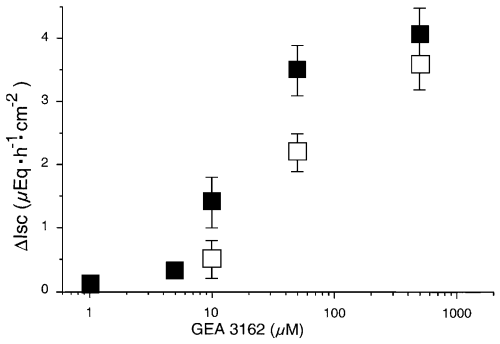

Fig. 1. Concentration-dependent stimulation of Isc by GEA 3162 (administered in a cumulative manner at the serosal side) in rat distal (filled squares, $n=11$) and proximal colon (open squares, $n=10$). Values are given as differences to the baseline prior to drug administration (ΔIsc) and are means (symbols) ± S.E.M. (error bars). The threshold, at which GEA 3162 induced a first significant increase in Isc, was 5×10^{-6} M in the distal and 5×10^{-5} M in the proximal colon.

small increase in Isc of 0.5 ± 0.2 μEq·h^{-1}·cm^{-2} ($n=6$, $P<0.05$ versus baseline), whereas in the presence of sodium nitroprusside at a maximally effective concentration of 5×10^{-4} M (at the serosal side), Isc increased by 2.0 ± 0.5 μEq·h^{-1}·cm^{-2} ($n=6$, $P<0.05$ versus baseline). The proximal colon exhibited a smaller sensitivity against GEA 3162 (Fig. 1). Therefore, we focused on the action of the NO-liberating compound GEA 3162 in the distal colon.

3.2. GEA 3162 possesses epithelial action sites

Previous data obtained at colonic epithelia revealed that the Isc, which was stimulated by sodium nitroprusside via NO liberation, was due to anion secretion (Wilson et al., 1993; Stack et al., 1995). The same result was observed for GEA 3162. In the absence of Cl^- ions in the bathing solution, GEA 3162 (5×10^{-5} M at the serosal side) caused only a small increase in Isc of 0.5 ± 0.1 μEq·h^{-1}·cm^{-2} ($n=6$), significantly lower compared to the Isc stimulated by GEA 3162 in the presence of Cl^- (3.0 ± 0.4 μEq·h^{-1}·cm^{-2}, $n=6$, $P<0.05$ versus response in the absence of Cl^-). Bumetanide (10^{-4} M at the serosal side), a blocker of the main entry pathway for Cl^- ions across the basolateral membrane, i.e., the Na^+–K^+—2 Cl^- cotransporter, abolished the GEA 3162-induced Isc (Fig. 2). After pretreatment of the tissues with the blocker of cystic fibrosis transmembrane regulator (CFTR) Cl^- channel, glibenclamide (5×10^{-4} M at the mucosal side, for baseline effect on Isc see Table 1), GEA 3162 (5×10^{-4} M at the serosal side) caused even a long-lasting decrease in Isc by -0.8 ± 0.1 μEq·h^{-1}·cm^{-2} ($n=9$, $P<0.05$). In four out of the nine tissues tested, this decrease was preceded by a transient increase in Isc, which amounted to 0.4 ± 0.1 μEq·h^{-1}·cm^{-2}. Taken together, these data reveal that the GEA 3162-induced current is due to Cl^- secretion.

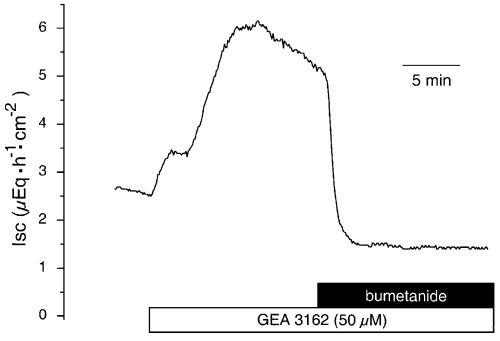

Fig. 2. GEA 3162 (5×10^{-5} M at the serosal side) induced an Isc, which was abolished by bumetanide (10^{-4} M at the serosal side). The recording is typical for six tissues, in which GEA 3162 (5×10^{-5} M at the serosal side) stimulated an increase in Isc by 2.2 ± 0.3 μEq·h^{-1}·cm^{-2} above the former baseline ($P<0.05$). Subsequent administration of bumetanide decreased Isc by 3.3 ± 0.4 μEq·h^{-1}·cm^{-2} ($P<0.05$).

Table 1
Baseline effects of different inhibitors on Isc in the rat distal colon

	ΔIsc (μEq·h^{-1}·cm^{-2})*	n
Glibenclamide	-1.4 ± 0.2 *	9
Indomethacin	-1.7 ± 0.4 *	6
L-NNA	-0.3 ± 0.0 *	7
Tetrodotoxin	-0.9 ± 0.2 *	10

Glibenclamide (5×10^{-4} M at the mucosal side), indomethacin (10^{-6} M at the serosal side), *N*-Ω-nitro-L-arginine (L-NNA, 10^{-3} M at both sides) and tetrodotoxin (10^{-6} M at the serosal side). Values are given as differences to the baseline prior drug administration (ΔIsc) and are means ± S.E.M.

* $P<0.05$ versus baseline.

Next we focused on the mechanism of GEA-induced secretion. In various cells, e.g., in parotid acini or the colonic HT29/B6 cell line (Watson et al., 1999; Bischof et al., 1995), it was shown that NO causes an increase of the cytoplasmic Ca^{2+} concentration. Therefore, the Ca^{2+}-dependence of GEA 3162-induced Cl^- secretion was investigated. As depicted in Fig. 3, a Ca^{2+}-dependent and -independent component of the GEA 3162 response emerged in relation to the concentration used. Under Ca^{2+}-free serosal condi-

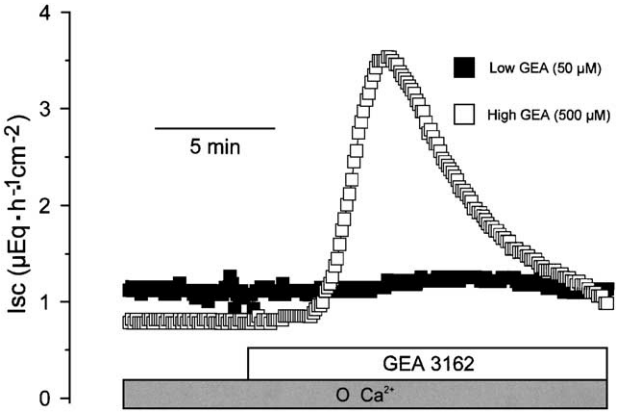

Fig. 3. Effect of GEA 3162 in a concentration of 5×10^{-5} M (filled squares) and 5×10^{-4} M (open squares) on Isc in the absence of serosal Ca^{2+}. The recordings are typical for six tissues for each concentration investigated. In total, GEA 3162 evoked a maximal increase in Isc of 0.3 ± 0.1 μEq·h^{-1}·cm^{-2} ($n=6$, not significant) at a concentration of 5×10^{-5} M and of 3.0 ± 0.4 μEq·h^{-1}·cm^{-2} ($n=6$, $P<0.05$ versus baseline) at a concentration of 5×10^{-4} M.

tions, a low concentration of GEA 3162 (5×10^{-5} M at the serosal side) stimulated Isc only by 0.3 ± 0.1 μEq·h^{-1}·cm^{-2} ($n=8$), much lower compared to 3.0 ± 0.4 μEq·h^{-1}·cm^{-2} in the presence of serosal Ca^{2+} ($n=6$, $P<0.05$ versus response in the absence of serosal Ca^{2+}). In contrast, when GEA 3162 was administered in a higher concentration (5×10^{-4} M at the serosal side), the drug stimulated a Ca^{2+}-independent Isc of 2.9 ± 0.5 μEq·h^{-1}·cm^{-2} ($n=8$, $P<0.05$, Fig. 3), not significantly different from the GEA 3162-induced current in the presence of serosal Ca^{2+}, suggesting at least two different action sites of the NO donor.

In the following set of experiments the contribution of subepithelial cells and the enteric nervous system to the GEA 3162-induced secretion was investigated. Both the neurotoxin tetrodotoxin (TTX, 10^{-6} M at the serosal side), as well as indomethacin (10^{-6} M at the serosal side, for baseline effects of the blockers see Table 1), reduced but did not abolish the action of GEA 3162 on Isc (Table 2).

3.3. Effect of GEA 3162 on isolated rat colonic crypt cells

Fura-2 experiments at isolated colonic crypts revealed that the Ca^{2+}-dependent Cl^- secretion observed in the Ussing chamber experiments was concomitant with a change in the intracellular Ca^{2+} concentration. In the presence of Ca^{2+} in the bathing solution, GEA 3162 (5×10^{-5} M) evoked an increase of the fluorescence ratio of fura-2 of 0.37 ± 0.06 ($n=6$, $P<0.05$, Fig. 4A) concordant with an increase in the intracellular Ca^{2+} concentration. This effect was completely dependent on the presence of extracellular Ca^{2+}. When the drug was applied in the absence of Ca^{2+} in the superfusion medium, GEA 3162 did no more induce an increase of the fluorescence ratio, but rather a small decrease by 0.09 ± 0.02 ($n=6$, $P<0.05$, Fig. 4B) was observed.

In order to find out whether the dependence of the GEA 3162 response from the presence of extracellular Ca^{2+} might be caused by an activation of the nonselective cation conductance mediating store-operated Ca^{2+} influx in rat colonic epithelium (Frings et al., 1999), patch-clamp studies were performed at isolated colonic crypts. In the nystatin-perforated whole cell configuration, the membrane potential, as well as membrane current, were altered by GEA 3162. In the presence of GEA 3162 (5×10^{-5} M), the membrane depolarized from -36 ± 4 to -30 ± 4 mV ($n=6$, $P<0.05$, Fig. 5A). This effect was concomitant with an increase in membrane current (Fig. 5B). The GEA 3162-induced current had a the reversal potential of -9 ± 8 mV, which was not significantly different from 0 mV as one should expect for a nonselective cation conductance with equal cation concentrations at both sides of the membrane. These data obtained at isolated crypt cells clearly show that the NO-liberating drug stimulates an increase of the cytoplasmic Ca^{2+} concentration, most probably mediated by a stimulation of the store-operated nonselective cation conductance.

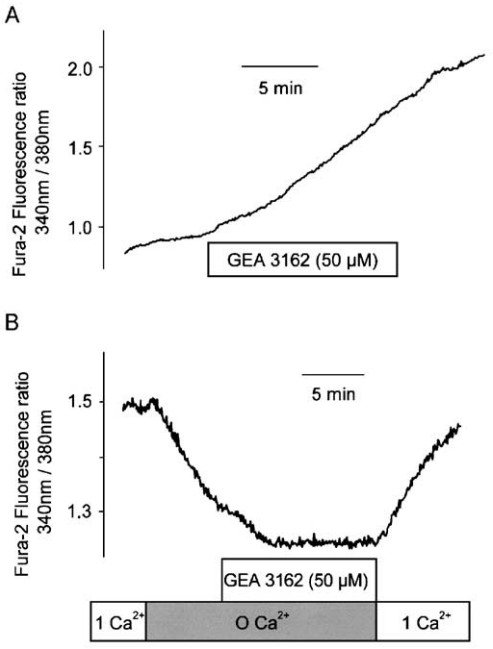

Fig. 4. Effect of GEA 3162 (5×10^{-5} M) on intracellular Ca^{2+} concentration, expressed as changes in the ratio of emission of fluorescent light by fura-2 when excited at 340 and 380 nm, respectively. (A) In the presence of extracellular Ca^{2+}, GEA 3162 evoked a rise in the intracellular Ca^{2+} concentration as indicated by an increase of the fluorescence ratio. This recording is typical for six experiments with similar results (for statistics, see text). (B) Changing the bathing solution to a nominally Ca^{2+}-free one caused a significant decrease of the fluorescence ratio, which was further strengthened in the presence of GEA 3162. This recording is typical for six experiments with similar results.

Table 2
Effects of the neurotoxin tetrodotoxin and indomethacin on the GEA 3162-induced Isc in the rat distal colon

	ΔIsc (μEq·h^{-1}·cm^{-2})		n
	GEA 3162 (5×10^{-5} M)	GEA 3162 (5×10^{-4} M)	
GEA 3162 alone	3.0 ± 0.4*	4.1 ± 0.4*	6–8
+ Tetrodotoxin	1.6 ± 0.2*,**	0.9 ± 0.3*,**	6–10
+ Indomethacin	3.1 ± 0.7*	2.1 ± 0.4*,**	6–7

GEA 3162 was administered in the indicated concentrations at the serosal side, indomethacin (10^{-6} M at the serosal side) and tetrodotoxin (10^{-6} M at the serosal side). Values are given as difference to the baseline prior drug administration (ΔIsc) and are means ± S.E.M.

* $P<0.05$ versus baseline.

** $P<0.05$ versus GEA 3162 alone.

3.4. Carbachol-induced increase in Isc is independent from production of NO

Is NO the physiological signal, which is responsible for the communication between the intracellular Ca^{2+} stores and the cation channels in the plasma membrane? In order to answer this question, the effect of the Ca^{2+}-dependent secretagogue, carbachol, on Isc was investigated in the presence of a broad blocker of NO synthases, i.e., *N*-Ω-nitro-L-arginine (L-NNA). As reported earlier (Strabel and

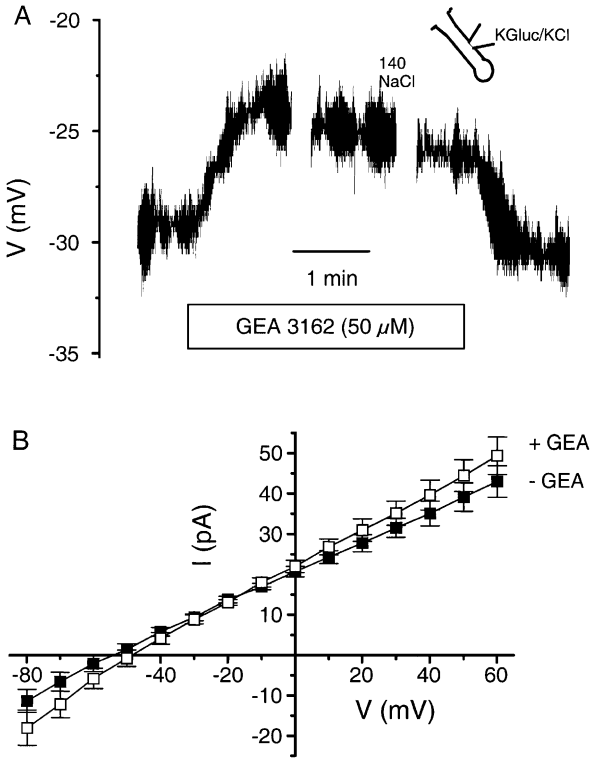

Fig. 5. Effect of GEA 3162 (5×10^{-5} M) on membrane potential and whole-cell current in isolated crypt cells using the nystatin-perforated whole-cell configuration. (A) Depolarization induced by GEA 3162 at a cell located at the middle region of a crypt as indicated by the schematic drawing. The interruptions in the voltage tracing are caused by measurements of IV relations in the voltage-clamp mode. (B) IV relation of the current stimulated by GEA 3162 (absence or presence of GEA 3162: filled squares and open squares, respectively). The inward current, measured at a holding potential of -80 mV, as well as the outward current, measured at a holding potential of $+60$ mV, increased significantly in the presence of GEA 3162 (5×10^{-5} M, $P<0.05$). Values are means (symbols) ± S.E.M. (error bars), $n=6$.

Diener, 1995), carbachol evokes a biphasic increase in Isc, a peak followed by a slowly decaying plateau. Under control conditions, the peak, i.e., the maximal Isc (I_{max}) induced by carbachol (5×10^{-5} M at the serosal side) amounted to 9.7 ± 0.9 μEq·h^{-1}·cm^{-2}, which during the plateau phase (measured 10 min after administration of the cholinergic agonist, I_{10}) had decayed to a value of 3.1 ± 0.5 μEq·h^{-1}·cm^{-2} ($n=6$, $P<0.05$ versus baseline for both data). In the presence of L-NNA (10^{-3} M at the mucosal and the serosal side, for effects of the drug on baseline see Table 1), I_{max} induced by carbachol was 9.2 ± 1.2 μEq·h^{-1}·cm^{-2} and declined to an I_{10} of 3.3 ± 0.5 μEq·h^{-1}·cm^{-2} ($n=7$). A similar failure to inhibit carbachol-evoked secretion has already been observed with another NO synthase inhibitor, *N*(g)-nitro-L-arginine methylester (L-NAME), or with lower concentrations of L-NNA (Schultheiss et al., 2001). Consequently, a production of NO is probably not involved in the physiological communication between the intracellular Ca^{2+} stores and the plasma membrane.

This conclusion was supported by experiments, in which a possible cross-desensitization between GEA 3162-and carbachol-induced Cl^- secretion was investigated. GEA 3162 (5×10^{-5} M at the serosal side) stimulated an increase in Isc of 1.1 ± 0.3 μEq·h^{-1}·cm^{-2} ($n=7$, $P<0.05$). When carbachol was administered about 10 min after GEA 3162, the cholinergic agonist still stimulated an increase in Isc amounting to 8.1 ± 1.8 and 2.1 ± 0.8 μEq·h^{-1}·cm^{-2} ($P<0.05$ to baseline) for I_{max} and I_{10}, respectively. This response was not significantly different from a control, in which carbachol was administered without prior application of GEA, in which carbachol stimulated an I_{max} of 8.0 ± 1.5 and an I_{10} of 2.8 ± 0.8 μEq·h^{-1}·cm^{-2} ($n=7$, $P<0.05$ versus baseline), respectively.

3.5. Additional action sites of GEA 3162

The above data demonstrate (see Fig. 3) that in a higher concentration, i.e., 5×10^{-4} M, GEA 3162 evokes a Ca^{2+}-independent secretion. In order to localize the action sites, either the apical or the basolateral membrane were selectively eliminated in Ussing chambers.

First, the basolateral membrane was electrically abolished by increasing the K^+ concentration of the basolateral bathing solution (Fuchs et al., 1977; Schultheiss and Diener, 1997). Isoosmolar replacement of Na^+ by K^+ on the basolateral side resulted in a decrease in Isc from 6.1 ± 0.7 to -4.5 ± 0.7 μEq·h^{-1}·cm^{-2} ($n=6$, $P<0.05$). In the presence of a serosally to mucosally directed Cl^- gradient (111.5 mM KCl solution at the serosal side, 107 mM NaGluc/4.5 KCl mM solution at the mucosal side), GEA 3162 (5×10^{-4} M, at the serosal side) induced an increase in Isc of 1.1 ± 0.2 μEq·h^{-1}·cm^{-2} ($n=6$, $P<0.05$, Fig. 6). This response could be totally prevented by pretreatment with glibenclamide (5×10^{-4} M, at the mucosal side, $n=8$, Table 3), a blocker of the apical CFTR Cl^- channel,

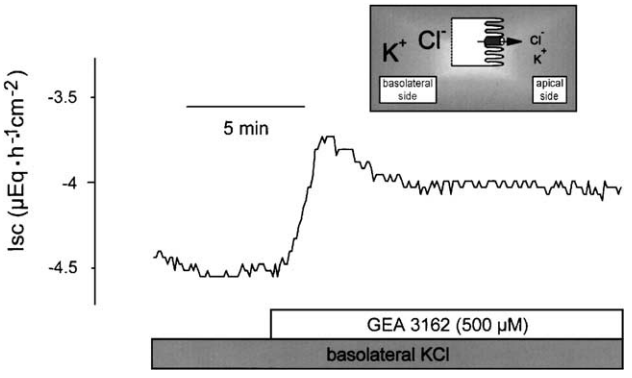

Fig. 6. Action of GEA 3162 (5×10^{-4} M at the serosal side) under conditions in which the basolateral membrane was depolarized by a high K^+ concentration (111.5 mmol·l^{-1} KCl solution at the serosal side; gray bar) in the presence of a mucosally directed Cl^- gradient (107 mmol·l^{-1} NaGluc/4.5 mmol·l^{-1} KCl solution at the mucosal side). The schematic drawing summarizes the experimental conditions. The line tracing is typical for six experiments with similar results (for statistics, see Table 3).

Table 3
Effects of GEA 3162 on Isc in the presence of inhibitors after basolateral depolarization of the rat distal colon

	ΔIsc (μEq·h^{-1}·cm^{-2})	n
GEA 3162 alone	1.1 ± 0.2*	6
Glibenclamide	− 0.5 ± 0.2	8
+GEA 3162	0.0 ± 0.1**	
Indomethacin	0.2 ± 0.3	5
+GEA 3162	1.6 ± 0.5*	
LY 83583	0.0 ± 0.0	10
+GEA 3162	0.4 ± 0.1*,**	
ODQ	0.3 ± 0.1	8
+GEA 3162	0.9 ± 0.2*	
KT 5823	0.5 ± 0.3	6
+GEA 3162	0.8 ± 0.1*	
Staurosporine	0.9 ± 0.2*	9
+GEA 3162	0.7 ± 0.1*	

GEA 3162 (5×10^{-4} M, at the serosal side), glibenclamide (5×10^{-4} M at the mucosal side), indomethacin (10^{-6} M at the serosal side), KT 5823 (10^{-6} M at both sides), ODQ (10^{-5} M at both sides), LY 83583 (10^{-5} M at both sides) and staurosporine (10^{-6} M at the serosal side). Values are means ± S.E.M.

* $P < 0.05$ versus baseline.

** $P < 0.05$ versus GEA 3162 alone.

indicating that the NO donor induces the opening of apical Cl^- channels. Interestingly, pretreatment with indomethacin (10^{-6} M, at the serosal side), which significantly inhibited GEA 3162-evoked Isc in normal tissues at this concentration (see above), did not affect the GEA 3162-induced Isc under basolateral depolarized conditions (Table 3). In order to investigate the messenger involved in the regulation of this apical Cl^- conductance, two blockers of the soluble guanylate cyclase, i.e., 6-anilino-5,8-quinolinequinone (LY 83583; Mülsch et al., 1989) and 1H-[1,2,4]oxodiazolo[4,3-a]quinoxalin-1-one (ODQ; Garthwaite et al., 1995), were used. LY 83583 (10^{-5} M, at both sides) did not change the baseline Isc, but significantly inhibited GEA 3162-stimulated increase in Isc (5×10^{-4} M at the serosal side, Table 3). In contrast to LY 83583, ODQ (10^{-5} M, at both sides) did not affect the GEA 3162-activated Isc (5×10^{-4} M at the serosal side, Table 3). Due to these divergent results, the involvement of cGMP-regulated enzymes, i.e., protein kinases was studied. However, neither the specific blocker of protein kinase G, KT 5823 (10^{-6} M at both sides), nor the unspecific protein kinase blocker staurosporine (10^{-6} M, at the serosal side) had any effect on the Cl^- current across the apical membrane stimulated by GEA 3162 (Table 3).

To study the effect of the NO donor on the basolateral membrane, the apical membrane was bypassed by the ionophore, nystatin (100 μg/ml at the mucosal side). In the presence of a mucosally to serosally directed K^+ gradient (98.5 mM NaCl/13.5 mM KCl solution at the mucosal side, 107 mM NaCl /4.5 mM KCl solution at the serosal side), nystatin induced a maximal increase in Isc of 17.6 ± 2.0 μEq·h^{-1}·cm^{-2} ($n = 7$, $P < 0.05$, Fig. 7A). When applied during the decaying phase of nystatin-induced Isc, GEA 3162 (5×10^{-4} M at the serosal side) evoked an increase in Isc by 1.0 ± 0.2 μEq·h^{-1}·cm^{-2} ($n = 6$, $P < 0.05$). This current might represent a current via basolateral K^+ channels, the basolateral Na^+–K^+-ATPase, or both. To distinguish between these possibilities, NaCl was replaced by *N*-methyl-D-glucamine ($NMDG^+$) Cl^-. Under these conditions, GEA 3162 (5×10^{-4} M at the serosal side) revealed a moderate but significant increase in Isc of 0.3 ± 0.1 μEq·h^{-1}·cm^{-2} ($n = 6$), indicating the activation of basolateral K^+ conductances. This effect cannot be ascribed to a local rise in the intracellular Ca^{2+} concentration, since even in the absence of serosal Ca^{2+} GEA 3162 (5×10^{-4} M at the serosal side) evoked a transient increase in Isc of 1.0 ± 0.4 μEq·h^{-1}·cm^{-2} under these conditions ($n = 5$, $P < 0.05$). The subsequent administration of Ca^{2+} (10^{-3} M) to the serosal bathing solution evoked a further transient

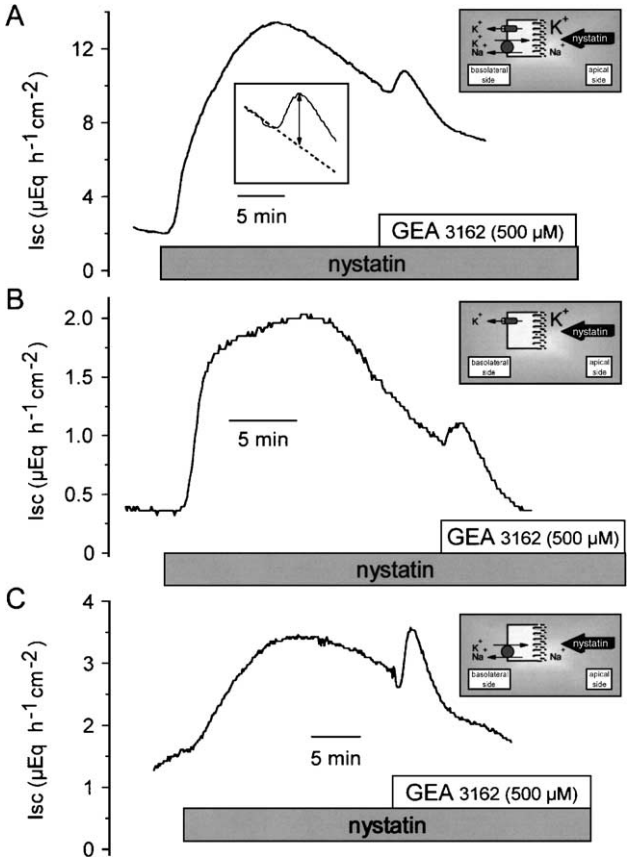

Fig. 7. Effects of GEA 3162 (5×10^{-4} M at the serosal side, white bars) on currents across the basolateral membrane in tissues, which were apically permeabilized by nystatin (100 μg·ml^{-1} at the mucosal side, gray bars). The schematic drawings summarize the individual experimental conditions. (A) Total response in Isc evoked by nystatin and GEA 3162 in the presence of mucosally Na^+ and a mucosally to serosally oriented K^+ gradient (carried by the Na^+–K^+-ATPase and by current across basolateral K^+ channels). The schematic drawing in the middle depicts the method for estimation of ΔIsc (for details see Section 2) (B) Response of Isc to nystatin and GEA 3162 in the presence of a K^+ gradient, but in the absence of Na^+ (= current across basolateral K^+ channels). (C) Isc evoked by nystatin and GEA 3162 in the presence of Na^+ but in the absence of a K^+ gradient (= current carried by the Na^+–K^+-ATPase). The original recordings are typical for six tissues with same results (for statistics, see text).

increase in Isc of 0.6 ± 0.3 μEq·h^{-1}·cm^{-2} which, however, did not reach statistical significance.

In the absence of a K^+ gradient and in the presence of Na^+ for investigating the effect on the Na^+–K^+-ATPase, GEA 3162 (5×10^{-4} M at the serosal side) also induced a pronounced increase of Isc which amounted to 1.4 ± 0.6 μEq·h^{-1}·cm^{-2} ($n = 6$, $P < 0.05$ versus baseline), suggesting the stimulation of the Na^+–K^+-pump by the NO donor.

4. Discussion

The results of the present study demonstrate that the NO donor GEA 3162 evokes a Cl^- secretion which, in dependence on the concentration used, is either Ca^{2+}-dependent or independent from the presence of extracellular Ca^{2+}. The action sites of the drug are a nonselective cation conductance, basolateral K^+ conductances, apical Cl^- channels and the Na^+–K^+-ATPase.

GEA 3162 releases NO in aqueous solutions (Kankaanranta et al., 1996; Holm et al., 1998), but the exact mechanism of this reaction is not yet totally solved (Malo-Ranta et al., 1994). Also, in isolated rat colonic crypts GEA 3162 releases NO as observed in experiments with the fluorescent dye, DAF-2 DA (data not shown), which responds to NO with an increase in its fluorescence signal (Berkels et al., 2000). Comparison with other NO-liberating substances revealed that this lipophilic compound was more effective in inducing an Isc than other, well known NO-liberating compounds, i.e., SNAP or sodium nitroprusside. Such a superior efficiency of GEA 3162 compared to other, conventional NO liberators has already been observed in other cells such as human lymphocytes (Kosonen et al., 1997) or smooth muscle (Favre et al., 1998).

In vivo NO is released from the guanidino group of L-arginine by NO synthases and virtually all mammalian cells are able to produce it. In the gut, NO originates from the enteric nervous system, the epithelium and subepithelial cells (for review, see, e.g., Alican and Kubes, 1996). In the rat colon, the constitutive form of NO synthase is present in the lamina propria and epithelial cells as shown by immunolocalization (Wilson et al., 1996). This form of the NO synthase is regulated by Ca^{2+}, calmodulin and oxidative cofactors and is very likely active in liberating NO under basal conditions in our preparation because the nonselective NO synthase blocker L-NNA at higher concentrations reduced the baseline Isc in the rat distal colon (see Table 1).

The effect of NO in rat colon is partially localized directly at the epithelial cells as revealed by experiments with isolated crypt cells and the partial resistance of the secretory response against TTX and indomethacin. At first glance, these data seem to be in conflict with the previous observation that simultaneous administration of TTX and the cyclooxygenase blocker piroxicam practically abolished the NO-induced Cl^- secretion in rat and human colon (Wilson et al., 1993; Stack et al., 1995), which had led to the conclusion that the NO response is mediated by the enteric nerves and prostanoid-producing cells. However, as shown previously, a combination of TTX together with a cyclooxygenase inhibitor suppresses completely any Cl^- secretion induced by Ca^{2+}-dependent secretagogues because their action is restricted to the opening of basolateral K^+ channels, leading only to a transepithelial anion secretion if a continuous production of cAMP, triggered by neurotransmitters and prostaglandins, keeps apical Cl^- channels open (Böhme et al., 1991; Strabel and Diener, 1995).

One action of the NO donor is the stimulation of Ca^{2+} influx from the extracellular medium into the cell. This is demonstrated by the dependence of the secretory response evoked by the low concentration (5×10^{-5} M) of GEA 3162 from the presence of extracellular Ca^{2+} in Ussing chambers (Fig. 3), and by fura-2 experiments, which reveal that the drug stimulates an increase in the cytosolic Ca^{2+} concentration only in the presence of extracellular Ca^{2+} (Fig. 4). Influx of Ca^{2+} in rat colonic epithelium can occur via a nonselective cation conductance (Frings et al., 1999), and apparently this conductance is stimulated by GEA 3162 as indicated by whole-cell patch-clamp experiments (Fig. 5). Similar results, i.e., an increase in cytoplasmic Ca^{2+} in the presence of a NO donor, sodium nitroprusside, have been obtained in the human colonic tumor cell line HT29/B6 (Bischof et al., 1995).

An alternative explanation for the activation of the store-operated nonselective cation conductance could be that GEA 3162, i.e., NO, might stimulate the release of Ca^{2+} from intracellular stores as it has been shown, e.g., for mouse parotid acini (Watson et al., 1999), neurosecretory PC12 cells (Clementi et al., 1996), hepatocytes or endothelial cells (for review, see Clementi, 1998) and the depleted stores generate an activating signal. However, if this would be the case, GEA 3162 still should cause an increase in the cytoplasmic Ca^{2+} concentration in the nominal absence of extracellular Ca^{2+}, which was never observed (Fig. 4B). Furthermore, L-NNA or L-NAME, i.e., blockers of different NO synthases, did not affect the secretory response evoked by carbachol (see also Schultheiss et al., 2001). Therefore, it seems to be quite unlikely that in rat colonic epithelium NO is a signal which transmits a decreased filling state of Ca^{2+} stores to activate the store-operated nonselective cation conductance as it was suggested for other epithelia such as HT29/B6 cells (Bischof et al., 1995) or for mouse parotid acini (Watson et al., 1999). Ca^{2+}-induced Cl^- secretion evoked by store depletion is characterized by a pronounced desensitization (see, e.g., Diener et al., 1989). However, pretreatment with GEA 3162 did not affect the carbachol response. Thus, NO seems to be a signaling mechanism in addition to the signal(s) produced by Ca^{2+}-depleted intracellular stores, which activates the same or a closely related Ca^{2+} entry from the extracellular side as it was previously described for smooth muscle cells DDT_1MF_2 (Van Rossum et al., 2000).

At higher concentrations, the NO donor exerts its secretory action via additional action sites located either at the apical, as well as the basolateral membrane. After permeabilization of the apical membrane with nystatin, GEA 3162 (5×10^{-4} M) stimulated a K^+ current across basolateral K^+ channels (Fig. 7B). This might be easily explained by the well-known Ca^{2+} dependence of cellular K^+ conductance (Böhme et al., 1991). However, also with a nominally Ca^{2+}-free serosal solution, GEA 3162 induced again a transient but significant increase in Isc. Thus, it is possible that NO activates a basolateral K^+ conductance in a Ca^{2+}-independent manner as it was described for vascular smooth muscle, in which a Ca^{2+}-dependent K^+ conductance was directly stimulated by NO (Bolotina et al., 1994). The third action site on the same membrane is the Na^+-K^+-ATPase. Fig. 7C clearly shows that in the presence of Na^+ and the absence of a K^+ gradient, GEA 3162 at the higher concentration revealed a pronounced transient increase in Isc. The mechanism behind this activation is unknown.

The last action site of GEA 3162 is the apical membrane, in which the drug stimulates a CFTR-like Cl^- conductance as indicated by experiments with basolateraly depolarized tissues (Fig. 6). Although NO liberation might stimulate the production of prostaglandins (Wilson et al., 1996), the activation of apical Cl^- channels was resistant against indomethacin. At first glance conflicting results emerged concerning the mechanism of activation by GEA 3162. NO, which is able to stimulate a soluble guanylate cyclase, could mediate its effect via an increase in cGMP, causing the activation of protein kinases and this could result in an increase in Cl^- conductance (for review, see, e.g., Mourad et al., 1999). The inhibitory effect of the blocker of soluble guanylate cyclase, LY 83583, points towards the contribution of this enzyme. However, neither ODQ nor inhibition of the subsequent enzyme in the cGMP cascade, i.e., the protein kinase, by KT 5823, a blocker specific for protein kinase G, or a nonselective protein kinase inhibitor, i.e., staurosporine, did reduce the NO-induced Cl^- secretion, suggesting a direct effect of NO on Cl^- conductances, e.g., by *S*-nitrosylation (Broillet, 1999). The explanation for these conflicting results between LY 83583 and ODQ, staurosporine or KT 5823 is very likely the metabolism of LY 83583, during which superoxide anions can be generated (Mülsch et al., 1989), leading to the formation of H_2O_2. This latter substance, however, was reported to inhibit the cAMP-induced Cl^- secretion in human colonic tumor cells T84 (Duvall et al., 1998). Thus, the apparent inhibitory effect of LY 83583 on the NO-induced Cl^- secretion must not necessarily be related to blockade of cGMP production. Consequently, a direct effect of NO an apical Cl^- conductance, similarly as reported for cyclic nucleotide-gated ion channels in olfactory transduction (Broillet, 1999, 2000), cannot be excluded.

Taken together, our present results indicate that the NO-liberating drug GEA 3162 concentration-dependently activates a Cl^- secretion in a Ca^{2+}-dependent and Ca^{2+}-independent manner involving the activation of nonselective cation conductances, K^+ conductances, Cl^- conductances and the Na^+-K^+-ATPase.

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