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Anion secretion evoked by *Pasteurella multocida* toxin across rat colon

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Abstract

Stimulation of muscarinic receptors is known to have a biphasic effect on colonic Cl^- secretion: a short-lasting activation, which is followed by a long-lasting inhibition. In order to find out, which role G_q proteins play in both processes, *Pasteurella multocida* toxin was used, a known activator of $G\alpha_q$. This toxin (1.5 μg/ml) had a dual action on short-circuit current (I_{sc}) across rat distal colon: it stimulated transiently I_{sc} and subsequently down-regulated the I_{sc} evoked by Ca^{2+}-dependent secretagogues such as acetylcholine or ATP. The inactive mutant (*P. multocida* toxin C1165S), which does not stimulate G^{α}_q, was ineffective. Cl^- dependence and sensitivity against bumetanide, a blocker of the Na^+–K^+–$2Cl^-$ cotransporter, confirmed that the increase in I_{sc} evoked by the toxin represented Cl^- secretion. The effect of *P. multocida* toxin was suppressed by YM-254890 (10^{-7} M), a blocker of $G\alpha_q$. Experiments with apically permeabilized tissues revealed that the secretory response to *P. multocida* toxin was concomitant with an increase in basolateral K^+ conductance as it is observed for other agonists inducing Ca^{2+}-dependent anion secretion. Consequently, these results suggest that G_q proteins are not only involved in the activation of secretion, e.g. after stimulation of muscarinic or purinergic receptors, but also play a central role in the long-term down-regulation of intestinal secretion after activation of these types of receptors.

Keywords: Cl^- secretion; G protein; Rat colon

1. Introduction

Pasteurella multocida is a pathogen responsible for several diseases such as fowl cholera or atrophic rhinitis in pigs (for review see Harper et al., 2006). Beside other virulence factors, strains of this gram-negative bacterium produce a 146 kDa single chain peptide, *P. multocida* toxin, known to act on G proteins of the type $G\alpha_q$ (Wilson et al., 1997), which are activated after endocytic uptake and translocation of the toxin into the cytosol whereas the closely related $G\alpha_{11}$ seems to be unaffected (Zywietz et al., 2001; Orth et al., 2005). Furthermore, *P. multocida* toxin also activates $G_{12/13}$ family protein and RhoA signalling (Orth et al., 2005). As the mechanism of *P. multocida*-induced diarrhea is still incompletely understood (Harper et al., 2006), it seemed to be of interest to investigate the action of *P. multocida* toxin on an intestinal model epithelium, i.e. rat distal colon, which is suitable to study the activation of intestinal Cl^- secretion (see e.g. Diener et al., 1988) as predominant mechanism underlying secretory diarrhea (for review see Binder and Sandle, 1994).

Furthermore, the action of secretagogues such as acetylcholine or its stable analogue, carbachol, which typically induce colonic Cl^- secretion via $G\alpha_q$, is characterized by a biphasic time course unique for the Ca^{2+}-signaling pathway. A transient activation of anion secretion, which can e.g. be recognized by a rapid increase in short-circuit current (I_{sc}), is followed by a long-lasting down-regulation of secretion (Warhurst et al., 1991). The mechanism underlying this inhibition of secretion is only incompletely understood. In colonic tumour cells such as T84 cells, the transactivation of the receptor for the epidermal growth factor (EGF) has been demonstrated to be involved in this response (Keely et al., 1998; Keely and Barrett, 2003), a mechanism, however, which is not observed in intact tissue

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such as rat colonic mucosa (Schultheiss and Diener, 2005). In this tissue, the stimulation of epithelial M1 and M3 receptors (Diener et al., 1989; Haberberger et al., 2006) leads to the stimulation of a phospholipase C (Bolt et al., 1993), the production of inositol 1,4,5-trisphosphate (IP_3) and the subsequent release of Ca^{2+} from intracellular stores via IP_3R2 and IP_3R3 receptors (Siefjediers et al., 2007). The predominant consequence is the activation of basolateral (and apical) Ca^{2+}-dependent K^+ channels, which hyperpolarizes the membrane and thereby favours Cl^- exit across the apical membrane (Böhme et al., 1991) via cAMP-dependent apical Cl^- channels of the CFTR (cystic fibrosis transmembrane regulator) type (Greger, 2000). This anion secretion is terminated by an inhibition of the apical Cl^- conductance in the prolonged presence of the agonist (Schultheiss et al., 2005). As the mechanism of this down-regulation is not really understood, it seemed to be of interest to use *P. multocida* toxin as tool to investigate whether the presumed activation of $G\alpha_q$ is sufficient to mimic both phases of Ca^{2+}-dependent anion secretion, i.e. the transient activation and the subsequent down-regulation.

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 (v/v); the pH was 7.4. In order to apply a mucosally to serosally directed K^+ gradient, the KCl concentration in the mucosal buffer solution was increased to 13.5 mM while reducing equimolarly the NaCl concentration in order to maintain isoosmolarity. For anion replacement experiments, a Cl^--free buffer solution was used containing (in mM): Na gluconate 107, K gluconate 4.5, $NaHCO_3$ 25, Na_2HPO_4 1.8, NaH_2PO_4 0.2, Ca gluconate 5.75, $MgSO_4$ 1, and glucose 12.

For the histochemical experiments, a 100 mM phosphate buffer was used containing 80 mM Na_2HPO_4 and 20 mM NaH_2PO_4, pH was 7.4. DAPI was dissolved in a phosphate-buffered saline (PBS) solution containing (in mM) sodium phosphate buffer 10, NaCl 120, KCl 2.7; pH was 7.4. Fixation was carried out using a 4% (w/v) paraformaldehyde containing 0.9% (w/v) NaCl/50 mM phosphate buffer (in mM: NaCl 154, Na_2HPO_4 40, NaH_2PO_4 10).

2.2. Animals and tissue preparation

Wistar rats were used with a weight of 180–200 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 a mucosa–submucosa preparation of the colon descendens. Two segments of the distal colon of each rat were prepared; in general, one was treated with a potential inhibitor of the *P. multocida* toxin response, the other served as control treated only with the solvent for the drug under investigation.

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 (I_{sc}) was continuously recorded and tissue conductance (G_t) was measured every min. I_{sc} is expressed as μEq/h/cm^2, i.e. the flux of a monovalent ion per time and area with 1 μEq/h/cm^2 = 26.9 μA/cm^2. Tissues were left for about 1 h to stabilize I_{sc}, before the effect of drugs was studied. The baseline in electrical parameters was determined as the mean over 3 min measured just before administration of a drug and effects of drugs were given as a difference in this baseline (ΔI_{sc}).

2.4. Morphology

The tissue was fixed overnight in a 4% (w/v) paraformaldehyde containing 0.9% (w/v) NaCl/50 mM phosphate buffer solution. Afterwards, the fixed tissue was rinsed in 0.9% (w/v) NaCl/50 mM phosphate buffer. The tissue was embedded in gelatin (gelatin type A from porcine skin; 100 g/l) and 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/l). Histochemical staining was performed using fluorescein isothiocyanate labelled phalloidin (phalloidin-FITC). After rehydration in phosphate buffer, the sections were incubated with $4 \cdot 10^{-7}$ M phalloidin-FITC in a phosphate buffer containing 2 ml/l Triton-X-100, 30 g/l BSA and 100 ml/l goat serum (Chemicon, Temecula, CA, USA) for 36 h at 4 °C. After a further rinse with phosphate buffer, the sections were incubated for 5 min with $3 \cdot 10^{-7}$ M 4′,6-diamidino-2-phenylindoldilactate (DAPI; Molecular Probes, Leiden, The Netherlands) dissolved in phosphate buffer saline. The preparations were examined on a fluorescence microscope (Nikon 80i). Digital images were taken with a B/W camera (DS-2M B/Wc) using the NIS Elements 2.30 software (all from Nikon, Düsseldorf, Germany) to finally adjust brightness, color and contrast.

2.5. Compounds

Recombinant *P. multocida* toxin and its inactive analogue, *P. multocida* toxin C1165S, were prepared by expression in *E. coli* and purified as GST fusion proteins as described previously (Busch et al., 2001). Bumetanide and indomethacin were dissolved in ethanol (final maximal concentration in the chamber 2.5 ml/l). Tetrodotoxin was dissolved in $2 \cdot 10^{-2}$ M citrate buffer. Acetylcholine chloride, adenosine 5′-triphosphate (ATP) disodium salt, and carbachol chloride were dissolved in aqueous stock solutions and diluted in salt buffer just before use. YM-254890 (a cyclic depsipeptide; for chemical structure, see Takasaki et al., 2004; generous gift of Dr. M. Taniguchi,

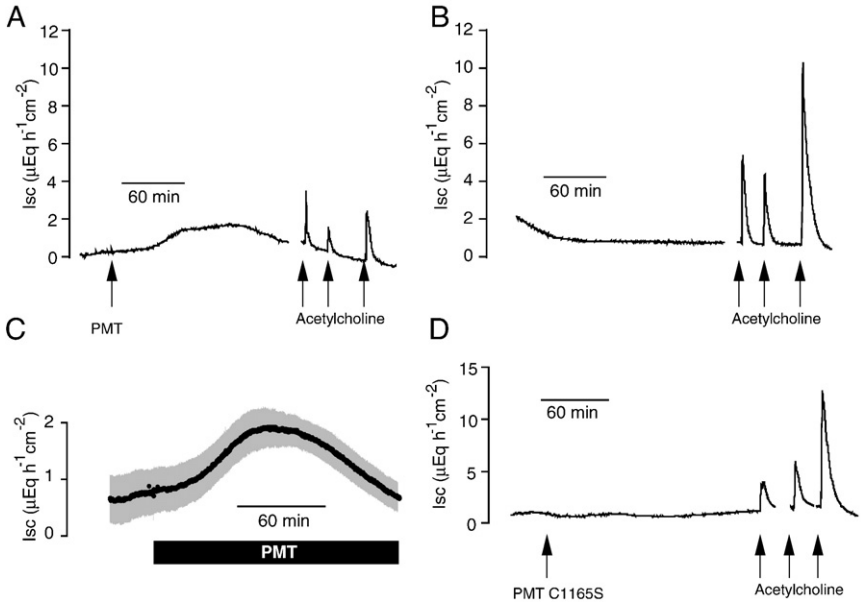

Fig. 1. A: Increase in I_{sc} evoked by *Pasteurella multocida* toxin (PMT; 1.5 µg/ml at the serosal side) and subsequent down-regulation of I_{sc} evoked by cumulative administration of acetylcholine (final concentrations: $5 \cdot 10^{-6}$ M, 10^{-5} M and 10^{-4} M at the serosal side). B: Time-dependent control. C: Averaged time course of the change in I_{sc} evoked by *P. multocida* toxin (1.5 µg/ml at the serosal side; black bar). Values are means (symbols)±S.E.M. (grey shaded area), $n=6$. D: The inactive analogue, *P. multocida* toxin C1165S (1.5 µg/ml at the serosal side) had no effect on basal I_{sc} nor on acetylcholine-evoked increase in I_{sc}. The tracings in A, B and D are original tracings representative for 6–8 experiments; for statistics of the acetylcholine responses, see Table 1.

Yamanouchi Pharmaceutical, Ibaraki, Japan) was dissolved in an aqueous stock solution containing 1 g/l bovine serum albumin (BSA). Nystatin and phalloidin-FITC were dissolved in dimethylsulphoxide (DMSO; final maximal DMSO concentration 2 ml/l). If not indicated differently, drugs were from Sigma, Deisenhofen, Germany.

2.6. Statistics

Results are given as means±one standard error of the mean (S.E.M.). Statistical comparisons were performed either by a Student's *t*-test (paired or unpaired as adequate) or by the Mann Whitney *U*-test as appropriate. $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Basal effects of P. multocida toxin and interaction with secretagogues

P. multocida toxin (1.5 µg/ml at the serosal side; this concentration was selected on the basis of published data, in which the ability of the toxin to stimulate the production of IP_3 in fibroblasts was measured; Zywietz et al., 2001) evoked a slow increase in I_{sc}, which started with a delay of at least 30 min (Fig. 1A). When measured at its maximum, the I_{sc} induced by the toxin amounted to 1.20±0.31 µEq/h/cm^2 above the former baseline ($P<0.05$ versus baseline I_{sc} prior administration of the toxin; Fig. 1C). Three hours after toxin administration, the I_{sc} had returned completely to baseline. Subsequently, acetylcholine was administered in a cumulative manner, i.e. the individual concentrations were applied without an intermediate washing step (in order to avoid a potential washing out of the *P. multocida* toxin), which causes a desensitization of the I_{sc}

Table 1
Effect of *Pasteurella multocida* toxin pretreatment on the acetylcholine-stimulated I_{sc}

	ACh ($5 \cdot 10^{-6}$ M)	ACh (10^{-5} M)	ACh (10^{-4} M)	*n*
	ΔI_{sc} (µEq/h/cm^2)			
No PMT	2.62±0.57[a]	1.67±0.44[a]	7.06±0.89[a]	8
After PMT	1.02±0.49	0.75±0.33	2.36±0.86[a,b]	6
No PMT C1165S	1.63±0.93	2.36±1.22	6.72±2.15[a]	6
After PMT C1165S	1.08±0.49	1.69±0.71	6.05±1.21[a]	6
PMT with Cl^-	2.85±0.69[a]	1.49±0.43[a]	3.92±0.77[a]	7
PMT Cl^--free	0.50±0.24[b]	0.19±0.07[a,b]	1.14±0.35[a,b]	7
PMT	2.91±1.43	1.50±0.73	4.24±1.92[a]	5
PMT+Bumetanide	0.43±0.05[a]	0.40±0.07[a]	1.63±0.28[a]	6
PMT	1.23±0.43[a]	0.48±0.11[a]	1.75±0.59[a]	6
PMT+Indomethacin	1.99±0.89	0.22±0.05[a]	0.94±0.39	6
PMT	3.80±0.84[a]	0.89±0.27[a]	4.40±1.19[a]	5
PMT+Tetrodotoxin	1.41±0.26[a,b]	0.50±0.14[a]	1.57±0.47[a]	6
PMT	0.93±0.47	0.53±0.15[a]	2.24±0.69[a]	6
PMT+YM-254890	0.09±0.02[a,b]	0.06±0.02[a,b]	0.24±0.06[a,b]	7

Pasteurella multocida toxin (PMT) and *P. multocida* toxin C1165S were applied in a concentration of 1.5 µg/ml at the serosal side. Inhibitor concentrations were: bumetanide (10^{-4} M at the serosal side), indomethacin (10^{-6} M at the mucosal and the serosal side), tetrodotoxin (10^{-6} M at the serosal side), YM-254890 (10^{-7} M at the serosal side). Replacement of Cl^- by gluconate was performed both at the mucosal and the serosal side. Values are given as difference to the baseline in I_{sc} just prior administration of acetylcholine (ΔI_{sc}) and are means± S.E.M. [a]$P<0.05$ versus baseline, [b]$P<0.05$ versus response of the same concentration of acetylcholine in the absence of *P. multocida* toxin or in the absence of the inhibitor under investigation, respectively.

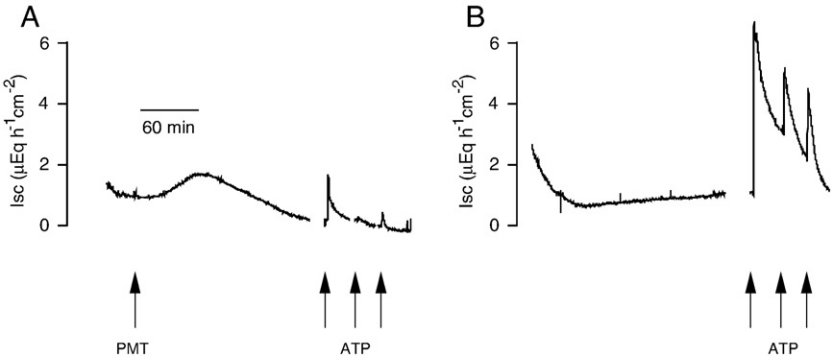

Fig. 2. A: Increase in I_{sc} evoked by *Pasteurella multocida* toxin (PMT; 1.5 µg/ml at the serosal side) and subsequent down-regulation of I_{sc} evoked by cumulative administration of ATP (final concentrations: $5 \cdot 10^{-4}$ M, 10^{-3} M and $5 \cdot 10^{-3}$ M at the serosal side). B: Time-dependent control. The tracings are original tracings representative for 8–9 experiments; for statistics of the ATP responses, see Table 2.

response resulting in an apparent non concentration-dependent effect of acetylcholine (Diener et al., 1989). After pretreatment with *P. multocida* toxin, the increase in I_{sc} evoked by the cholinergic agonist was strongly reduced (cf. Fig. 1A and B; for statistics, see Table 1). *P. multocida* toxin C1165S (1.5 µg/ml at the serosal side), an inactive mutant (Busch et al., 2001), had no effect, neither on basal I_{sc} nor did it down-regulate acetylcholine-induced I_{sc} (Fig. 1B).

In order to clarify, whether the down-regulation of ion secretion by long-term treatment with *P. multocida* toxin was restricted to activation of muscarinic receptors, ATP was used, which stimulates anion secretion across rat colonic mucosa via P2Y receptors (Leipziger et al., 1997). ATP (0.5–5 mM at the serosal side) induced a strong increase in I_{sc} during cumulative administration, which was significantly reduced by pretreatment with *P. multocida* toxin for 3 h (Fig. 2, Table 2).

3.2. Ionic nature of the P. multocida toxin response

In order to identify the ionic nature of the *P. multocida* toxin-evoked increase in I_{sc}, anion replacement and transport blocker experiments were carried out. Substitution of Cl^- by the impermeable anion, gluconate, significantly inhibited the increase in I_{sc} induced by the toxin by more than two thirds (Fig. 3, $P<0.05$). In addition, bumetanide, an inhibitor of the Na^+–K^+–$2Cl^-$ cotransporter involved in basolateral Cl^- uptake during Cl^- secretion, inhibited the response to *P. multocida* toxin. In the presence of bumetanide (10^{-4} M at the serosal side; for concentration–response data, see Schultheiss et al., 1998), *P. multocida* toxin (1.5 µg/ml at the serosal side) evoked only an increase in I_{sc} of 0.37±0.11 µEq/h/cm^2 ($n=6$), which was significantly smaller ($P<0.05$) versus the response in the absence of the transport blocker, which amounted to 1.25±0.38 µEq/h/cm^2 in this experimental series ($n=5$). Consequently, the increase in I_{sc} induced by the bacterial toxin represents predominantly a secretion of chloride anions.

3.3. Action sites of P. multocida toxin

Intestinal Cl^- secretion can be evoked either by actions of secretagogues directly at the epithelium or by indirect effects of secretagogues at submucosal cells releasing e.g. neurotransmitters or prostaglandins, which indirectly affect the enterocytes (see e.g. Diener et al., 1988). Therefore, the effects of drugs interfering with neurotransmitter release or production of eicosanoids on the *P. multocida* toxin response were investigated. The neurotoxin, tetrodotoxin (10^{-6} M at the serosal side; for concentration–response data, see Andres et al., 1985), which inhibits the propagation of action potentials via blockade of neuronal voltage-dependent Na^+ channels (Catterall, 1980), did not affect the action of *P. multocida* toxin. In the presence of tetrodotoxin, *P. multocida* toxin (1.5 µg/ml at the serosal side), induced an increase in I_{sc} of 0.96±0.25 µEq/h/cm^2 ($n=6$), which was not significantly different from the response to *P. multocida* toxin in a parallely performed control series, where the toxin evoked an increase in I_{sc} of 0.98±0.16 µEq/h/cm^2 ($n=5$).

In contrast, pretreatment with indomethacin (10^{-6} M at the mucosal and the serosal side; for concentration–response data, see Bridges and Rummel, 1983), reduced the I_{sc} response to *P. multocida* toxin significantly ($P<0.05$) by about two thirds (Fig. 4), suggesting a contribution of cyclooxygenase products in the response to the bacterial toxin. However, it is known that the action of Ca^{2+}-dependent secretagogues depends on a stimulation of the enterocytes by agonists of the cAMP-pathway, which is necessary to keep the CFTR channel, i.e. the predominant anion channel in the apical membrane of the enterocytes (Greger, 2000), in an open state (Strabel and Diener, 1995). Therefore, the ability of a low concentration of forskolin ($5 \cdot 10^{-7}$ M at the mucosal and the serosal side) was tested to

Table 2
Effect of *Pasteurella multocida* toxin pretreatment on the ATP-stimulated I_{sc}

	ATP ($5 \cdot 10^{-4}$ M)	ATP (10^{-3} M)	ATP ($5 \cdot 10^{-3}$ M)	n
	ΔI_{sc} (µEq/h/cm^2)			
No PMT	1.85±0.67[a]	0.68±0.23[a]	1.79±0.35[a]	9
After PMT	1.17±0.34[a]	0.03 ±0.12[b]	0.59±0.13[a,b]	8

Values are given as difference to the baseline in I_{sc} just prior administration of acetylcholine (ΔI_{sc}) and are means±S.E.M. [a]$P<0.05$ versus baseline, [b]$P<0.05$ versus response of the same concentration in the absence of *Pasteurella multocida* toxin (PMT).

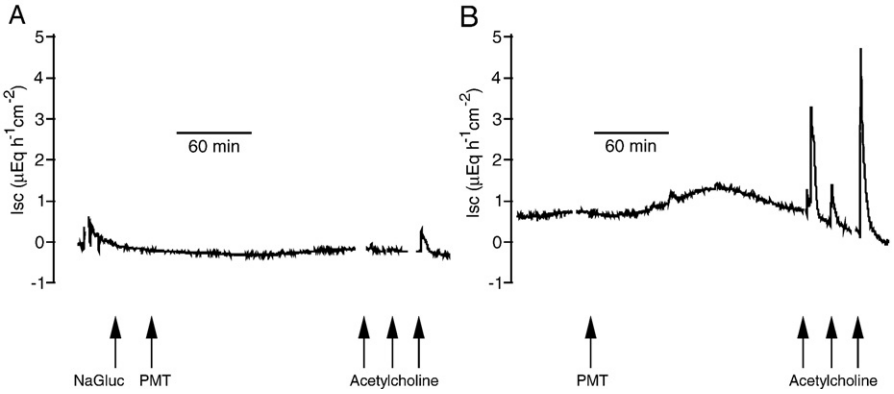

Fig. 3. In the absence of chloride (replacement of NaCl by Na gluconate (NaGluc) at the mucosal and the serosal side), the action of *Pasteurella multocida* toxin (PMT; 1.5 µg/ml at the serosal side) is suppressed. B: Control response in the presence of Cl^-. In average, *P. multocida* toxin evoked a ΔI_{sc} of 1.03±0.27 µEq/h/cm^2 (n=7) in the presence and 0.35±0.10 µEq/h/cm^2 in the absence of Cl^- (n=7, P<0.05 versus response in the presence of Cl^-). The tracings are original tracings representative for 7 experiments in each series; for statistics of the acetylcholine responses, see Table 1.

overcome the inhibition by indomethacin. In the combined presence of forskolin and indomethacin, the toxin evoked again an increase in I_{sc} of 1.16±0.58 µEq/h/cm^2 (n=9), i.e. a response of similar amplitude as under control conditions, indicating that the anion secretion evoked by the bacterial toxin is not mediated by the release of prostaglandins but instead depends on the production of cAMP within the epithelium stimulated by the continuous release of prostaglandins from the submucosal tissue.

P. multocida toxin is thought to activate $G\alpha_q$ proteins, but has in addition the ability to interfere with $G\alpha_{12/13}$ proteins (Orth et al., 2005). Therefore, the effect of YM-254890, a peptide $G\alpha_q$ inhibitor (Takasaki et al., 2004), on the *P. multocida* toxin response was tested. In order to establish the necessary concentration for YM-254890, control experiments with carbachol were performed, which activates a Ca^{2+}-dependent secretion via stimulation of muscarinic M_3 (and M_1) receptors at the colonic epithelium (Diener et al., 1989; Haberberger et al., 2006). At a concentration of 10^{-7} M, applied 30 min prior administration of carbachol, the G_q inhibitor suppressed the action of carbachol (Fig. 5).

Therefore, this concentration of YM-254890 was used to study the presumed participation of $G\alpha_q$ at the I_{sc} response evoked by *P. multocida* toxin.

In the presence of YM-254890 (10^{-7} M at the serosal side), the activation of I_{sc} by the bacterial toxin was reduced by more than 70% (Fig. 6, P<0.05), and (as had to be expected) the subsequent administration of acetylcholine was nearly ineffective (Table 1). Therefore, the effect of *P. multocida* toxin on rat colonic epithelium seems to be mediated by a $G\alpha_q$ protein.

Activation of G_q proteins is generally coupled to the stimulation of a phospholipase C and followed by the release of stored Ca^{2+} (Abdel-Latif, 1986). The subsequent opening of Ca^{2+}-dependent basolateral K^+ channels hyperpolarizes the membrane and thereby increases the driving force for Cl^- exit across apical Cl^- channels (Böhme et al., 1991). Thus, in a final series of experiments it was tested, whether the stimulation of Cl^- secretion, i.e. the increase in I_{sc} evoked by *P. multocida* toxin, might be caused by the opening of basolateral K^+ channels. Therefore, tissues were pretreated for 90 min with *P. multocida* toxin (1.5 µg/ml at the serosal side), i.e. for a time period, during which in general a maximal stimulation of I_{sc} by

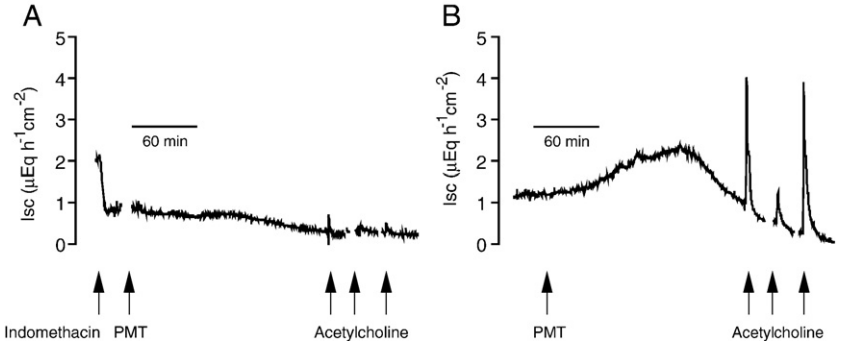

Fig. 4. A: Indomethacin (10^{-6} M at the mucosal and the serosal side) prevents the action of *Pasteurella multocida* toxin (PMT; 1.5 µg/ml at the serosal side). B: Time-dependent control. In average, *P. multocida* toxin evoked a ΔI_{sc} of 1.29±0.27 µEq/h/cm^2 (n=6) in the absence and 0.43±0.11 µEq/h/cm^2 in the presence of indomethacin (n=6, P<0.05 versus response in the absence of the inhibitor). The tracings are original tracings representative for 6 experiments in each series; for statistics of the acetylcholine responses, see Table 1.

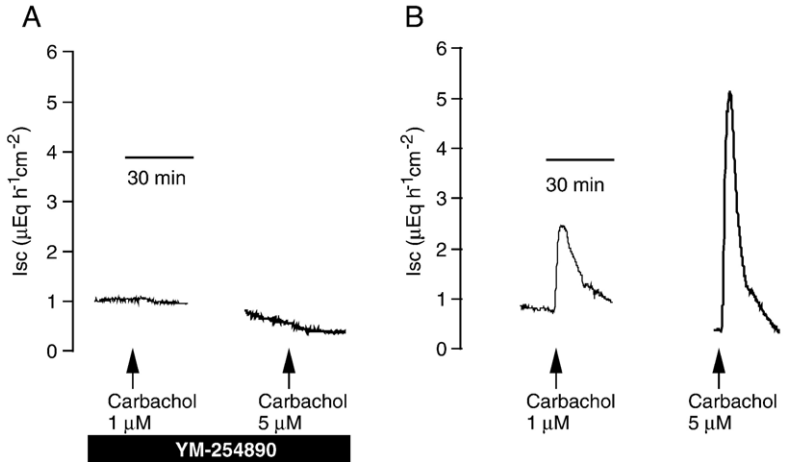

Fig. 5. A: Pretreatment with YM-254890 (10^{-7} M at the serosal side, administered 30 min before the first administration of carbachol and then again after the washing period) suppresses the response to carbachol (10^{-6} M and $5 \cdot 10^{-6}$ M, administered at the serosal side). The line interruption indicates the washing of the serosal compartment (3× with 5 times the chamber volume), before the next concentration of carbachol was tested. B: Control response to carbachol in the absence of YM-254890. In average, the increase in I_{sc} evoked by 10^{-6} M carbachol amounted 2.61±0.31 µEq/h/cm^2 in the absence and 0.15±0.067 µEq/h/cm^2 in the presence of YM-254890. At a concentration of $5 \cdot 10^{-6}$ M, carbachol induced an increase in I_{sc} of 4.99±0.92 µEq/h/cm^2 in the absence and 0.077±0.055 µEq/h/cm^2 in the presence of YM-254890 (means±S.E.M., $n=6$, $P<0.05$ for both concentrations versus response to the respective concentration in the absence of the inhibitor).

P. multocida toxin could be evoked (cf. Fig. 1C). Then the apical membrane was permeabilized by the ionophore, nystatin (100 µg/ml at the mucosal side) in the presence of a mucosal-to-serosal K^+ gradient in order to drive a K^+ current across the basolateral membrane. Pretreatment with *P. multocida* toxin caused an increase in this current by about 90% ($P<0.05$) compared to a control, that did not receive the bacterial toxin (Fig. 6).

3.4. Effect on the actin cytoskeleton

In other cells such as e.g. fibroblasts, *P. multocida* toxin is known to exert strong effects on the actin cytoskeleton (Zywietz et al., 2001). Therefore, the actin cytoskeleton was stained with FITC-labelled phalloidin after 3 h treatment of the mucosa with *P. multocida* toxin (1.5 µg/ml at the serosal side). However, no obvious change in the actin cytoskeleton was visible, neither after 3 h *P. multocida* toxin (Fig. 8) nor after a shorter incubation time of 90 min (data not shown).

4. Discussion

The present results demonstrate that the protein toxin, *P. multocida* toxin, evokes a transient increase in I_{sc} (Fig. 1), which is dependent on the presence of Cl^- (Fig. 3) and is inhibited by a blocker of the Na^+–K^+–$2Cl^-$ cotransporter, bumetanide (Table 1), responsible for the basolateral uptake of Cl^- during Cl^- secretion (Binder and Sandle, 1994), indicating that this current is caused by a stimulation of Cl^- secretion. *P. multocida* toxin is known to be incorporated by endocytosis (Rozengurt et al., 1990), which explains the delay between *P. multocida* toxin administration and the observed increase in I_{sc} of 30–60 min (Fig. 1A).

The consequence of *P. multocida* toxin uptake is the activation of $G\alpha_q$ inducing the formation of IP_3 via phospholipase C (Baldwin et al., 2003). The inactive mutant form *P. multocida* toxin C1165S, which lacks the ability to stimulate G proteins, was ineffective (Fig. 1B), which is in accordance with the presumed mechanism of action of the bacterial toxin.

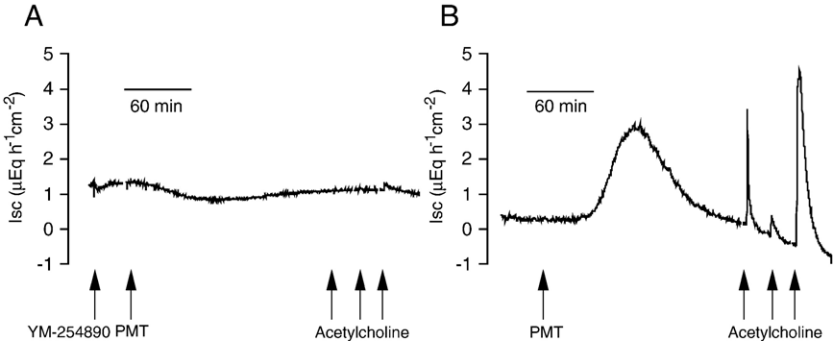

Fig. 6. YM-254890 (10^{-7} M at the serosal side) prevents the action of *Pasteurella multocida* toxin (PMT; 1.5 µg/ml at the serosal side). B: Control response in the absence of YM-254890. In average, *P. multocida* toxin evoked a ΔI_{sc} of 1.87±0.60 µEq/h/cm^2 ($n=6$) in the absence and 0.55±0.18 µEq/h/cm^2 in the presence of YM-254890 ($n=7$, $P<0.05$ versus response in the absence of the inhibitor). The tracings are original tracings representative for 6–7 experiments; for statistics of the acetylcholine responses, see Table 1.

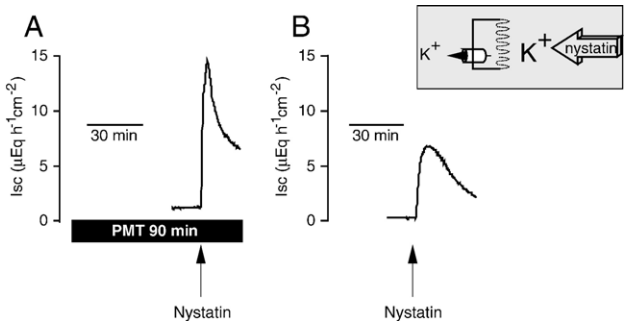

Fig. 7. A: Pretreatment with *Pasteurella multocida* toxin (1.5 µg/ml at the serosal side; black bar) for 90 min potentiates the I_{sc} evoked by permeabilization of the apical membrane with nystatin (100 µg/ml at the mucosal side) in the presence of a mucosal-to-serosal K^+ gradient as indicated by the schematic inset. B: Control response to nystatin without *P. multocida* toxin pretreatment. In average, nystatin evoked an increase in I_{sc} (ΔI_{sc}) of 6.83±1.53 µEq/h/cm^2 (n=6) in the absence and of 12.9±2.99 µEq/h/cm^2 (n=6, P<0.05 versus response without pretreatment with *P. multocida* toxin) in the presence of the bacterial toxin.

However, *P. multocida* toxin has been shown to stimulate also G proteins of the type $G\alpha_{12/13}$ (Orth et al., 2005). Therefore, YM-254890 was used as a specific inhibitor of $G\alpha_q$ (Takasaki et al., 2004). A concentration of 10−7 M YM-254890, which nearly suppressed the effect of muscarinic receptor stimulation (Fig. 5), also strongly reduced the action of the bacterial toxin confirming the central role of $G\alpha_q$ in its mechanism of action.

P. multocida toxin shares its mechanism, by which it induces Cl^- secretion, with other agonists of $G\alpha_q$ such as the cholinergic agonist, carbachol, whose dominant action is the activation of basolateral Ca^{2+}-dependent K^+ channels. The increase in the basolateral K^+ conductance hyperpolarizes the cell to increase driving force for apical Cl^- exit (Böhme et al., 1991), an action, which is supported by the transient opening of Ca^{2+}-dependent apical K^+ and Cl^- channels (Schultheiss et al., 2005). The increase in basolateral K^+ conductance by the toxin was indeed observed in experiments, where the apical membrane was permeabilized by the ionophore, nystatin, with an external K^+ gradient driving a K^+ current across the basolateral membrane (Fig. 7). Furthermore, *P. multocida* toxin shows the typical dependence of secretagogues acting on the Ca^{2+}-signaling pathway on the prestimulation of the mucosa by prostaglandins. When basal production of eicosanoids, released from subepithelial cells, was suppressed, the action of *P. multocida* toxin was strongly inhibited (Fig. 4). This does, however, not indicate that the action of the toxin is mediated by eicosanoids, as the administration of forskolin, an activator of the adenylate cyclase, was able to overcome the block by indomethacin. Consequently, similarly as it has been observed e.g. for carbachol (Strabel and Diener, 1995), the continuous stimulation of cAMP production within the enterocytes is necessary to keep the dominant anion channel in the apical membrane, the CFTR, in an open state so that the hyperpolarization of the membrane due to the activation of K^+ channels can stimulate Cl^- efflux.

When acetylcholine and ATP, known to stimulate muscarinic and P2Y receptors, respectively, at the colonic epithelium, were administered 3 h after *P. multocida* toxin, i.e. when the I_{sc} evoked by the toxin had already decreased, a strong down-regulation of the secretory responses evoked by these agonists was observed (Figs. 1 and 2). Consequently, the activation of $G\alpha_q$ by *P. multocida* toxin is sufficient not only to mimic the stimulatory action of e.g. carbachol on Cl^- secretion, but in addition to induce a long-lasting down-regulation of secretion as it is the case for carbachol or other Ca^{2+}-dependent secretagogues (see e.g. Warhurst et al., 1991; Barrett et al., 1998; Schultheiss et al., 2001). The mechanism of the down-regulation is not fully understood and may exhibit cell-type specificity. In T84 human colonic tumour cells, a transactivation of the EFG receptor via calmodulin has been shown to be involved in this down-regulation (Keely and Barrett, 2003). However, at rat colon, tyrphostin AG1478, an inhibitor of the EGF receptor protein tyrosine kinase, PD 98059, an inhibitor of MAP kinase, and wortmannin, a blocker of the phosphatidylinositol-3-kinase, did not affect the action of carbachol on transepithelial I_{sc} (Schultheiss and Diener, 2005), making this signaling pathway unlikely to be involved. In this epithelium, however, fatty acids released after stimulation of a Ca^{2+}-dependent phospholipase A_2 seem to mediate in part the inhibition, which, however, must involve also other factors because an inhibitor of the cytosolic phospholipase A_2 only slowed, but did not prevent, the run-down of carbachol-stimulated I_{sc} (Schultheiss et al., 2001). In other cells such as e.g. fibroblasts, *P. multocida* toxin is known to exert strong effects on the actin cytoskeleton (Zywietz et al., 2001), probably through interaction with the small G protein

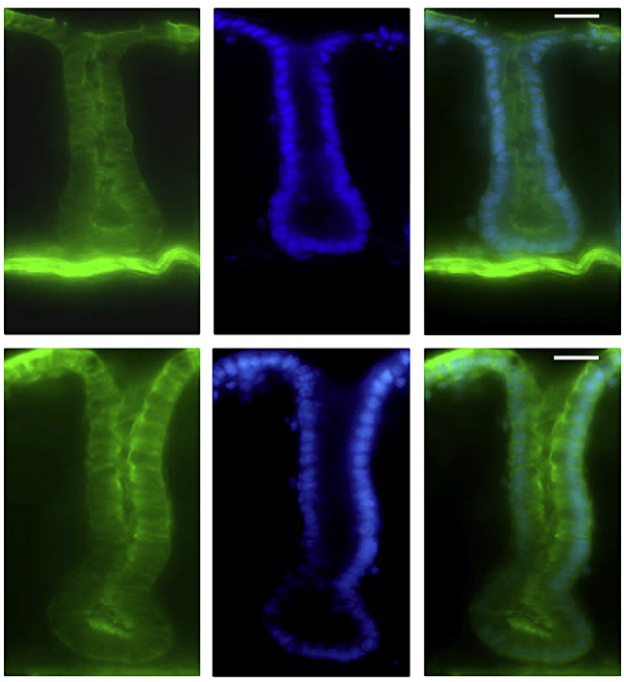

Fig. 8. Morphology of the colonic epithelium after 3 h treatment with *Pasteurella multocida* toxin (1.5 µg/ml at the serosal side, upper row) or under control conditions (lower row). The orientation of each picture is: lower part: muscularis mucosae; upper part: surface region of the colonic epithelium. Left colum: staining of the actin cytoskeleton with FITC-labelled phalloidin; middle column: nuclear staining with DAPI, right column: overlay of both. Bars: 50 µm. Typical pictures for 3 independent experiments under each condition.

Rho. The cytoskeleton is involved in the regulation of many ion channels such as e.g. CFTR (Prat et al., 1995) and seems to play a role in the mediation of the actions of cytoplasmic Ca^{2+} on apical ion channels in rat colon (Hennig et al., in press). However, there were no obvious changes in the actin cytoskeleton visible even after 3 h treatment with *P. multocida* toxin (Fig. 8).

Taken together, these results demonstrate that *P. multocida* toxin is able to induce Cl^- secretion across a typical secretory epithelium such as rat colon via stimulation of $G\alpha_q$ and activation of basolateral K^+ channels. Consequently, it seems likely to conclude that this ability may contribute to the diarrhea which can be evoked by this toxin.

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