RESEARCH COMMUNICATION

Cyclic AMP-induced mucin exocytosis is independent of Cl^- movements in human colonic epithelial cells (HT29-Cl.16E)

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The human colonic epithelial goblet cell line HT29-Cl.16E was used to test whether stimulated Cl^- transport is involved in the mucin exocytotic response to an increase in intracellular cyclic AMP by measuring in parallel the short-circuit current (Isc) and mucin exocytosis. Addition of 50 μM forskolin to HT29-Cl.16E cells resulted in a 2-fold stimulation of mucin release and an increase in Isc by 20 $\mu A/cm^2$. To evaluate the requirement for cosecretion of Cl^-, the Cl^- flux was altered by three different manipulations : (1) Cl^- in the medium was replaced by the poorly transported anion gluconate; (2) basolateral Cl^- influx through the Na^+–K^+–$2Cl^-$ cotransporter was inhibited by bumetanide; and (3) an inward Cl^- flux through the apical plasma membrane was generated by reversing the Cl^- gradient. These manipulations did not change the forskolin-stimulated mucin release and thereby provide evidence that Cl^- movements are not required for fusion of mucin granules with the plasma membrane.

INTRODUCTION

Electrolyte secretion and macromolecular exocytosis are important functions of the lining cells of many epithelial organs. Macromolecular exocytosis is defined as the process by which secretory products sequestered in membrane-bound granules are released at the plasma membrane [1]. The release of the contents comprises granule fusion and physical movement of the contents out of the granules after fusion. It has been proposed that electrolyte fluxes play a role in driving both the fusion process and the physical release of the granule contents. Until recently, electrolyte and macromolecular secretions were considered to be carried out by phenotypically distinct cell types. However this view has been challenged by several studies, which have demonstrated that macromolecule-secreting exocrine cells possess the same or similar electrolyte secretory pathways as other epithelial cells. For example, pancreatic acinar cells contain protein kinase A (PKA)-activated as well as calcium-activated Cl^- channels for Cl^- secretion [2,3]. Finally, Cl^- and macromolecule secretions are coupled in macromolecule-secreting epithelial cells by virtue of the presence of Cl^- channels in the membranes of secretory granules [4]. The coupling of these two secretory processes has been shown in pancreatic acinar cells, but could also be considered in tracheobronchial and intestinal epithelial cells secreting mucins. In such epithelial cells, a logical extension of these proposals is to provide a possible explanation for the pathophysiology of cystic fibrosis (CF) exocrinopathy, primarily due to a defect in PKA-regulated Cl^- channels, and in which many of the clinical problems appear to be caused by an abnormally viscous mucus resulting from abnormalities in macromolecular secretions [5]. As detailed above, lack of cyclic AMP (cAMP)-dependent Cl^- and fluid secretion across the granule membrane of macromolecule-secreting epithelial cells might profoundly impair either the exocytotic fusion process or the flushing-out, or both processes, and result in abnormal mucin secretion.

With the availability of the human colonic goblet cell line HT29-Cl.16E, a clonal derivative from HT29 cell line [6], it is now possible to explore in a given cell type, i.e. goblet cells, whether Cl^- movements are involved in mucin exocytosis stimulated by an increase in intracellular cAMP. Indeed, these cells, when cultured to confluency on porous membranes, form homogenous monolayers of mucous-secreting cells, each cell being able to secrete high amounts of both mucin and Cl^- when stimulated with secretagogues [7–10]. In HT29-Cl.16E cells, the recent finding of a cAMP-induced Cl^- secretion [9,10] is in favour of the presence of the CF transmembrane regulator protein, and is in line with the fact that the CF gene has been found to be expressed both in parental HT29 cells [11,12] and in their clonal derivative HT29-Cl.16E cells (C. Bou-Hanna, unpublished work).

Specifically, this study was intended to inhibit the Cl^- flux generated by an increase in intracellular cAMP levels or to reverse its direction, and to examine in parallel cAMP-stimulated mucin exocytosis. To this end, we measured the effects: (1) of a low-Cl^--containing medium, in which Cl^- was substituted with the non-transported ion gluconate, on forskolin-stimulated mucin exocytosis and chloride secretion; (2) of a specific inhibitor of the Na^+–K^+–$2Cl^-$ cotransporter, which mediates concentrative uptake of Cl^- across the basolateral membrane [13]; and (3) of establishing an inward Cl^- flux across the apical plasma membrane through a Cl^- gradient between mucosal and serosal compartments (mucosal > serosal) and permeabilization of the basolateral plasma membrane with amphotericin B. Altogether, our results show that cAMP-induced Cl^- movements can be specifically blocked in HT29-Cl.16E cells without affecting cAMP-increased mucin exocytosis.

MATERIALS AND METHODS

Cell culture

HT29-Cl.16E monolayers were formed by plating 1.2×10^6 cells on vitrogen-coated Millicell CM nitrocellulose filter inserts

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Abbreviations used: Isc, short-circuit current; CS, cystic fibrosis; cAMP, cyclic AMP; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; DIDS, 4,4′-di-isothiocyanostilbene-2,2′-disulphonate; PKA, protein kinase A.

Table 1 Composition of mucosal and serosal solutions in experiments with two different Cl^- gradients

The composition of the Ringer and low-Cl^- media was as follows. Ringer solution comprises: 4 mM KCl, 114 mM NaCl, 1.25 mM $CaCl_2$, 1 mM $MgCl_2$, 22.98 mM $NaHCO_3$ and 25 mM glucose. Low-Cl^- medium: 4 mM K-gluconate, 114 mM Na-gluconate, 1.25 mM $CaCl_2$, 1 mM $MgCl_2$, 22.98 mM $NaHCO_3$ and 25 mM glucose.

	Incubation time (min)	Mucosal bath	Serosal bath
$[Cl]_M < [Cl]_S$	15	Ringer	Ringer
	30	Low-Cl^- solution	Ringer
	40	Low-Cl^- solution	Ringer + amphotericin B (20 μM)
	10	Low-Cl^- solution	Ringer + amphotericin B (20 μM) + ouabain (100 μM)
	15	Forskolin (50 μM)	Forskolin (50 μM)
$[Cl]_M > [Cl]_S$	15	Ringer	Ringer
	30	Ringer	Low-Cl^- solution
	40	Ringer	Low-Cl^- solution + amphotericin B (20 μM)
	10	Ringer	Low-Cl^- solution + amphotericin B (20 μM) + ouabain (100 μM)
	15	Forskolin (50 μM)	Forskolin (50 μM)

(0.6 cm^2; Millipore). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS). Cells formed confluent monolayers of goblet cells after about 7 days in culture, as judged by low conductance and the release of visible mucus into the mucosal compartment. Experiments were conducted with cells between day 8 and 12 post-seeding.

Electrophysiology

Cl^- secretion was measured electrophysiologically and quantified as short-circuit current (Isc), as previously described [10].

Mucin measurements

Mucin secretion into the mucosal compartment was measured after a 24 h metabolic incorporation with D-[6-^{3}H]glucosamine hydrochloride (4 μCi/ml in DMEM/10% FCS; specific activity 20–40 mCi/mmol; Amersham, Paris, France), as previously described [7]. Filters were exposed to the secretagogue forskolin (50 μM; Calbiochem, Coger, Paris, France) for 15 min. The [^{3}H]mucins secreted into the mucosal compartment of control or forskolin-treated monolayers were collected after pipetting the medium several times over the monolayers. The mucin exocytotic response was quantified by using a specific and sensitive 'electrophoretic assay' for secretory mucins, as previously described [7].

To test whether forskolin-stimulated mucin exocytosis and Cl^- secretion are linked or independent processes in HT29-Cl.16E cells, three types of experiments were carried out. (1) HT29-Cl.16E monolayers were extensively washed for 4 × 15 min in a low-Cl^- solution in which Cl^- ions were substituted with the non-transported ion gluconate, to eliminate Cl^- fluxes. The cells were then stimulated in a low-Cl^- solution, with 50 μM forskolin added to the serosal compartment, and assessed for Isc or [^{3}H]mucin measurement. Control monolayers were washed in normal Ringer solution and incubated for 15 min with forskolin in normal Ringer solution. The composition of the Ringer solution and low-Cl^- medium are indicated in Table 1. (2) Bumetanide (10^{-4} M; Sigma), a specific inhibitor of the Na^+–K^+–$2Cl^-$ cotransporter, was added to the serosal bath 5 min before the addition of forskolin (15 min) which was added to both serosal and mucosal solutions, for the determination of the release of [^{3}H]mucin. For Isc measurements, bumetanide was added to the serosal bath when the Isc provoked by forskolin was maximal. (3) To elucidate the importance of Cl^- fluxes in mucin exocytosis, Cl^- movements were manipulated to effect a reversal of the normal direction of the Cl^- flux across the luminal plasma membrane, into the cell. The following protocol was used: (i) HT29-Cl.16E monolayers were permeabilized with 20 μM amphotericin B (Sigma) at the serosal side, as previously described [10]; (ii) a Cl^- gradient was established between the mucosal and serosal sides ($[Cl^-]_M < [Cl^-]_S$); (iii) the apical Cl^- channels were opened by adding 50 μM forskolin, and Isc was measured. Then, the Cl^- gradient was reversed ($[Cl^-]_M > [Cl^-]_S$), resulting in an absorptive secretory Cl^- flux. The composition of the media used in these experiments is summarized in Table 1.

Statistical analysis

Data are expressed as mean ± S.E.M of at least three monolayers. The significance of differences was assessed by Student's t test, whereby $P < 0.05$ was considered significant.

RESULTS

Post-confluent filter-grown HT29-Cl.16E cells form homogenous monolayers of goblet cells, continuously secreting mucins at their apical surface (baseline mucin secretion). The baseline electrical conductance was 4.4 ± 0.5 mS/cm^2 ($n = 9$) and the Isc was 2.8 ± 0.9 μA/cm^2 ($n = 9$). As shown in Table 2, forskolin induced a 2-fold increase in mucin exocytosis and a sustained Isc increase of about 7-fold, up to 20 μA/cm^2, in standard incubation medium.

First, we examined the effects of a low-Cl^- medium on both forskolin-mediated Cl^- and mucin secretory processes. When HT29-Cl.16E cells were stimulated in gluconate-containing medium, the Isc response to forskolin dropped dramatically (ΔIsc in Ringer medium: 19.8 ± 0.8 μA/cm^2; ΔIsc in gluconate-containing medium: 1.1 ± 0.7 μA/cm^2, i.e. 94% inhibition versus control, $n = 6$), whereas the mucin exocytotic response remained unchanged (Table 2). Next, we examined the action of bumetanide, a well-known inhibitor of the Na^+–K^+–$2Cl^-$ cotransporter. As shown in Figure 1, bumetanide abolished the Isc response due to forskolin (92% inhibition), but it did not modify forskolin-increased mucin exocytosis (Table 2). These manipulations to inhibit the Cl^- flux did not damage the cells, in that inhibition of Isc was reversed when low-Cl^- medium was returned to normal

Table 2 Effects of Cl^- substitution by gluconate and of the Na^+–K^+–2Cl— cotransporter inhibitor bumetanide on forskolin-stimulated mucin exocytosis in HT29-Cl.16E cells

Mean ± S.E.M. of six monolayers. Bumetanide was added to the serosal side of monolayers 5 min before adding forskolin (50 μM) to both mucosal and serosal reservoirs. [^{3}H]Mucins were collected 15 min after forskolin addition.

	[3H]Mucin exocytosis (d.p.m./10^6 cells)			
	Effect of Cl^- substitution by gluconate		Action of bumetanide	
	Ringer solution	Low Cl^- solution (gluconate)	Without bumetanide	With bumetanide (10^{-4} M)
Baseline	4787 ± 940	5778 ± 509	4104 ± 539	4556 ± 598
+ Forskolin	8957 ± 1175	10124 ± 1841	9207 ± 756	10035 ± 1819

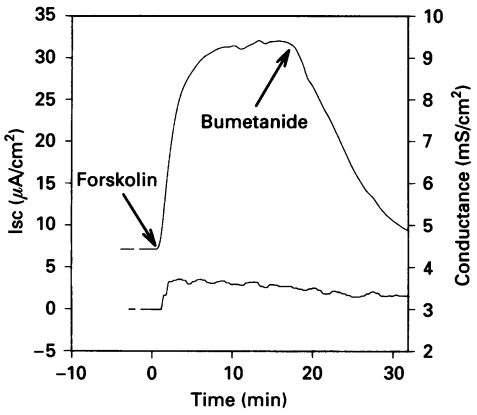

Figure 1 Effects of bumetanide on cAMP-induced Cl^- secretion in HT29-Cl.16E cells

Bumetanide (10^{-4} M) was added to the serosal bath of monolayers when Isc provoked by forskolin (50 μM) was maximal. Isc (upper tracing) and conductance (lower tracing) are representative of three experiments.

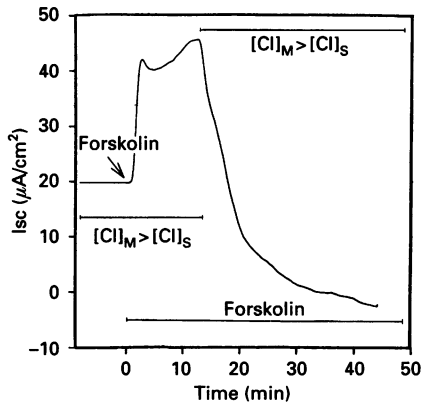

Figure 2 Effects of different Cl^- gradients across the apical plasma membrane of HT29-Cl.16E cells on Cl^- flux as measured by Isc

The basolateral plasma membrane of HT29-Cl.16E cells was permeabilized with 20 μM amphotericin B in the serosal compartment, as described in the Materials and methods section. The Isc was monitored continuously. The Cl^- gradient across the apical membrane was varied as indicated by the horizontal bars: initially, the concentration of mucosal Cl^- was lower than concentration of serosal and cytosolic Cl^- ($[Cl]_M < [Cl]_S$); after stimulation with 50 μM forskolin, added to both mucosal and serosal baths, the Cl^- gradient was reversed, with mucosal Cl^- higher than serosal Cl^- ($[Cl]_M > [Cl]_S$). For the exact composition of solutions see Table 1. Tracing is representative of three experiments.

Ringer solution or when bumetanide was washed out (results not shown). The monolayer conductance decreased from 4.6 ± 0.31 mS/cm² in Ringer solution to 2.4 ± 0.67 mS/cm² (n = 12) in low-Cl^- medium, as expected with tight junctions of much lower permeability to gluconate than to Cl^-. When bumetanide was added after stimulation by 50 μM forskolin, Isc gradually declined over 5 min to baseline values, consistent with gradual depletion of intracellular Cl^-. In addition, neither low-Cl^- medium nor bumetanide significantly affected baseline mucin exocytosis (Table 2). In the last set of experiments the Cl^- flux across the apical membrane was reversed from the normal secretory direction to an absorptive one. Monolayers were permeabilized with 20 μM amphotericin B in the serosal bath, and a Cl^- gradient was established between the mucosal and serosal compartments ($[Cl]_M < [Cl]_S$). After forskolin (50 μM) addition, the cAMP-dependent mucosal Cl^- conductance increased, resulting in normal secretory Cl^- current, as measured by Isc (Figure 2). Then, the direction of the gradient was reversed with $[Cl]_M > [Cl]_S$, which gave rise to an absorptive Cl^- current (Figure 2). The forskolin-stimulated mucin release occurred to the same extent, regardless of the presence or direction of the Cl^- gradient, or whether the gradient was abolished with equally high concentrations of Cl^- on both sides (Table 3).

Table 3 Effects of the inversion of Cl^- gradients at the apical membrane of HT29-Cl.16E cells on forskolin-stimulated mucin exocytosis

Confluent HT29-Cl.16E monolayers were metabolically labelled for 24 h with [^{3}H]glucosamine, then treated as described in the Materials and methods section and Table 1, and stimulated with forskolin for 15 min. [^{3}H]Mucins were measured with a specific electrophoretic assay as reported in the Materials and methods section. Results are mean ± S.E.M. of four monolayers. * Not significantly different from results obtained under the other two conditions.

	Stimulation over baseline secretion (%)
$[Cl]_M = [Cl]_S$	173 ± 6*
$[Cl]_M > [Cl]_S$	183 ± 18*
$[Cl]_M < [Cl]_S$	169 ± 17*

DISCUSSION

A general approach to elucidating the possible involvement of Cl^- in macromolecular exocytosis has been based on agents that were assumed to block anion transport, but can be non-specific,

such as stilbene derivatives. 4,4′-Di-isothiocyanostilbene-2,2′-disulphonate (DIDS) and/or 4-acetimido-4′-isothiocyanostilbene-2,2′-disulphonate have previously been shown to inhibit a number of exocytotic secretory processes, including insulin secretion by islet cells, neutrophil degranulation and mucin secretion from T84 intestinal cells [14]. In the T84 cell line, inhibition of mucin secretion by DIDS was observed with vasoactive intestinal peptide-stimulated mucin secretion [14]. However, the specific mode of action of DIDS remains largely speculative since in another work DIDS was reported to have no effect on apical cAMP-induced Cl^- secretion [15]. Finally the complexity of DIDS action is well exemplified in a recent report showing that DIDS has a dual effect in HT29 cells: at low concentrations it enhances K^+ conductance and at higher concentrations it inhibits Cl^- conductance [16]. In view of these findings, a different approach was adopted: the Cl^- flux was altered without interfering with the cAMP-dependent change in Cl^- permeability. Cl^--ion substitution with a poorly transported ion (gluconate) allowed us to gain insight into (1) the nature of the electrolytes secreted under stimulation elicited by an intracellular increase in cAMP levels; and (2) the mechanisms involved in electrolyte and macromolecular cosecretions in mucous cells. The finding that forskolin-induced Isc is abolished in gluconate-containing medium strongly suggests that this current is due to Cl^- secretion. In addition, the maintenance of a cAMP-induced mucin exocytotic response in gluconate-containing medium is in line with the concept that the exocytotic fusion of granules is independent of cAMP-dependent Cl^- transport either at the plasma membrane or the organelle membrane.

The same type of interpretation applies to the bumetanide and absorptive (reverse from normal secretory) Cl^- flux experiments. The importance of the bumetanide-sensitive Na^+–K^+–$2Cl^-$ co-transporter for maintenance of cellular $Cl^-$is well established for epithelial cells in general and for HT29-Cl.19A and T84 in particular [13]. Thus, blocking of this transporter depletes the cell of Cl^- within 5 min, as judged by the decrease in Isc (Figure 1). Finally, all results indicate that the cAMP-dependent direction and magnitude of the Cl^- flux do not influence the mucin exocytotic response. Therefore, the fusion machinery must contribute a target for the cAMP messenger system which is independent from CFTR/cAMP-activated Cl^- conductance.

Our assay *in vitro* was not designed to evaluate the effects of Cl^- flux on the flushing out of mucin under physiological conditions. The procedure of washing cells for the purpose of collecting mucin probably flushed out the contents from all fused granules so that the collected mucin was a measure of the extent of granule fusion. In other words, our results do not exclude that Cl^- and resulting fluid fluxes have effects on mucin release in the absence of washings, i.e. under more physiological conditions. Mucin hydration and solvation may be important determinants of mucin release in the absence of washings, i.e. when driven by fluid secretion in glands and crypts *in situ*.

Finally, when one considers the abnormally viscous mucus observed in CF exocrinopathy in connection with our findings, one would suggest that abnormalities in macromolecular secretions resulting from a defect in cAMP-induced Cl^- transport are likely to represent a 'post-fusion' defect in the exocytotic process, i.e. an improper flushing-out of macromolecular secretions.

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REFERENCES

1 Almers, W. (1990) Annu. Rev. Physiol. **52**, 607–624
2 Kopelman, H. and Gauthier, C. (1991) Pediatr. Res. **29**, 529–533
3 Petersen, C. C., Toescu, E. C. and Petersen, O. H. (1991) EMBO J. **10**, 527–533
4 De Lisle, R. C. and Hopfer, U. (1986) Am. J. Physiol. 250, G489–G496
5 Sturgess, J. M. and Imrie, J. (1982) Am. J. Pathol. **106**, 303–311
6 Augeron, C. and Laboisse, C. L. (1984) Cancer Res. **44**, 3961–3969
7 Augeron, C., Voisin, T., Maoret, J. J., Berthon, B., Laburthe, M. and Laboisse, C. (1992) Am. J. Physiol. **262**, G470–G476
8 Merlin, D., Augeron, C., Laboisse, C. L. and Hopfer, U. (1992) Gastroenterology **102**, A935 (abstract)
9 Merlin, D., Jarry, A., Hopfer, U. and Laboisse, C. L. (1993) Gastroenterology **104**, A1052 (abstract)
10 Merlin, D., Augeron, C., Tien, X. Y., Guo, X., Laboisse, C. L. and Hopfer, U. (1994) J. Membr. Biol. **137**, 137–149
11 Sood, R., Bear, C., Auerbach, W., Reyes, E., Jensen, T., Kartner, N., Riordan, J. R. and Buchwald, M. (1992). EMBO J. **11**, 2487–2494
12 Morris, A. P., Cunningham, S. A., Benos, D. J. and Frizell, R. A. (1992) J. Biol. Chem. **267**, 5575–5583
13 Matthews, J. B., Awtrey, C. S. and Madara, J. L. (1992) J. Clin. Invest. **90**, 1608–1613
14 Marcon, M. A., McCood, D., Forstner, J. and Forstner, G. (1990) Biochim. Biophys. Acta **1052**, 17–23
15 Anderson, M. P., Rich, D. P., Gregory, R. J., Smith, A. E. and Welsh, M. J. (1991) Science **251**, 679–682
16 Kubitz, R., Warth, R., Allert, N., Kunzelmann, K. and Greger, R. (1992) Pflügers Arch. **421**, 447–454

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