Pflügers Arch – Eur J Physiol (1997) 433:638–647 © Springer-Verlag 1997

ORIGINAL ARTICLE

H.J. Epple · K.M. Kreusel · C. Hanski
J.D. Schulzke · E.O. Riecken · M. Fromm

Differential stimulation of intestinal mucin secretion by cholera toxin and carbachol

Received: 10 June 1996 / Received after revision: 19 September 1996 / Accepted: 30 September 1996

Abstract Cholinergic stimulation triggers the secretion of apically stored, preformed mucin from goblet cells but the pathway of cAMP-stimulated mucin secretion is not known. In this study the effect of cholera toxin on mucin secretion in the human colonic goblet cell line HT-29/B6 was investigated and compared to the action of carbachol. PAS staining of mucin blotted onto nitrocellulose served to quantify the secretion of total mucin. Metabolic labelling was used to evaluate the secretion of newly synthesized mucin. The mucinous nature of the detected material was confirmed with an immunoblot employing a well-characterized polyclonal antibody reacting with MUC2-mucin. Cholera toxin caused a 116-fold increase of intracellular cAMP and strongly stimulated the secretion of both preformed and newly synthesized mucin for more than 20 h. Carbachol only triggered the release of preformed mucin immediately after addition. The secretory response to cholera toxin could be partly inhibited by the protein kinase A inhibitor H8 and the microtubule inhibitor colchicine. The action of carbachol was not affected by these agents. In conclusion, we demonstrate a direct cAMP-dependent effect of cholera toxin on mucin secretion by intestinal goblet cells. In contrast to carbachol, the action of cholera toxin involves de novo synthesis of mucin molecules and microtubule-mediated secretion. There seem to be distinct secretion pathways for muscarinic or cAMP-dependent stimulation of mucin secretion.

H.J. Epple · K.M. Kreusel · M. Fromm (✉)
Institut für Klinische Physiologie,
Universitätsklinikum Benjamin Franklin, Freie Universität Berlin,
Hindenburgdamm 30, D-12200 Berlin, Germany

H.J. Epple · C. Hanski · J.D. Schulzke · E.O. Riecken
Medizinische Klinik und Poliklinik,
Abt. f. Innere Medizin mit Schwerpunkt Gastroenterologie,
Universitätsklinikum Benjamin Franklin, Freie Universität Berlin,
Hindenburgdamm 30, D-12200 Berlin, Germany

K.M. Kreusel
Augenklinik und Poliklinik,
Universitätsklinikum Benjamin Franklin, Freie Universität Berlin,
Hindenburgdamm 30, D-12200 Berlin, Germany

Key words Mucin secretion · Colon cells · HT-29/B6 cells · Cholera toxin · Carbachol · Compound exocytosis

Introduction

The viscous mucus gel that covers most of the mucosal surfaces of the gastrointestinal tract is mainly constituted of extremely large glycoproteins called mucins. Although different mucin genes have been identified as well as several factors altering mucin gene expression [8], our understanding of the regulation of mucin secretion still remains fragmentary. According to the general principles of protein secretion, two distinct pathways have been proposed for mucin secretion [4]. In analogy to the constitutive protein secretion of other cell types, continuous baseline mucin secretion is supposed to be unregulated, i.e. lacking receptor-mediated regulation [4]. Regulated mucin secretion, on the other hand, is described as release of apically stored, preformed mucin granules which may or may not be visible as compound exocytosis under the microscope [32]. This latter process is controlled by Ca^{2+}-dependent agonists such as acetylcholine but not by cyclic nucleotides [24, 26, 33]. Therefore, until the end of the 1980s, cyclic nucleotides were not believed to be intracellular mediators of stimulated mucin secretion [23]. Since then, evidence has increased that secretagogues dependent upon cyclic adenosine monophosphate (cAMP) are potent and independent stimulators of intestinal mucin secretion [6, 11, 18, 30]. However, few data are presently available on the mechanism of cAMP-induced mucin secretion and its relationship to Ca^{2+}-dependent exocytosis of preformed mucin granules.

As to the stimulatory properties of cholera toxin, it is well established that, besides Cl^- and fluid secretion, cholera toxin also induces intestinal mucin secretion. The stimulated mucin secretion leads to the “rice water” consistency of the stools of cholera patients and to substantial enteric protein and carbohydrate losses [31, 36]. The stimulus-secretion coupling mechanisms of cholera-

toxin-induced Cl^- secretion have been extensively studied. It could be shown that there are direct and indirect effects of cholera toxin on Cl^- secretion. Direct activation of cAMP-dependent Cl^- secretion follows binding of cholera toxin to ganglioside GM_1 on the apical surface of enterocytes. After translocation, the toxin's active A_1-peptide catalyses ADP-ribosylation of the α-subunit of the stimulatory heterotrimeric G-protein ($G_{s\alpha}$), leading to permanent stimulation of adenylate cyclase with excess cAMP formation and hence to permanent stimulation of cAMP dependent Cl^- secretion [1, 2]. It seems reasonable to attribute at least part of cholera toxin's mucin secretory action to direct stimulation of cAMP-dependent mucin secretion from goblet cells. However, several studies employing highly differentiated mucin-secreting cell clones have reported conflicting results regarding the mucin secretory effect of cholera toxin. Whereas Jarry et al. [10] and Roumagnac and Laboisse [29] found a strong secretory response to cholera toxin, two other studies failed to demonstrate a direct stimulatory action on mucin secretion [15, 18]. The results of the latter studies led to the hypothesis that cholera toxin does not stimulate intestinal mucin secretion directly at epithelial cells but only by neural mediation [21]. Furthermore, cholera toxin elicited a mucin secretory response in rat small intestine while other cAMP-dependent agonists did not, indicating a mediation pathway independent of a rise in cAMP [28].

In the present study, the effect of cholera toxin on mucin secretion by HT-29/B6 cells was investigated. HT-29/B6 is a highly differentiated colonic cell line derived from HT-29 cells after glucose deprivation [12]. Unlike their parent cells, they form polarized monolayers of goblet cells when grown on permeable supports. After proper stimulation they secrete Cl^- and mucin [12, 13]. In a recent paper it could be shown that, like two other human colonic cell lines (i.e. T84 and LS180 cells), the HT-29/B6 clone produces MUC2-mucin, the major secretory mucin of the colon [20]. Thus, HT-29/B6 cells are an excellent model for studying direct effects of secretagogues on intestinal Cl^- and mucin secretion. We used this model in order to answer the following questions. First, does cholera toxin stimulate intestinal mucin secretion directly at epithelial cells and, if so, how can the aforementioned contradictory effects of cholera toxin in prior cell line studies be explained? Second, can the secretory response to cholera toxin be attributed to the toxin's effect on cAMP formation or should other second messengers be considered?

Furthermore, we compared the stimulatory action of cholera toxin on mucin secretion by HT-29/B6 cells to that of carbachol. As it transpired that cholera toxin unequivocally stimulated mucin secretion by HT-29/B6 cells via elevation of cAMP, the differences found between the actions of cholera toxin and carbachol can be taken to indicate fundamental differences in cAMP-dependent as opposed to Ca^{2+}-dependent mucin secretion.

Materials and methods

Cell culture

HT-29/B6 cells were routinely cultured in 25-cm^2 culture flasks in RPMI 1640 medium containing 2% stabilized L-glutamine and supplemented with 10% fetal calf serum (FCS) at 37°C in a 95% air, 5% CO_2 atmosphere. For mucin secretion studies, cells were seeded on Millicell filters fixed at the bottom of culture plate inserts (effective membrane area 0.6 cm^2; Millipore, Eschborn, Germany). Three inserts were placed together into one conventional culture dish (O.D. 60 mm). After confluence, the monolayers formed an epithelial barrier between the apical (mucosal) compartment within the culture plate insert (600 µl) and the basolateral (serosal) compartment in the culture dish which contained 8 ml of medium. Measurement of transepithelial resistance (R^t) was used to test for confluence. Monolayers were used for the experiments between 14 and 16 days after seeding if R^t exceeded 250 $\Omega \cdot cm^2$.

Sampling of secreted mucin

Prior to secretion studies, loosely attached mucus was removed from the apical surface of the monolayers by a standardized rinsing procedure followed by removal of the apical medium. Rinsing was performed by drawing back and forth the apical medium with a pipette. The distance between the tip of the pipette and the monolayers was kept constant by means of a laboratory-built holding device in order to minimize variations of rinsing intensity. The whole procedure was performed 1 h and immediately prior to the experiments. Then the experiment was started by exchanging the apical or basolateral medium with medium containing the respective secretagogue at the desired concentration. Since the secretagogues were applied either apically or basolaterally, the other compartment was filled with agonist-free medium. In order to avoid interference of serum glycoproteins with the PAS stain, the apical medium did not contain FCS. For radiolabelling of secreted mucin, 140 $kBq \cdot ml^{-1}$ [^{3}H]glucosamine or 10 $kBq \cdot ml^{-1}$ [^{14}C]threonine was added to the basolateral medium simultaneously with the secretagogue. After the incubation period, the rinsing procedure was performed and the apical compartment was removed for mucin determination. Quantification of mucin secreted into the apical sample was achieved by PAS stain and by β-counting (filter count), and the secretion rate during the incubation period was calculated.

Mucin secretion assays

PAS staining of secreted mucin was performed according to the method of Thornton et al. [37]. Samples were blotted onto nitrocellulose (pore diameter 0.2 mm) in a slot blot Minifold II apparatus (Schleicher and Schüll, Dassel, Germany). The nitrocellulose membrane was removed, washed in Tris-buffered saline (TBS) (10 mM Tris, 150 mM NaCl, pH 7.4) and incubated in 1% (w/v) sodium periodate, 3% (v/v) acetic acid for 30 min at room temperature. It was then washed twice in 0.1% (w/v) sodium disulphite in 1 mM HCl (washing solution), followed by development in Schiff's reagent. Then the membrane was repetitively rinsed with washing solution and dried. The colour intensity of each slot was measured with a scanning densitometer (Hoefer GS 300, Hoefer, San Francisco, Calif., USA). Pooled mucin secreted by confluent HT-29/B6 monolayers in culture flasks was used for standardization. The mucin concentration in the standard pool was determined using a mucin standard obtained from J. K. Sheehan, University of Lund, Sweden. Standard curves were constructed using a range of from 50 ng to 3200 ng of mucin protein from the standard. Regression analysis of the data regularly resulted in a 2nd degree polynomial with excellent correlation to the data ($r > 0.98$). The function obtained was used to calculate the amount of mucin in the unknown samples. For quantification of radiolabelled mucin (in the following termed "filter count"), the mucin secreted into

the apical compartment was separated from free label by a filtration method employing nitrocellulose filters (pore diameter 0.2 mm) in a filtration manifold (Schleicher and Schüll). The filters were first moistened with 1 ml of TBS. After application and suction-aided filtration of the samples, the filters were rinsed three times with TBS. Filters were then dissolved in liquid scintillation cocktail (Packard, Groningen, The Netherlands) and labelled mucin was quantified by β-counting. In additional experiments, the activity in the filtrate was counted in order to determine the amount of free label present in the apical compartment.

Evaluation of mucin detection methods

CsCl-density centrifugation was performed using pooled apical samples and lysed monolayers in guanidine-HCl buffer [guanidine-HCl 4 M, Tris 100 mM, ethylenediaminetetraacetate (EDTA) 5 mM, phenylmethylsulphonyl fluoride (PMSF) 1 mM]. Starting density was 1.39 $g \cdot ml^{-1}$. After 27 h of centrifugation at an average relative centrifugal force of 201 060 *g* (50 000 rpm using a TV850 vertical rotor in a Kontron ultracentrifuge), fractions were collected and aliquots from each fraction were taken for mucin quantification by PAS stain, filter count or immunoassay. A total of 20 equally treated monolayers was used for the comparison of intra- and extracellular mucin before and after cholera toxin stimulation. Initially the apical medium was harvested from ten monolayers, which were then cut from the plastic inserts with a scalpel and lysed by a 24-h incubation in the guanidine-HCl buffer. Simultaneously the other ten monolayers were incubated with cholera toxin. After 20 h of incubation the apical medium from these monolayers was also harvested and their cells were lysed with guanidine-HCl buffer.

The immunoassay for secreted mucin employing a polyclonal antibody against human intestinal mucin was performed as described by McCool et al. [18]. Samples were blotted onto nitrocellulose (pore diameter 0.2 mm) in a slot blot Minifold II apparatus. After blockade of the remaining binding sites with 3% bovine serum albumin (BSA, essentially fatty-acid free), the nitrocellulose sheets were incubated for 24 h at 4°C in antimucin antibody (obtained from McCool) in TBS containing 3% BSA. Excess antibody was removed by repeated washing with TBS and the nitrocellulose sheet was incubated for 90 min at room temperature in protein A–horseradish peroxidase conjugate diluted 1:3000 in TBS containing 3% BSA. Excess conjugate was removed by repeated washing with TBS. Then, 30 mg of protein A–horseradish peroxidase colour development reagent (4-chloro-1-naphthol) was dissolved in 10 ml of methanol in the dark, and 50 ml TBS and 30 ml ice-cold 30% H_2O_2 were added to complete the development solution. The nitrocellulose sheet was incubated for 10 min in the dark in the development solution. After washing with distilled water and air drying of the nitrocellulose sheet, the colour intensity of each slot was determined with a scanning densitometer. Pooled mucin secreted by HT-29/B6 cells was used for standardization, as described for the PAS stain. Regression analysis of the data using the log concentrations resulted, in this assay, in a 1st degree polynomial, again with excellent correlation to the data ($r > 0.97$). The equation obtained was used to calculate the amount of mucin in the unknown samples.

Electrophysiological studies

Monolayers were apically incubated with cholera toxin (1 μg/ml) as for mucin secretion studies. For short-circuit current (I_{sc}) measurements, the intact culture plate inserts were removed from the medium and placed into modified Ussing chambers as described earlier [12]. Both the mucosal and the serosal compartment were filled with 10 ml of a modified Ringer's solution containing (in mM): 151 Na^+, 5 K^+, 1.7 Ca^{2+}, 0.9 Mg^{2+}, 130.4 Cl^-, 28 HCO_3^-, 1 $H_2PO_4^-$, 0.9 SO_4^{2-}, 25 D(+)-glucose. The solution was stirred and oxygenated via bubble lift. The pH at 95% O_2 and 5% CO_2 was 7.4. The temperature was kept constant at 37°C by means of temperature-controlled water jackets. I_{sc} and R^t were determined after a 15-min equilibration period by a computerized automatic clamp device (Fiebig Hard- and Software, Berlin, Germany).

cAMP measurement

Filter-grown monolayers were incubated with cholera toxin (1 $\mu g \cdot ml^{-1}$) as for mucin secretion studies. Cells were assayed for intracellular cAMP 2 h, 6 h, 12 h and 24 h after incubation. Untreated controls were assayed in parallel immediately before and after the incubation period. The filters were placed into iced 95% ethanol in order to stop cAMP formation and to extract cAMP. The cAMP present in the air-dried samples was determined by a commercially available cAMP radioimmunoassay kit (Amersham, Braunschweig, Germany). The protein content of ten representative filter-grown monolayers was determined with a commercially available protein assay kit (Pierce Europe, OudBeijerland, The Netherlands).

Chemicals

All chemicals, if not otherwise stated, were from Sigma (St. Louis, Mo., USA). Radionuclides were from Amersham. Cell culture components were from Biochrom (Berlin, Germany).

Statistics

All results are given as mean±SEM. Data were tested for significance by means of the unpaired two-tailed *t*-test. Significances were denoted * = $P<0.05$, ** = $P<0.005$, *** = $P<0.001$.

Results

Two methods were routinely used to evaluate mucus secretion by HT-29/B6 cells. Labelled precursors of mucin synthesis were added together with the tested secretagogues at the beginning of each experiment. The labelled mucin detected by β-counting (filter count) must therefore have been newly synthesized and secreted during the time of the experiment. With PAS staining, on the other hand, secretion of both newly synthesized and preformed mucin was monitored. The mucinous nature of the PAS-stained and labelled glycoproteins was confirmed by their high density in CsCl-density gradient and by a solid-phase immunoassay.

Evaluation of mucin detection methods

After CsCl-density gradient centrifugation of pooled apical samples from cholera-toxin-treated cells (1 $\mu g \cdot ml^{-1}$, apical side), one identical peak with a buoyant density of 1.37 $g \cdot ml^{-1}$ was found by PAS stain as well as by filter count (Fig. 1). In order to identify the material in the peak, a solid-phase immunoassay was used employing a characterized antibody directed against human intestinal mucin [17, 18, 27]. The immunoassay detected a high-density peak (1.37 $g \cdot ml^{-1}$) and a second peak of lower density (1.25 $g \cdot ml^{-1}$). The peaks found by PAS stain and filter count corresponded exactly to the high-density immunoreactive peak.

In order to rule out an overestimation of labelled mucin in the filter count, the binding of free [^{3}H]glucos-

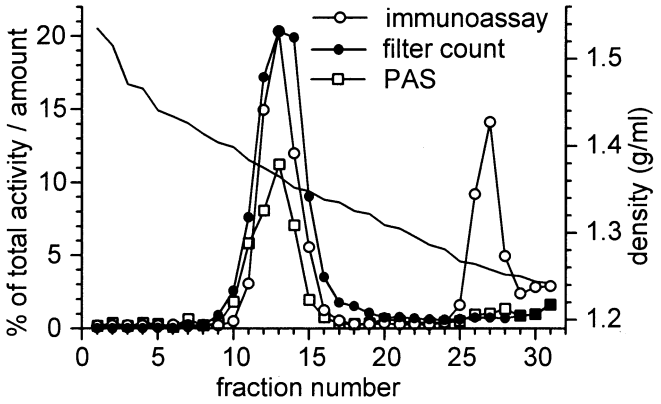

Fig. 1 Buoyant density of mucin secreted by HT-29/B6 cells. CsCl-density centrifugation was performed using 2 ml of pooled apical samples from cholera-toxin-treated monolayers (1 μg·ml^{-1}, apical side). Aliquots from each fraction were taken for mucin determination by immunoassay, filter count and PAS stain (*left ordinate*). The density of each fraction was determined as the mean from two aliquots (*solid line*; *right ordinate*)

amine to the filter was tested in selected experiments. When basolateral medium containing [^{3}H]glucosamine was passed through the filter, 0.39 ± 0.05% (n = 34) of the free tracer attached. The activity of filter-bound [^{3}H]glucosamine in mucin secretion assays was calculated from the activity in the filtrate. As it was always less than 5% of the total filter activity, a correction for free label bound to the filter was not performed. All of the mucin was retained by the filters, since no mucin could be detected in the filtrate of apical samples with either PAS stain or the highly sensitive immunoassay (n = 6, data not shown).

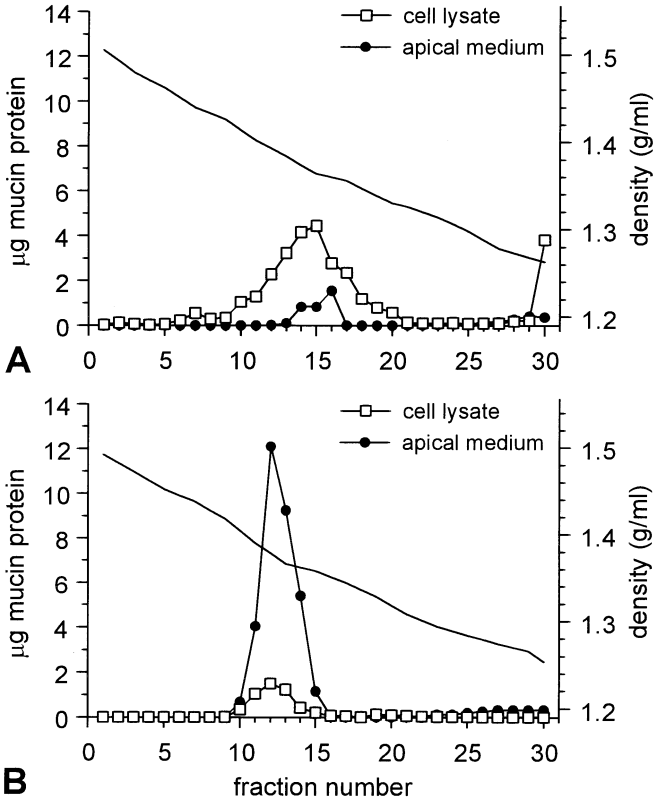

Fig. 2A, B Buoyant density of intracellular and extracellular (secreted) mucin produced by HT-29/B6 cells. CsCl-density centrifugation was performed using 5 ml of pooled apical samples from ten monolayers as well as cell lysates from the same monolayers. The mucin content in the fractions was determined by PAS stain. **A** Intracellular mucin (cell lysates) and secreted mucin (apical medium) from untreated monolayers. **B** Intracellular mucin (cell lysates) and secreted mucin (apical medium) from monolayers incubated for 20 h with cholera toxin (1 μg·ml^{-1}, apical side)

Effect of cholera toxin on intestinal mucin secretion

A comparison of intra- and extracellular mucin before and after the 20-h cholera toxin treatment is depicted in Fig. 2. After cholera toxin treatment the mucin content in the apical medium (= secreted mucin) was increased to more than sevenfold above the preincubation level. This increase was almost exclusively due to increased secretion of the high-density glycoproteins (buoyant density 1.38 g·ml^{-1}) which have been characterized before (compare Fig. 1). A significant amount of glycoproteins with lower density was only detected in cell lysates from untreated monolayers. Assuming that these low-density glycoproteins represent less glycosylated, immature mucins, they must have been further processed under the influence of cholera toxin. The intracellular mucin (mature and immature) amounted to 86% of total mucin (intra- plus extracellular mucin) before cholera toxin stimulation, but decreased to only 13% thereafter. The extracellular mucin increased correspondingly from 14% to 87% of total mucin, reflecting the mucin secretion induced by cholera toxin. The parallel decrease in the intracellular mucin content indicates release of intracellular mucin by the action of cholera toxin. Total mucin increased by 13%, indicating a considerable degree of mucin synthesis during the 20-h incubation time.

The stimulatory action of cholera toxin on mucin secretion was studied in more detail by PAS stain and filter count. Depending on the detection method, cholera-toxin-stimulated mucin secretion amounted to either 800% (PAS stain, P<0.001), 260% (filter count, [^{3}H]glucosamine, P<0.005) or 440% (filter count, [^{14}C]threonine, P<0.001) of untreated controls (Fig. 3). As the cell culture is devoid of neural and mesenchymal components, this effect must have been due to a direct interaction of cholera toxin with the cultured colon epithelial cells. The increase in mucin secretion induced by cholera toxin was not significantly different from that elicited by the cAMP-mobilizing agent forskolin (10^{-5} M, basolateral side). Surprisingly, the mucin secretion by HT-29/B6 cells was markedly higher after incubation with cholera toxin or forskolin than after muscarinic stimulation with carbachol (10^{-4} M, basolateral side). Furthermore, a stimulatory effect of carbachol on mucin secretion could only be detected in the PAS stain assay (1.7 times unstimulated mucin secretion by control monolayers,

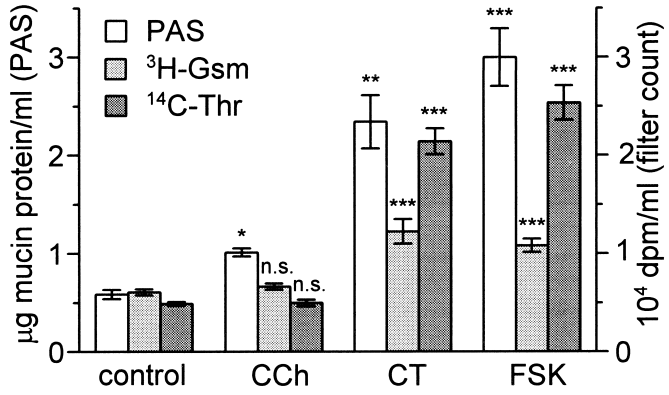

Fig. 3 Stimulation of mucin secretion by HT-29/B6 cells by cholera toxin. Mucin secretion was determined in untreated control monolayers, after 20 h of incubation with carbachol (*CCh*, 100 µM, basolateral side), cholera toxin (*CT*, 1 µg·ml^{-1}, apical side), and forskolin (*FSK*, 10 µM, basolateral side). Quantification was performed by PAS stain (*open bars, left ordinate*), filter count with [^{3}H]glucosamine (*^{3}H-Gsm, light bars, right ordinate*) or filter count with [^{14}C]threonine (*^{14}C-Thr, dark bars, right ordinate*). For better comparability, the filter count values for [^{14}C] are given as disintegrations per 2 min time. Significance is given versus controls. Values are means ± SEM of 8 experiments

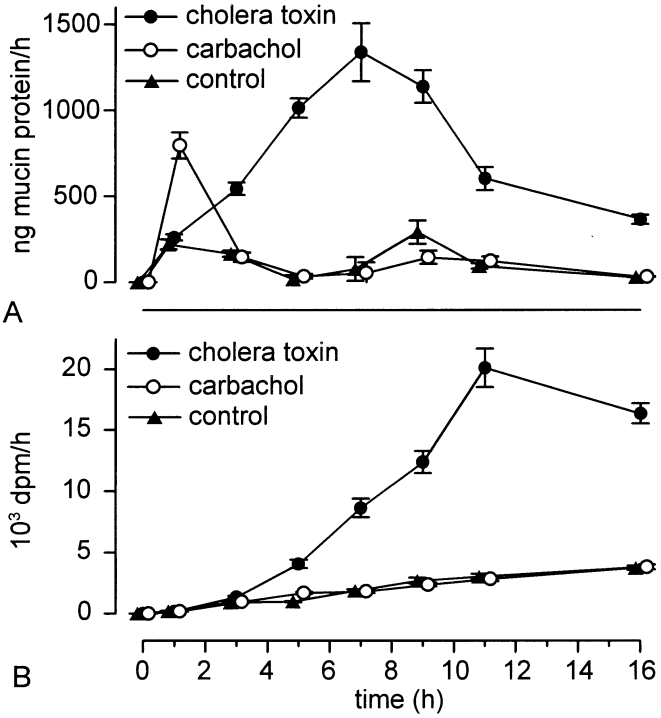

Fig. 4A, B Time course of cholera-toxin- and carbachol-stimulated mucin secretion. Samples were collected at 0, 2, 4, 6, 8, 10, 12 and 20 h after incubation with cholera toxin (1 µg·ml^{-1}, apical side) or carbachol (100 µM, basolateral side). [^{3}H]Glucosamine was used as a metabolic label for the filter count. Data were determined by PAS stain (**A**) or filter count (**B**) and were assigned to half of the respective sampling time. Untreated monolayers served as control. Values are means ± SEM of data from 8 monolayers

P<0.001), but not by filter count (Fig. 3). Therefore, carbachol only triggered release of preformed mucin whereas cholera toxin and forskolin also stimulated secretion of newly synthesized mucin.

Time course of stimulated mucin secretion

Mucin secretion induced by cholera toxin or carbachol exhibited different time courses (Fig. 4). The response to cholera toxin (1 µg·ml^{-1}, apical side) started after a lag time of 2 h (PAS stain) or 3 h (filter count) and reached maximum secretion after 8 h (PAS stain) and 12 h (filter count). After 20 h, the mucin secretion by cholera-toxin-treated monolayers was still significantly increased. Like cholera toxin, forskolin (10^{-5} M, basolateral side) also substantially stimulated mucin secretion during the whole incubation period (data not shown). Unlike cholera toxin and forskolin, carbachol (10^{-4} M, basolateral side) only stimulated secretion of preformed mucin immediately after incubation (Fig. 4A). Also, with the better time resolution of these experiments, no effect of carbachol on the secretion of newly synthesized mucin was found (Fig. 4B).

Combined effect of carbachol and cholera toxin or forskolin on mucin secretion

As shown before (Fig. 4), there is a considerable degree of variety in the mucin secretion rate during the first 20 h of cholera toxin incubation. Therefore, the combined effect of carbachol and cholera toxin was investigated using monolayers pretreated for 20 h with cholera toxin. Then the mucin secretion by cholera-toxin-pretreated monolayers – although higher than that of controls – remained stable during the observed time period (Fig. 5A). As in control monolayers, carbachol exerted its stimulatory action on cholera-toxin-pretreated cells only immediately after its addition. It induced a 1.9-fold higher secretion in monolayers pretreated with cholera toxin than in controls (P < 0.001, Fig. 5A), indicating an additive or synergistic action of cholera toxin and carbachol on mucin secretion by HT-29/B6 cells.

In order to decide whether synergy was present we performed additional experiments using forskolin instead of cholera toxin, assuming that both substances influenced the same mucin secretion pathway. Forskolin was used for these experiments, because – as known from Cl^- secretion experiments – its action starts without latency, and quantitative regulation of mucin (and Cl^-) secretion was easier by varying forskolin doses rather than those of cholera toxin. We used a low forskolin concentration (10^{-7} M, serosal side), the effect of which on mucin secretion did not become apparent until the action of carbachol was complete. The carbachol-stimulated secretion was enhanced by 180% by forskolin (Fig. 5B), indicating a synergistic action of carbachol and forskolin.

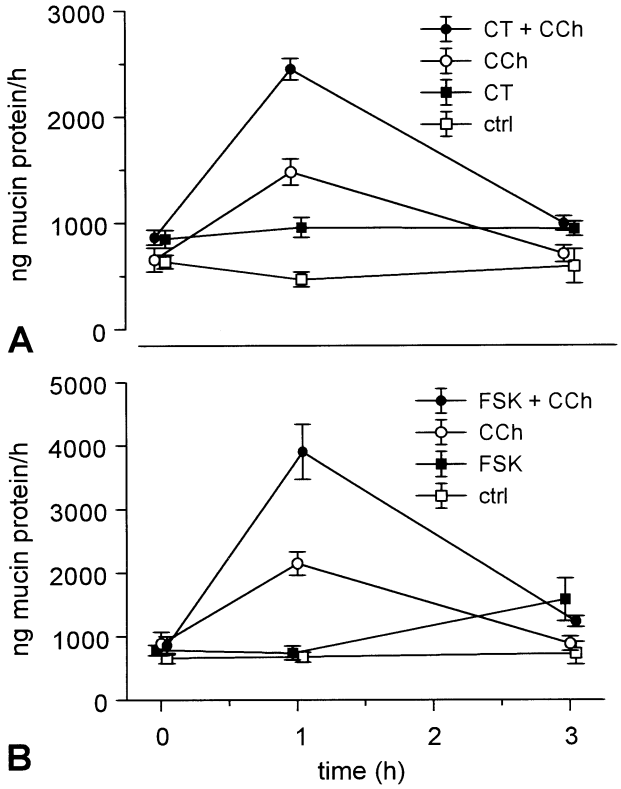

Fig. 5A, B Combined effect of carbachol and cholera toxin or forskolin on mucin secretion. Samples were collected at 0, 2 and 4 h after incubation with carbachol or forskolin. Data were determined by PAS stain and were assigned to half of the respective sampling time. Values are means ± SEM. **A** A total of 12 monolayers were preincubated with cholera toxin toxin (*CT*, 1 μg·ml^{-1}, apical side). Carbachol was added to six cholera-toxin-treated monolayers (*CT + CCh*) and to six untreated monolayers (*CCh*). A total of six untreated monolayers served as control (*ctrl*). **B** Six monolayers were incubated with carbachol (100 μM, basolateral side) and forskolin (10^{-7} M, serosal side) either alone (*CCh*, *FSK*) or together (*FSK + CCh*). Six untreated monolayers served as control (*ctrl*)

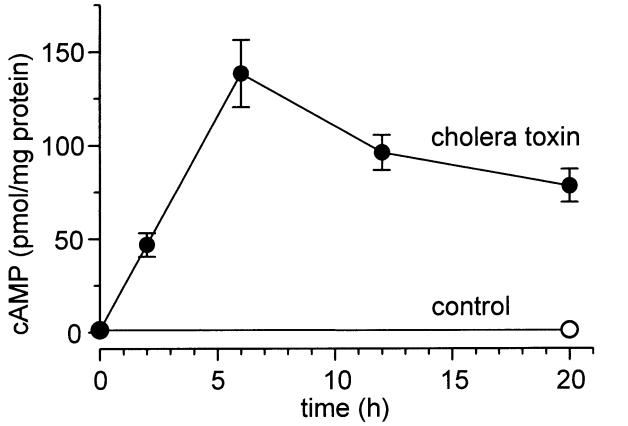

Fig. 6 cAMP content in HT-29/B6 cells after stimulation with cholera toxin. Intracellular cAMP was determined 2, 6, 12 and 20 h after incubation with cholera toxin (1 μg·ml^{-1}, apical side, *closed circles*). Intracellular cAMP in untreated monolayers (*control*) was determined immediately before and after the incubation period. Ten filters were taken for protein determination at the beginning of the experiment. cAMP values are means ± SEM of data from six monolayers

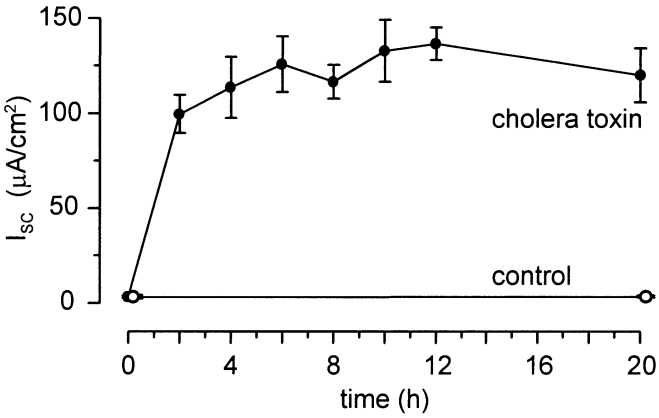

Fig. 7 Cholera-toxin-induced short-circuit current (I_{SC}) of HT-29/B6 monolayers. For I_{SC} measurement, monolayers were mounted in modified Ussing chambers 2, 4, 6, 8, 10, 12 and 20 h after incubation with cholera toxin (1 μg·ml^{-1}, apical side, *filled circles*). I_{SC} was determined after a 10-min equilibration period within the chambers. Untreated monolayers served as the control (*open circles*). Values are means ± SEM of six experiments

Mediation of cholera-toxin-induced secretion

The intracellular cAMP concentration during 20 h of incubation with cholera toxin (1 μg·ml^{-1}, apical side) is shown in Fig. 6. Cholera toxin caused a more than 100-fold increase of intracellular cAMP between 6 h and 12 h after incubation. After 20 h, the intracellular cAMP content in the cholera-toxin-treated cells was still 65 times higher than that of untreated controls. Comparison of Figs. 4A and 6 illustrates the good correlation between the time courses of intracellular cAMP and mucin secretion after stimulation of HT-29/B6 cells with cholera toxin.

At the epithelial level, cholera-toxin-induced Cl^- secretion is generally thought to be mediated by intracellular cAMP. Therefore, we performed electrophysiological experiments in order to investigate the effect of cholera toxin on Cl^- secretion by HT-29/B6 cells (Fig. 7). Cl^- secretion was determined as I_{SC} after incubating HT-29/B6 cells with the enterotoxin. As expected, cholera toxin had a strong stimulatory effect on I_{SC}. Inhibition of cholera-toxin-stimulated I_{SC} by serosal 100 μM bumetanide (75 ± 9% inhibition, n = 6) as well as by serosal 5 mM Ba^{2+} (77 ± 4% inhibition, n = 6) (data not shown) revealed electrogenic Cl^- secretion as the cause of I_{SC}. Maximum values of I_{SC} were reached as early as 2 h after incubation and were maintained over the entire 20-h incubation time.

To further characterize the signal transduction pathway of cholera-toxin-induced mucin secretion, experiments using a protein kinase inhibitor, i.e. *N*-[2-(methylamino)ethyl]-5-isoquinoline-sulphonamide hydrochloride (H8), were performed [9]. First we tried to define

Table 1 Effect of the protein kinase inhibitor H8 on stimulated mucin secretion. Mucin secretion was determined after 20 h of incubation with phorbol 12-myristate 13-acetate (*PMA*, 50 nM, basolateral side), *carbachol* (100 μM, basolateral side), *forskolin* (10 μM, basolateral side) and *cholera toxin* (1 μg · ml^{-1}, apical side) alone or in the presence of H8 (100 μM, basolateral side). The protein kinase inhibitor H8 was added 30 min prior to the respective secretagogues. [^{3}H]Glucosamine was used as a metabolic label for the filter count. Data are given as % mucin secretion by control monolayers without H8 (n = 5–6)

	PAS		Filter count	
	No H8	Plus H8	No H8	Plus H8
Control	100 ± 8	79 ± 9 n.s.	100 ± 5	71 ± 5 **
PMA	1033 ± 110	824 ± 88 n.s.	202 ± 11	217 ± 12 n.s.
Carbachol	202 ± 25	177 ± 18 n.s.	105 ± 8	71 ± 9 n.s.
Forskolin	659 ± 60	433 ± 78 *	197 ± 16	127 ± 9 **
Cholera toxin	701 ± 63	347 ± 47 ***	188 ± 14	119 ± 10 ***

the action of H8 in our experimental model. Carbachol, forskolin, and phorbol 12-myristate 13-acetate (PMA) served as positive controls for stimulation of Ca^{2+}-, cAMP-, and protein-kinase-C-dependent secretion. The effect of H8 on cGMP-dependent protein kinase was not investigated because a membrane-permeable cGMP analogue, dibutyryl-cGMP (10^{-4} M, serosal side), did not have any effect on Cl^- and mucin secretion by HT-29/B6 cells (n = 6, data not shown), ruling out a significant regulatory role of cGMP-dependent protein kinase on mucin secretion by these cells. Whereas H8 had some inhibitory potency on basal secretion of newly synthesized mucin, the secretory response to carbachol as well as to the protein-kinase-C-stimulating agent PMA ($5 \cdot 10^{-8}$ M, basolateral side) was not affected by H8 (Table 1). On the other hand, H8 had a strong inhibitory effect on the mucin secretion stimulated by the cAMP-mobilizer forskolin (10^{-5} M, basolateral side). Thus, we proved the specificity of H8 as a protein-kinase-A-inhibiting agent in our experimental model. Mucin secretion induced by cholera toxin (1 μg·ml^{-1}, apical side) was also strongly inhibited by H8, and the degree of inhibition was the same as that of the secretory response induced by forskolin.

Effect of colchicine on stimulated mucin secretion

The microtubule depolymerizing agent colchicine (10^{-4} M, basolateral side) inhibited basal secretion of newly synthesized mucin (Fig. 8B, control). This result is in agreement with the conventional concept of microtubule-dependent baseline secretion of newly synthesized mucin [4, 20, 23]. The secretion of preformed mucin on the other hand was enhanced by colchicine, indicating facilitated release of apically stored mucin granules (Fig. 8A, control). Although the reason for this action is not clear, it closely resembles the stimulatory effect of a Golgi function inhibitor, monensin, on baseline mucin secretion by LS180 cells [20]. After treatment

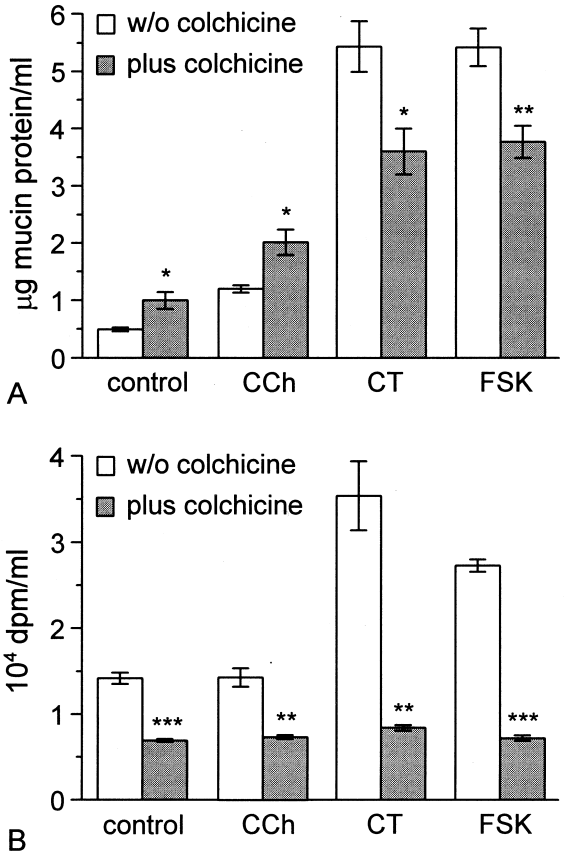

Fig. 8A, B Effect of colchicine on mucin secretion. Mucin secretion was determined by PAS stain (**A**) and by filter count (**B**) in the absence (*open bars*) or presence of colchicine (100 μM, basolateral side, *closed bars*). Data represent controls and 20-h incubations with carbachol (*CCh*, 100 μM, basolateral side), cholera toxin (*CT*, 1 μg·ml^{-1}, apical side) or forskolin (*FSK*, 10 μM, basolateral side). Colchicine was added to the medium 2 h prior to the respective secretagogues. [^{3}H]Glucosamine was used as a metabolic label for the filter count. Values are means ± SEM of six experiments

with colchicine, cholera toxin and forskolin failed to enhance secretion of newly synthesized mucin (Fig. 8B, CT, FSK). The residual secretion of newly synthesized mucins was the same in control monolayers as in those treated with cholera toxin or forskolin. In control experiments we used a stereo-isomer of colchicine, β-lumicolchicine, which has no effect on the microtubular cytoskeleton [7]. In these experiments cholera toxin stimulated mucin secretion by 280% (PAS) and 260% (filter count). In the presence of β-lumicolchicine (10^{-4} M, serosal side) the respective values were 340% (PAS) and 270% (filter count), and there were no significant differences in the stimulatory action of cholera toxin either with or without β-lumicolchicine (PAS: P = 0.26, filter count: P = 0.858; n = 6; data not shown). An intact microtubule function therefore seems to be important for unstimulated baseline mucin secretion as well as for the mucin secretion stimulated by cholera toxin or forskolin. In contrast, carbachol and colchicine exerted an additive action on the secretion of preformed mucin (Fig. 8A,

CCh), suggesting a microtubule-independent mechanism of carbachol-induced secretion.

Discussion

In the present paper, two different methods were routinely used for mucin measurement. The blotting procedure applied for the PAS stain assay has previously been shown to achieve a good separation between high- and low-molecular-weight glycoproteins [37]. As the pore size of the nitrocellulose filters was the same in the filter count and PAS stain assays, the filter count should achieve a similar discrimination. In addition, the amounts of filter-bound free label as well as filtrated mucin were shown to be negligibly small. The metabolic label was given together with the respective secretagogue at the beginning of each experiment. This labelling technique, in combination with the PAS stain assay, permitted, to a certain degree, the differential quantification of preformed and newly synthesized mucin within the apical sample.

The detection of mucin by the PAS stain and filter count methods was confirmed by CsCl-density gradient centrifugation. With this method a separation of mucin molecules from attached low-molecular-weight glycoproteins can be achieved [25]. Using the PAS stain method as well as the filter count, only one single peak was found within the typical density expected for mucin molecules [18, 25]. Thus, low-molecular-weight glycoproteins do not interfere with our mucin assays. The mucinous nature of the material forming this single peak was further confirmed by a highly specific immunoassay employing a well-characterized antibody directed against human intestinal mucin [17, 18, 27]. Recently, it was demonstrated that this antibody recognizes MUC2-mucin as the major antigen in the human colonic cell line LS180 [19]. Furthermore, this antibody precipitated a single antigen from cell lysates and supernatants of HT-29/B6 cells. Immunoprecipitates by this antibody as well as by an antibody raised against a tandem repeat of MUC2-mucin showed identical behaviour on SDS-PAGE with antigen derived from either HT-29/B6 or LS180 cells [20]. These data indicate that in HT-29/B6, as in LS180 cells, MUC2 is the major antigen detected by this antibody. We found two immunoreactive peaks after CsCl-density centrifugation of pooled apical samples. This is in agreement with a study by McCool et al. [18], who used the same antibody to detect mucin secreted from T84 cells. These investigators also found two immunoreactive peaks after CsCl-density centrifugation, the high-density peak of which was shown to consist of mucin. In our experiment, the high-density (= mucin) peak coincided exactly with the peak found by the PAS stain and filter count methods. Thus, the material forming this major peak detected by three independent mucin assays was identified as mucin and most probably contained MUC2-mucin. The second immunoreactive peak contained only small amounts of [^{3}H]glucosamine-labelled or PAS-positive material. Concluding from its density, this peak was most probably caused by weakly glycosylated antigenic mucin fragments, but its biochemical composition was not investigated.

Direct action of cholera toxin on epithelial cells

We clearly demonstrate by three different assays that cholera toxin is able to stimulate mucin secretion by a direct action on colon epithelial cells. The differences in the magnitude of mucin secretion, as measured by our three assays, underline the need to measure mucins using a variety of techniques. For these methodological reasons, we feel that a direct stimulatory effect of cholera toxin on mucin secretion by intestinal epithelial cells has not yet been unequivocally established. Both of the previous studies reporting a stimulatory effect of cholera toxin on mucin secretion by cultured human intestinal cells employed only a single method to evaluate mucin secretion [10, 29]. Their results contradict those of two other studies performed on cultured mucin-secreting epithelia, which failed to find enhanced mucin secretion after cholera toxin stimulation despite stimulatory effects by other cAMP-increasing [18] or Ca^{2+}-dependent [15, 18] agonists. However, in the latter studies, the colonocyte monolayers were incubated with cholera toxin for only 30 min [18] or 60 min [15] before mucin secretion was assessed. From our time course studies it is obvious that there is a significant time lag before the stimulatory action of cholera toxin becomes apparent. This latency is most likely due to the process of toxin binding, translocation of its active subunit and stimulation of adenylate cyclase following ADP-ribosylation of $G_{s\alpha}$ [16]. Thus, although cholera toxin does not stimulate rapid or accelerated mucin secretion, as correctly stated in the aforementioned studies [15, 18], it exerts a strong stimulatory effect on mucin secretion by colonocytes after incubations of longer than 1 h.

Mediation of cholera-toxin-stimulated mucin secretion

Three possibilities have to be considered regarding the signal transduction pathway of cholera-toxin-induced mucin secretion: indirect pathways involving nonepithelial cells, direct stimulation of epithelial cells via the cAMP cascade, and direct stimulation involving second messengers independent of cAMP. Differing actions of cholera toxin and other cAMP-dependent agonists on rat intestinal mucin secretion have led to the hypothesis that the mucin response to cholera toxin may not be controlled by the adenylate cyclase–cAMP system [28]. Indirect pathways, for example via enterochromaffin-like cells or the enteric nervous system, are, of course, beyond the scope of this cell culture study. However, our results provide evidence that the *direct* stimulatory action of cholera toxin on intestinal mucin secretion is mediated by intracellular cAMP, in analogy to the toxin's

direct effect on Cl^- secretion. First, all effects of cholera toxin on mucin secretion were mimicked by forskolin, which is a potent postreceptor adenylate-cyclase-stimulating agent. Second, apical incubation of HT-29/B6 cells with cholera toxin resulted in a sustained and long-lasting elevation of intracellular cAMP. Third, cholera-toxin- and forskolin-induced secretion could be partly inhibited by H8, which displayed protein kinase A inhibitory action in our experimental model. These results are supported by recently published studies performed on HT-29/B6 cells and HT-29cl.19A cells. Cholera toxin (as well as forskolin) caused a 40-fold increase in intracellular cAMP in HT-29cl.19A cells, whereas protein kinase C activity remained unaffected [22], and incubation of HT-29/B6 cells with forskolin did not alter their intracellular Ca^{2+} concentration [3]. Since all the features of mucin secretion investigated in our study were identical after stimulation of the HT-29/B6 cells with either forskolin or cholera toxin, it is very unlikely that intracellular Ca^{2+} participates in the action of cholera toxin but not in that of forskolin.

Pathways of regulated mucin secretion

At present, mucin secretion from goblet cells is thought to take place in two distinct pathways involving different intracellular mucin compartments [4]. The so-called regulated pathway is characterized by a pulsatile, rapid release of apically located, preformed mucin granules. Mucin secreted in this way consists only of preformed mucin, and microtubule function does not play a role in this secretory pathway [20]. The properties of carbachol-induced mucin secretion found in our experiments are very compatible with this secretory pathway, confirming the known effect of cholinergic stimulation on mucin secretion [18, 26, 32, 33].

A different secretion pathway has been described for baseline mucin secretion. This pathway is characterized by continuous mucin synthesis in the Golgi region and continuous, microtubule-mediated, peripheral vesicle transport to the apical surface followed by continual single-granule exocytosis of the mucinous content of the vesicles [4, 23]. Since secretagogues triggering release of preformed mucin did not affect the rate of baseline mucin secretion, this secretory pathway has tentatively been called unregulated [4, 20]. However, our results strongly suggest a regulatory role of cAMP in this secretory pathway. In contrast to carbachol, cholera-toxin-induced secretion contained newly synthesized mucin. The stimulatory action of cholera toxin on the secretion of newly synthesized mucin became apparent after a time lag of 4 h. This delay fits in well with the estimated 4-h transit time of mucins from the Golgi to the apical cell membrane in baseline mucin secretion [35]. To achieve significant labelling of the mucin storage pool, much longer incubation times (>24 h) with labelled mucin precursor would be necessary [4]. Therefore, the labelled (i.e. newly synthesized) mucin found in the apical sample after cholera toxin stimulation must have bypassed the mucin storage compartment. Mucin vesicles released during baseline mucin secretion are also thought to bypass the storage pool, and their travel to the apical membrane is mediated by microtubules. Microtubule depolymerization by colchicine not only inhibited baseline but also cholera-toxin-induced mucin secretion, indicating the importance of intact microtubules for both baseline and cAMP-stimulated mucin secretion. Finally, the time course of the secretory response to cholera toxin is much more compatible with the upregulation of continuously ongoing mucin secretion rather than with a pulsatile release of stored mucin granules as triggered by carbachol. This two-compartment model also provides a good explanation for the interesting result that, even after prolonged incubation of HT-29/B6 cells in cholera toxin with subsequent depletion of intracellular mucin, carbachol still elicited its secretory response.

Based on our results, we suggest the following regulation of intestinal mucin secretion: Ca^{2+}-dependent agonists cause accelerated mucin secretion by rapid release of preformed, apically stored mucin granules. Elevation of cAMP on the other hand stimulates the continuous secretion of preformed and newly synthesized mucin, bypassing the Ca^{2+}-regulated storage pool. This model is corroborated by the stimulatory action of cAMP on mucin RNA expression and glycoprotein synthesis found in earlier studies [5, 10, 14]. It is a significant expansion of the present concept of regulated mucin secretion, because secretagogue-evoked stimulation of goblet cells is currently thought to result mainly in compound exocytosis, whereas continual single-granule exocytosis is opposed to this process as constitutive secretion without receptor-mediated regulation [4, 20]. We suggest that both types of mucin secretion are differentially regulated via classic signal transduction pathways.

Acknowledgements The excellent technical assistance of U. Lempart is gratefully acknowledged.

References

1. Cassel D, Pfeuffer T (1978) Mechanism of cholera toxin action: covalent modification of the guanyl nucleotide-binding protein of the adenylate cyclase system. Proc Natl Acad Sci USA 75:2669–2673
2. De Jonge HR (1976) Cyclic nucleotide dependent phosphorylation of intestinal epithelium proteins. Nature 262:590–593
3. Fischer H, Kreusel KM, Illek B, Machen TE, Hegel U, Clauss W (1992) The outwardly rectifying Cl^- channel is not involved in Cl^- secretion in HT-29 cells: evidence for a very low conductance Cl^- channel. Pflügers Arch 422:159–167
4. Forstner GG (1995) Signal transduction, packaging and secretion of mucins. Annu Rev Physiol 57:585–605
5. Forstner GG, Shih M, Lukie B (1973) Cyclic AMP and intestinal glycoprotein synthesis. The effect of β-adrenergic agents, theophylline and dibutyryl cyclic AMP. Can J Physiol Pharmacol 51:122–129
6. Forstner GG, Zhang Y, McCool D, Forstner J (1994) Regulation of mucin secretion in T84 adenocarcinoma cells by forskolin: relationship to Ca^{2+} and PKC. Am J Physiol 266: G606–G612

7. Fuller CM, Bridges RJ, Benos DJ (1994) Forskolin- but not ionomycin-evoked Cl^- secretion in colonic epithelia depends on intact microtubules. Am J Physiol 266:C661–C668
8. Gendler JG, Spicer AP (1995) Epithelial mucin genes. Annu Rev Physiol 57:607–634
9. Hidaka H, Inagaki M, Kawamoto S, Sasaki Y (1984) Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochemistry 23:5036–5041
10. Jarry A, Merlin, D, Velcich A, Hopfer U, Augenlicht LH, Laboisse CL (1994) Interferon-γ modulates cAMP-induced mucin exocytosis without affecting mucin gene expression in a human colonic goblet cell line. Eur J Pharmacol 267: 95–103
11. Keates AC, Hanson PJ (1990) Regulation of mucus secretion by cells isolated from the rat gastric mucosa. J Physiol (Lond) 423:397–409
12. Kreusel KM, Fromm M, Schulzke JD, Hegel U (1991) Cl^- secretion in epithelial monolayers of mucus-forming human colon cells. Am J Physiol 261:C574–C582
13. Kreusel KM, Rapp K, Hegel U (1992) Effect of acetylcholine on secretion of mucin and chloride in the epithelial cell line HT-29/B6. Pflügers Arch 420:R59
14. LaMont JT, Ventola AS (1977) Stimulation of colonic glycoprotein synthesis by dibutyryl cyclic AMP and theophylline. Gastroenterology 72:82–86
15. Lencer WI, Reinhardt FD, Neutra MR (1990) Interaction of cholera toxin with cloned human goblet cells in monolayer culture. Am J Physiol 258:G96–G102
16. Lencer WI, de Almeida JB, Moe S, Stow JL, Ausiello DA, Madara JL (1993) Entry of cholera toxin into polarized human intestinal epithelial cells. Identification of an early brefeldin A sensitive event required for A1-peptide generation. J Clin Invest 92:2941–2951
17. Mantle M, Forstner GG, Forstner JF (1984) Biochemical characterization of the component parts of human intestinal mucin from patients with cystic fibrosis. Biochem J 224:345–354
18. McCool DJ, Marcon MA, Forstner JF, Forstner GG (1990) The T84 human colonic adenocarcinoma cell line produces mucin in culture and release it in response to various secretagogues. Biochem J 267:491–500
19. McCool DJ, Forstner JF, Forstner GG (1994) Synthesis and secretion of mucin by the human colonic tumour cell line LS180. Biochem J 302:111–118
20. McCool DJ, Forstner JF, Forstner GG (1995) Regulated and unregulated pathways for MUC2 mucin secretion in human colonic LS180 adenocarcinoma cells are distinct. Biochem J 312:125–133
21. Moore BA, Sharkey KA, Mantle M (1993) Neural mediation of cholera toxin-induced mucin secretion in the rat small intestine. Am J Physiol 265:G1050–G1056
22. Nath SK, Huang X, L'helgoualc'h A, Rautureau M, Bisalli A, Heyman M, Desjeux-JF (1994) Relation between chloride secretion and intracellular cyclic adenosine monophosphate in a cloned human intestinal cell line HT-29 cl 19A. Gut 35:631–636
23. Neutra MR, Forstner JF (1987) Gastrointestinal mucus: synthesis, secretion and function. In: Johnson LR (ed.) Physiology of the gastrointestinal tract, 2nd edn. Raven, New York, pp 975–1009
24. Neutra MR, O'Malley LJ, Specian RD (1982) Regulation of intestinal goblet cell secretion. II. A survey of potential secretagogues. Am J Physiol 242:G380–G387
25. Niv Y, Byrd JC, Ho SB, Dahiya R, Kim YS (1992) Mucin synthesis and secretion in relation to spontaneous differentiation of colon cancer cells. Int J Cancer 50:147–152
26. Phillips TE, Phillips TL, Neutra MR (1984) Regulation of intestinal goblet cell secretion. III. Isolated intestinal epithelium. Am J Physiol 247:G674–G681
27. Qureshi R, Forstner GG, Forstner JF (1979) Radioimmunoassay of human intestinal goblet cell mucin. J Clin Invest 64: 1149–1156
28. Roomi N, Laburthe M, Fleming N, Crowther R, Forstner JF (1984) Cholera-induced mucin secretion in rat small intestine: lack of effect of cAMP, cycloheximide, VIP, and colchicine. Am J Physiol 247:G140–G148
29. Roumagnac I, Laboisse CL (1989) A simple immunofiltration assay for mucins secreted by a human colonic epithelial cell line. J Immunol Methods 122:265–271
30. Seidler U, Sewing KF (1989) Ca^{2+}-dependent and independent secretagogue action on gastric mucus secretion in rabbit mucosal explants. Am J Physiol 256:G739–G746
31. Sherr HP, Mertens BR, Broock R (1979) Cholera toxin-induced glycoprotein secretion in rabbit small intestine. Gastroenterology 77:18–25
32. Specian RD, Neutra MR (1980) Mechanism of rapid mucus secretion in goblet cells stimulated by acetylcholine. J Cell Biol 85:626–640
33. Specian RD, Neutra MR (1982) Regulation of intestinal goblet cell secretion. I. Role of parasympathetic stimulation. Am J Physiol 242:G370–G379
34. Specian RD, Neutra MR (1984) Cytoskeleton of intestinal goblet cells in rabbit and monkey. The theca. Gastroenterology 18:1313–1325
35. Specian RD, Oliver MG (1991) Functional biology of intestinal goblet cells. Am J Physiol 260:C183–C193
36. Steinberg SE, Banwell JG, Yardley JH, Keusch GT (1968) Comparison of secretory and histological effects of *Shigella* and cholera enterotoxins in rabbit jejunum. Gastroenterology 56:1047–1052
37. Thornton DJ, Holmes F, Sheehan JK, Carlstedt I (1989) Quantitation of mucus glycoproteins blotted onto nitrocellulose membranes. Anal Biochem 182:160–164