Role of calcium in carbachol- and neurotensin-induced mucin exocytosis in a human colonic goblet cell line and cross-talk with the cyclic AMP pathway

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The mechanisms of Ca^{2+} -induced mucin secretion were examined in monolayers of the differentiated epithelial colon cell line Cl.16E by combined measurements of free intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) using a fluorescence indicator and mucous secretion using a specific and sensitive electrophoretic assay. Carbachol, a cholinergic agonist, induced an initial concentration-dependent $[\text{Ca}^{2+}]_i$ peak increasing from 129 ± 3 nM (basal $[\text{Ca}^{2+}]_i$) to 608 ± 101 nM at 1×10^{-4} M carbachol with an ED_{50} of $7 \mu\text{M}$, and this was followed by a lower-level plateau. These biphasic effects were reversed by the muscarinic-receptor antagonist atropine. In the absence of extracellular Ca^{2+} , the initial $[\text{Ca}^{2+}]_i$ peak was maintained while the sustained plateau was abolished. The regulatory peptide neurotensin caused a monophasic transient rise in $[\text{Ca}^{2+}]_i$ followed by a very rapid return to baseline. The neurotensin-induced rise in $[\text{Ca}^{2+}]_i$ was concentration-dependent with an ED_{50} of 4 nM, and was maximal at 1×10^{-6} M (598 ± 127 nM). The $[\text{Ca}^{2+}]_i$ response to neurotensin was not

significantly affected by extracellular Ca^{2+} depletion. Carbachol-induced mucin exocytosis was concentration-dependent with an ED_{50} of $15 \mu\text{M}$, and was inhibited by 35% upon removal of extracellular Ca^{2+} . Neurotensin caused a concentration-dependent rise in mucous secretion with an ED_{50} of 36 nM, not significantly affected upon removal of extracellular Ca^{2+} . Together our results suggest that while the mucin secretory response to carbachol depends on both the release of Ca^{2+} from intracellular stores and a Ca^{2+} influx from external medium, the secretory response to neurotensin is based solely on intracellular Ca^{2+} mobilization. Finally, evaluation of the cross-talk between the cyclic AMP pathway stimulated by vasoactive intestinal peptide (VIP) and the Ca^{2+} pathway stimulated by neurotensin or carbachol led to the conclusion that the potentiated secretory response elicited by the combined action of carbachol and VIP requires extracellular Ca^{2+} .

INTRODUCTION

Like other exocrine cells, mucous intestinal cells store their secretory products in granules and release them by stimulus-secretion-coupling mechanisms. These mechanisms generally involve a receptor-mediated effect on intracellular second messengers. Several lines of investigation suggest that Ca^{2+} -dependent mechanisms play a major role in eliciting a secretory response from mucous cells. The long-standing observation of a cholinergic regulation of intestinal goblet cells both 'in vivo' and 'in vitro' [1], together with the demonstration that cholinergic agonists evoke an increase in intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in a variety of epithelial cells [2], provided the first indication that cellular Ca^{2+} mobilization may regulate mucin exocytosis. More direct evidence indicating a Ca^{2+} -dependent secretagogue action on mucin secretion has been obtained by using rabbit mucosal explants in organ culture [3]. In the latter system the stimulatory effects of the Ca^{2+} ionophore A23187 on glycoprotein secretion were abolished by removal of extracellular Ca^{2+} and the stimulatory effect of acetylcholine was abolished by depletion of intracellular Ca^{2+} stores [3]. More recently, in an effort to design a reductionist approach to correlate the secretagogue-induced secretory response with changes in the concentration of putative intracellular second messengers in the secretory cell, Seidler and Pfeiffer have investigated the acetylcholine-induced changes in $[\text{Ca}^{2+}]_i$ in isolated and highly purified rabbit gastric mucous cells [4]. Interestingly they found

that acetylcholine stimulated a cell-membrane Ca^{2+} -entry mechanism in addition to release of Ca^{2+} from intracellular stores.

One limitation of this approach, however, resides in the fact that the interdependence of the Ca^{2+} signal and secretory function could not be assessed in this system of highly purified mucous cells because of a low yield of secretory mucins.

In view of these difficulties, we were prompted to study Ca^{2+} -dependent secretion in mucous cells by using an approach based on the stably differentiated clonal derivative Cl.16E [5] of the colonic cancer cell line HT29 [6]. Cl.16E cell line was selected as a model system because it represents a homogeneous population of mucous-secreting epithelial cells responding to carbachol [7]. In addition, this cell line responds to neurotensin [8], another Ca^{2+} -linked secretagogue. Based on this cell line, used in combination with a specific and sensitive assay for secretory mucins, our specific aims were to investigate (1) the acetylcholine- and neurotensin-induced changes in $[\text{Ca}^{2+}]_i$ in colonic mucous cells, and (2) the stimulus-secretion coupling upon stimulation of colonic mucous secretion by acetylcholine and neurotensin.

MATERIALS AND METHODS

Cell line and culture methods

Cl.16E, a stably differentiated, mucous-secreting cell clone of the HT-29 human carcinoma cell line, has previously been described and characterized [5]. Cl.16E cells were routinely cultured in 25 cm^2 plastic flasks (Falcon) in Dulbecco's modified Eagle's

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; $[\text{Ca}^{2+}]_o$, extracellular Ca^{2+} concentration; VIP, vasoactive intestinal peptide; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal-calf serum; BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-*NNN'*-tetra-acetic acid acetoxymethyl ester; DMSO, dimethyl sulphoxide.

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medium (DMEM, Gibco) supplemented with 10% (v/v) heat-inactivated fetal-calf serum (FCS, Gibco). Mycoplasma contamination was tested for periodically by the method of Chen [9].

Filter cultures giving improved access of the basolateral membrane of the cells to the secretagogues were established as follows: cells were plated on nitrocellulose filters (2×10^6 cells/filter) (Millipore, HAHY; $0.45 \mu\text{m}$ pore diam.; diam. 1.3 cm) that had been previously sealed on the bottom of wells of multiwell tissue-culture plates (Falcon, 24-well Multiwell tissue-culture plates). At day 1, each filter was transferred to a 35 mm diam. culture dish in 2.5 ml of culture medium. The culture medium was changed every other day starting from day 3. Experiments were conducted between day 8 and day 14 after seeding.

Metabolic labelling of secretory glycoproteins and measurement of mucins

Secreted mucins were quantified using a previously described electrophoretic assay [8]. Briefly, confluent monolayers on filters were incubated with $10 \mu\text{Ci}$ of D-[6- ^3H]glucosamine hydrochloride (Amersham, TRK 398; specific radioactivity: 20–40 Ci/mmol) in 2.5 ml of DMEM containing 10% (v/v) FCS for 24 h. Then the filters were washed three times in a serum-free medium composed of a 1:1 mixture of Ham F12 medium (Gibco) and DMEM, this medium being referred to as Ham–DMEM, and transferred into the wells of a multiwell culture plate containing $600 \mu\text{l}$ of Ham–DMEM, with or without secretagogues in the presence of 0.01% BSA. After 15 min the incubation medium was removed using a pipette and the monolayer was rinsed with the spent medium to remove adherent mucin. Then the incubation medium was dialysed for 36 h against several changes of deionized water at 4°C and lyophilized. The secretory glycoproteins were separated by SDS/PAGE on 3% (w/v) polyacrylamide gels. The band at the stacking/running gel interface, which contains the mucins, was cut out, placed into a borosilicate vial, and incubated overnight at 40°C in the presence of 0.5 ml of Soluene 350. Radioactivity was determined by liquid-scintillation counting.

Effects of extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$) and $[\text{Ca}^{2+}]_i$ chelation on mucous secretion of Cl.16E cells

To chelate extracellular Ca^{2+} the filter-grown cells were stimulated in S-Minimum Essential Medium (S-MEM, Gibco) supplemented with 0.5 mM EGTA. The control cells in these experiments underwent the same procedure except for the fact that the medium (Ham–DMEM) contained Ca^{2+} and no EGTA. The cell-permeable Ca^{2+} chelator, BAPTA/AM [1,2-bis(2-aminophenoxy)ethane-*NNN'*-tetra-acetic acid acetoxymethyl ester] (Calbiochem) was used to chelate intracellular Ca^{2+} . Filter-grown cells were labelled for 24 h with [^3H]glucosamine, then rinsed three times in standard incubation medium (Ham–DMEM), and incubated for 30 min at 37°C with $50 \mu\text{M}$ BAPTA/AM in CaCl_2 -free MEM (S-MEM, Gibco). BAPTA/AM was dissolved in dimethyl sulphoxide (DMSO) (final concentration 0.1%). The cells were then rinsed three times in S-MEM medium without CaCl_2 plus 0.5 mM EGTA (Fluka) to chelate extracellular Ca^{2+} , then transferred to fresh Ca^{2+} -free medium and stimulated as described above.

Measurements of $[\text{Ca}^{2+}]_i$ with Fura-2/AM

Stock Fura-2 acetomethoxy ester (Fura-2/AM) was dissolved in 100% DMSO to a concentration of 1 mM and stored at -20°C (Molecular Probe).

Cl.16E monolayers were dissociated into monodispersed cell suspensions by incubation in 1 mM EDTA for 8 min, followed by a very short incubation in a few drops of a 0.5 mM EDTA solution containing 0.25% trypsin. The cells were then resuspended in DMEM containing 10% (v/v) FCS and incubated in the dark with $3 \mu\text{M}$ Fura-2/AM in 10 ml of DMEM/10% (v/v) FCS, for 20 min at 37°C . The cells were then washed twice in a medium composed of 116 mM NaCl, 5.6 mM KCl, 1.2 mM MgCl_2 , 1.2 mM NaH_2PO_4 , 1.8 mM CaCl_2 , 25 mM Hepes, 1 g/l glucose, without Phenol Red, adjusted to pH 7.4. The cells were resuspended to 1.5×10^6 cells per ml and left at 4°C in the dark until used. When indicated, EGTA was added to the incubation medium at a final concentration of 4 mM just before adding the secretagogue in order to chelate extracellular Ca^{2+} . For all measurements, cells were temperature-equilibrated in a thermostatically controlled quartz cuvette (37°C) equipped with a magnetic microstirrer. Ca^{2+} movements were measured from the observed changes in the fluorescence of the Ca^{2+} -sensitive dye Fura-2. Observations were made with a Jobin Yvon JY3D spectrofluorimeter. The free Ca^{2+} concentration was calculated from the dye fluorescence level using the apparent dissociation constant of 220 nM for the Ca^{2+} –Fura-2 complex and estimation of the Ca^{2+} released by the cells was performed according to the method of Grynkiewicz et al. [10].

The emission wavelength was set at 505 nm with a bandwidth of 10 nm, while the excitation wavelength was 340 nm with a bandwidth of 4 nm. The cells were allowed to equilibrate for about 5 min in the cuvette, then the secretagogue was added. Maximal reading time for one sample was 20 min. A calibration was then performed for each sample by lysing the cells with $40 \mu\text{M}$ digitonin, recording the fluorescence for at least 3 min, then adding 10 mM EGTA and recording the signal for another 2 min while waiting for a stable level.

To test the effects of $[\text{Ca}^{2+}]_i$ and $[\text{Ca}^{2+}]_o$ chelation on $[\text{Ca}^{2+}]_i$, Quin-2 or BAPTA was used to chelate intracellular Ca^{2+} . The dissociated cells were incubated for 20 min at 37°C with $75 \mu\text{M}$ Quin-2/AM, in the dark, in a Krebs medium (116 mM NaCl, 5.6 mM KCl, 1.2 mM MgCl_2 , 1.2 mM NaH_2PO_4 , 1 g/l glucose) containing $80 \mu\text{M}$ CaCl_2 and $250 \mu\text{M}$ EGTA, then rinsed and resuspended in the same medium but in which 25 mM Na_2HCO_3 was replaced by 25 mM Hepes (pH 7.4). Different secretagogues were then tested as described above. Autofluorescence was not a problem and was usually less than 0.1%.

Statistical analysis

Values are given as means $\pm$ S.E.M. Student's *t* test for paired samples was used for statistical evaluation.

RESULTS

In our experiments with mucous cells maintained in suspension there was a small amount of leakage of Fura-2 into the extracellular media over time. This commonly described problem can lead to spuriously high values of calculated $[\text{Ca}^{2+}]_i$. In fact, comparison of recordings of resting $[\text{Ca}^{2+}]_i$ in an incubation medium containing Ca^{2+} (1.8 mM) with those of resting $[\text{Ca}^{2+}]_i$ in an incubation medium devoid of Ca^{2+} (0.1 μM) revealed a very reproducible overestimation of 60 nM. This artifact was corrected in the results reported below. In resting Cl.16E cells the average $[\text{Ca}^{2+}]_i$ was 129 ± 3 nM ($n = 109$).

Changes in $[\text{Ca}^{2+}]_i$ during stimulation by carbachol or neurotensin

Carbachol

As shown in Figure 1(a), carbachol (1×10^{-4} M) stimulated a

Figure 1 Characterization of carbachol- and neurotensin-mediated mobilization of $[Ca^{2+}]_i$ in CI.16E cells maintained in suspension

The $[Ca^{2+}]_i$ was measured fluorimetrically in Fura-2-loaded cells. (a) Carbachol (1×10^{-4} M) induced a biphasic rise in $[Ca^{2+}]_i$. Both components of this rise were inhibited by atropine (results not shown). (b) Effect of removing extracellular Ca^{2+} on carbachol-induced $[Ca^{2+}]_i$ changes. Although an initial transient was observed in Ca^{2+} -free buffer, the sustained plateau was abolished by removal of extracellular Ca^{2+} . (c) Neurotensin (1×10^{-6} M) induced a monophasic rise in $[Ca^{2+}]_i$ with a very rapid return to baseline level. (d) Removal of extracellular Ca^{2+} did not affect neurotensin-induced $[Ca^{2+}]_i$ changes.

rapid and transient elevation of $[Ca^{2+}]_i$ followed by a sustained plateau, which was inhibited by the selective muscarinic antagonist atropine (see below). As shown in Figure 2(a), carbachol induced a dose-dependent rise in $[Ca^{2+}]_i$ with maximal effect observed at 1×10^{-4} M ($[Ca^{2+}]_i = 608 \pm 101$ nM, $P < 0.001$ compared with control unstimulated cells, $n = 18$) with one-half maximal effect seen at about 7μ M.

We examined the effects of extra- and intra-cellular Ca^{2+} chelation on variations of $[Ca^{2+}]_i$ induced by carbachol. When all extracellular Ca^{2+} was chelated by the addition of 4 mM EGTA a few seconds before the addition of the secretagogue, the initial rapid rise in $[Ca^{2+}]_i$ remained unchanged, as there was no significant difference of the average peak $[Ca^{2+}]_i$ between Ca^{2+} -containing and Ca^{2+} -free external medium (521 ± 89 nM, $n = 14$ in the Ca^{2+} -free medium) upon stimulation with 1×10^{-4} M carbachol. Finally the ED_{50} (10μ M) of this dose-related $[Ca^{2+}]_i$ peak was not significantly different from that obtained in the presence of extracellular Ca^{2+} (Figure 2a). However, in Ca^{2+} -free incubation medium, the plateau was depressed to baseline levels, indicating that the $[Ca^{2+}]_i$ plateau is solely due to Ca^{2+} entry from the extracellular space into the cell (Figure 1b). Upon restoration of $[Ca^{2+}]_o$ to 1 mM, $[Ca^{2+}]_i$ rapidly returned to the plateau value; this return was prevented by addition of atropine before the addition of Ca^{2+} . When 1×10^{-6} M atropine was added after the addition of Ca^{2+} , $[Ca^{2+}]_i$ rapidly fell to baseline values (Figure 3). Together these results suggest that the Ca^{2+} -entry pathway remains active as long as carbachol binds to the receptor.

In cells loaded with an excess of Quin-2/AM and in the absence of extracellular Ca^{2+} , to suppress the intracellular Ca^{2+} movements, carbachol did not induce any effect on $[Ca^{2+}]_i$ (results not shown).

Neurotensin

Upon addition of neurotensin, a rapid and transient increase in $[Ca^{2+}]_i$ occurred in mucous cells, followed by a rapid decline and return to the resting level (Figure 1c), the maximally effective concentration of neurotensin being 1×10^{-6} M (598 ± 127 nM, $P < 0.001$ compared with non-stimulated cells, $n = 16$). Neurotensin induced a dose-dependent rise in $[Ca^{2+}]_i$ with half-maximal stimulation at 4 nM (Figure 2b).

As shown in Figure 1, EGTA chelation of extracellular Ca^{2+} did not alter $[Ca^{2+}]_i$ variations induced by neurotensin. In fact, in Ca^{2+} -free medium, values for maximal elevation of $[Ca^{2+}]_i$ at 1×10^{-6} M neurotensin ($[Ca^{2+}]_i = 555 \pm 78$ nM, $n = 13$), as well as for half-maximal stimulation (7.5 nM neurotensin) (Figure 2), were not significantly different from those obtained in Ca^{2+} -containing medium (1.8 mM). Upon stimulation with neurotensin in Ca^{2+} -free medium, the increase in $[Ca^{2+}]_i$ was followed by a return to the baseline level, which was not altered by restoring $[Ca^{2+}]_o$ to 1 mM (Figure 3). Together these findings show that neurotensin did not stimulate an influx of extracellular Ca^{2+} in addition to the release of Ca^{2+} from intracellular stores.

Finally, chelation of intracellular Ca^{2+} abolished $[Ca^{2+}]_i$ mobilization (results not shown).

Interdependence of the $[Ca^{2+}]_i$ signal and secretory response to carbachol and neurotensin in mucous cells

Carbachol

The stimulatory effect of carbachol on mucous secretion as measured by $[^3H]$ mucin release over a 15 min incubation period paralleled that of carbachol on $[Ca^{2+}]_i$ (Figure 4). Carbachol

Figure 2 Concentration-response relationships of carbachol- (a) and neurotensin- (b) stimulated initial rise in $[Ca^{2+}]_i$ in the presence (●, 1.8 mM) or absence (○, 0.1 μ M) of $[Ca^{2+}]_o$.

The initial Ca^{2+} peak that occurred within seconds of carbachol or neurotensin addition was measured fluorimetrically in Fura-2-loaded cells. Values of $[Ca^{2+}]_i$ are means $\pm$ S.E.M. of eight to 24 determinations, and are expressed as a percentage of maximum stimulation.

Figure 3 Demonstration of a carbachol-activated Ca^{2+} entry mechanism in Cl.16E cell membrane

In Ca^{2+} -free medium (the Ca^{2+} chelator EGTA was added a few seconds before stimulation to a final concentration of 4 mM), carbachol (Carb) caused a transient rise in $[Ca^{2+}]_i$ due to release from intracellular stores. When Ca^{2+} was added to the medium after $[Ca^{2+}]_i$ had returned to baseline, $[Ca^{2+}]_i$ rose to the plateau value observed during stimulation in Ca^{2+} -containing buffer. This rise was prevented by atropine, indicating persistent cholinergic activation of a Ca^{2+} entry mechanism (a). The same experiments were conducted with neurotensin (b), and showed that neurotensin (Neu) did not stimulate an influx of extracellular Ca^{2+} .

dose-dependently stimulated mucin exocytosis with a maximal stimulatory effect being obtained at 1×10^{-4} M carbachol and with an ED_{50} of 15 μ M, these values being in line with those

Figure 4 Concentration-response relationship of carbachol-stimulated mucin secretion in Cl.16E cells

Cl.16E monolayers were prelabelled with $[^3H]$ glucosamine for 24 h, then exposed for 15 min to carbachol at the indicated concentrations. Mucin release was quantified as described in the Materials and methods section. Values of mucin secretion are means $\pm$ S.E.M. of six monolayers with two determinations per monolayer.

reported for $[Ca^{2+}]_i$. At its maximally effective concentration (1×10^{-4} M), carbachol stimulated $[^3H]$ mucin secretion approx. 4–5-fold. Finally, the addition of atropine together with carbachol abolished the exocytotic response.

To examine directly the interdependence between cholinergically stimulated glycoprotein secretion and intracellular second-messenger events, we examined the effects of extra- and intra-cellular Ca^{2+} chelation on mucous exocytosis, i.e. Ca^{2+} dependency of secretagogue action. When the mucous secretory response to carbachol was examined in a Ca^{2+} -free medium, it was found to be inhibited by 34.5% ($P < 0.05$, compared with stimulated cells in standard conditions) (Figure 5). It is worth noting that baseline secretion in Ca^{2+} -free conditions was higher than that in standard medium. However, carbachol still exerted a secretagogue effect as the value for mucous secretion of stimulated cells was significantly higher than that of control unstimulated cells in Ca^{2+} -free medium.

Finally, after Ca^{2+} -depletion of cells by a 30 min incubation in the presence of BAPTA/AM, the mucin secretory response to carbachol was abolished (Figure 5).

Neurotensin

The previously reported values for neurotensin-stimulated mucin secretion after $[^3H]$ glucosamine incorporation compare well with the concentration-response relationships for the initial $[Ca^{2+}]_i$ peak. In fact, half-maximal stimulation was at 36 ± 5.2 nM for mucous secretion. In addition 1×10^{-6} M neurotensin stimulated maximally both $[Ca^{2+}]_i$ and mucous secretion.

Just as EGTA chelation of extracellular Ca^{2+} did not affect neurotensin-induced variations of $[Ca^{2+}]_i$ (Figure 2b), it was

Figure 5 Interdependence of the $[Ca^{2+}]_i$ signal and secretory response to carbachol and neurotensin

Cl.16E cells were prelabelled with $[^3H]$ glucosamine for 24 h. Control cells were incubated for 30 min at 37 °C in a Ca^{2+} -containing medium (Ham-DMEM) and exposed for 15 min to carbachol (1×10^{-3} M) or to neurotensin (NT) (1×10^{-6} M). For stimulation in Ca^{2+} -free external medium, the cells were incubated for 30 min in a Ca^{2+} -containing medium and then stimulated with carbachol (1×10^{-3} M) or NT (1×10^{-6} M) for 15 min in a Ca^{2+} -free medium (S-DME) containing 0.5 mM EGTA. Finally, for stimulation in the absence of extracellular and intracellular Ca^{2+} , the cells were incubated for 30 min in a Ca^{2+} -free medium (S-DME) containing 0.5 mM EGTA and 50 μ M BAPTA/AM and then stimulated with carbachol (1×10^{-3} M) or NT (1×10^{-6} M) for 15 min in S-DME containing 0.5 mM EGTA. Values for mucin secretion are means $\pm$ S.E.M. of three to 12 experiments, with three monolayers per experiment, and three determinations per monolayer.

without effect on neurotensin-stimulated $[^3H]$ mucin exocytosis (99.35% of control, $n = 4$) (Figure 5).

Finally, chelation of intracellular Ca^{2+} abolished the mucous secretory response to neurotensin (Figure 5).

Cross-talk between the Ca^{2+} -dependent secretory response and cyclic AMP pathway

Previous studies have shown that activation of the cyclic AMP pathway, by vasoactive intestinal peptide (VIP), forskolin or dibutyryl cyclic AMP, together with activation of the Ca^{2+} pathway by carbachol, produced a secretory response over the basal level that was significantly greater than the sum of the individual responses, thus indicating the presence of a potentiating interaction. By contrast, the combination of VIP and neurotensin resulted in an additive effect on mucous secretion. The $[Ca^{2+}]_i$ response to VIP (1×10^{-6} M to 1×10^{-11} M) combined with carbachol or with neurotensin did not differ from the $[Ca^{2+}]_i$ response to carbachol or neurotensin employed alone. Together these results predicted that Ca^{2+} influx, a component of the Ca^{2+} pathway specific to carbachol's action, would be involved in the potentiated secretory response to VIP and carbachol. To test this prediction the mucous secretory response to several secretagogues used alone, or in combination, was examined in Ca^{2+} -free medium by comparison with that obtained in normal medium. As expected, the response to VIP and to neurotensin, used alone or in combination, was not significantly affected in Ca^{2+} -free

Figure 6 Cross-talk between Ca^{2+} and cyclic AMP pathways

Cl.16E cells were preincubated for 24 h with $[^3H]$ glucosamine. The cells were then incubated for 15 min either in a Ca^{2+} -containing medium (1.8 mM, open bars), or in a Ca^{2+} -free medium (0.1 μ M, shaded bars) in the presence of VIP (1×10^{-6} M), carbachol (1×10^{-3} M), or neurotensin (1×10^{-6} M) alone or in the presence of VIP combined either with carbachol or with neurotensin. Values are means $\pm$ S.E.M. of three experiments, with three monolayers per experiment, and three determinations per monolayer.

medium. Carbachol's action on mucous secretion was significantly reduced in Ca^{2+} -free medium. Finally the combined effect of VIP and carbachol was only additive in Ca^{2+} -free medium, while it was significantly higher than additive in Ca^{2+} -containing medium (Figure 6).

DISCUSSION

To investigate Ca^{2+} -mobilizing receptors, several studies have focused on HT29, a human colonic epithelial cell line. HT29 cells are a useful model because studies with these cells have demonstrated the presence of muscarinic acetylcholine receptors linked to Ca^{2+} mobilization [11]. In addition these cells possess receptors for neurotensin coupled to polyphosphoinositide turnover [12,13] and intracellular free- Ca^{2+} mobilization [13,14].

The major limitation of the HT29 parent line resides in its absence of polarization which is likely to result from missorting of apical proteins [15,16]. Therefore this model does not provide a suitable approach to examining stimulus-secretion coupling in vectorially secreting cells. In this context, it is worth emphasizing the importance of the two stably differentiated clonal cell derivatives [5] Cl.19A and Cl.16E which represent homogeneous populations of Cl⁻-secreting (Cl.19A) [17] or mucous-secreting (Cl.16E) [5,7,17,18] cells responding to both cyclic AMP-linked (e.g. forskolin) or Ca^{2+} -linked (e.g. carbachol) secretagogues [7,19–21]. Previous studies having demonstrated the power of combining a homogeneous mucous-secreting cell line (Cl.16E) with cyclic AMP measurement techniques to study the role of cyclic AMP in mediating the action of VIP [22], we utilized basically the same approach to explore the Ca^{2+} -dependent exocytotic function of goblet cells. In addition, unique to the homogenous goblet cell line Cl.16E is its ability to work around the difficulty of accurately measuring the mucins secreted by

explant cultures of mucosa or by isolated cells. In this context, Cl.16E goblet cell line offers a unique advantage (i) to correlate the secretagogue-induced gastrointestinal secretory response with $[Ca^{2+}]_i$ changes in the secretory cells, (ii) to examine the Ca^{2+} -dependence of secretagogue action by chelating extracellular and/or intracellular Ca^{2+} , and (iii) to decipher the interactions of mediators resulting in a potentiated secretory response.

Carbachol and neurotensin elicited peak $[Ca^{2+}]_i$ and mucin exocytosis with the same efficacy in Cl.16E cells. However, in contrast with the dual-component rise in mucous cell $[Ca^{2+}]_i$ stimulated by carbachol, neurotensin induced a transient rise in $[Ca^{2+}]_i$. Our finding of distinct $[Ca^{2+}]_i$ profiles elicited by carbachol and neurotensin is in line with previous studies on parental HT29 cells by Turner et al. [13], who showed that while neurotensin produced a rapid and transient increase in $[Ca^{2+}]_i$, the initial increase in response to carbachol was followed by a sustained elevated level of $[Ca^{2+}]_i$ that returned to baseline only with the addition of atropine. By contrast, in another study based on simultaneous whole-cell current and Ca^{2+} recordings in HT29 cells, Morris et al. [23] found that neurotensin-induced peak $[Ca^{2+}]_i$ responses were followed by a sustained increase in $[Ca^{2+}]_i$; this effect was variable in size and present in only 60% of cells bathed in 1 mM $[Ca^{2+}]_o$. This result points to the heterogeneity of the parental HT29 cells, and suggests that variations in culture conditions which are likely to occur over long-term culture may select for particular subpopulations with distinct $[Ca^{2+}]_i$ profiles upon stimulation by neurotensin. In this context it is worth noting that a clonal derivative such as the Cl.16E cell line provides a tool by which to circumvent the drawback of using a heterogeneous culture.

The fact that the Ca^{2+} -entry process elicited by carbachol is critical for the secretory response of several epithelial cell types is largely based on experiments in which cells were stimulated in Ca^{2+} -free medium [24]. For example, it has been reported that the effect of carbamoylcholine on amylase secretion from perfused rat parotid acinar cells is decreased markedly by Ca^{2+} omission from extracellular fluid [25]. Recently, Melvin et al., using an 'in situ' vasculature isolated sublingual gland perfusion system, have reported the requirement for Ca^{2+} entry during sustained muscarinic-induced fluid and electrolyte secretion [26]. Finally, in their study of glycoprotein secretion from rabbit gastric mucosal explants, Seidler and Sewing found that removal of Ca^{2+} from the medium at the time of secretagogue addition decreased the effect of acetylcholine [3]. In this context the present work confirms and extends these previous studies by providing direct evidence that, in goblet cells, an elevation of $[Ca^{2+}]_i$, as a result of both release from intracellular stores and activation of a membrane entry mechanism for Ca^{2+} , is a central event in stimulus-secretion coupling during cholinergic stimulation. In contrast with the findings obtained with carbachol, neither the $[Ca^{2+}]_i$, nor the mucin secretory response was affected when Cl.16E cells were stimulated with neurotensin in Ca^{2+} -free incubation medium.

The validity of the methodology we used is largely based on the demonstration that removal of extracellular Ca^{2+} did not impair the secretory response to VIP, a result demonstrating that in Ca^{2+} -free medium the exocytotic response to secretagogues is not non-specifically affected. It is noteworthy, however, that baseline mucous secretion was obviously augmented upon incubation in Ca^{2+} -free medium. A possible explanation takes into account that some proteins such as annexins [27], involved in the exocytotic machinery, are Ca^{2+} -sensitive. Therefore their function is likely to be affected by quantitative variations in Ca^{2+} in their immediate environment. Finally chelation of intracellular Ca^{2+} resulted in (i) a major increase in baseline mucous secretion and

(ii) a non-specific abolition of the exocytotic response to all secretagogues tested. Given the fact that intracellular Ca^{2+} is essential to key cellular functions, it comes as no surprise that its chelation brought about non-specific effects. In this context, when considering the regulation of exocytotic function of epithelial cells, one should be very cautious in interpreting results based solely on the manipulation of $[Ca^{2+}]_i$.

Thus the above reported findings have provided enough clues to enable us to propose a possible mechanism for the potentiation of carbachol's secretagogue action by the cyclic AMP pathway. Previous work based on Cl.16E cells has shown that the efficacy of VIP for stimulation of mucous secretion is less than that of carbachol [8]. However, VIP was found to potentiate the mucin secretory response to carbachol. Furthermore, forskolin or dibutyryl cyclic AMP mimicked the action of VIP [8], while A23187, a Ca^{2+} ionophore, mimicked the effect of carbachol (C. Bou-Hanna and C. L. Labois, unpublished work). Together these results suggested that potentiation is caused by a co-ordinated interaction between the cyclic AMP system and the Ca^{2+} system at a step distal to second-messenger generation. The same conclusion was reached by Yoshimura and Nezu, who studied the interaction between Ca^{2+} and cyclic AMP messenger systems in perfused rat parotid acinar cells [25]. Surprisingly, however, such a potentiating effect was not observed between VIP and neurotensin [8]. Our present findings may provide an explanation for these apparently paradoxical results if we consider that Ca^{2+} entry which is unique to the carbachol signalling pathway is possibly implicated in the cross-talk between cyclic AMP and the Ca^{2+} signalling system. This predicts that the potentiating effect of VIP on carbachol's action would be abolished in Ca^{2+} -free incubation medium. This work provides experimental evidence confirming our hypothesis. From this we conclude that a sustained elevation in $[Ca^{2+}]_i$ resulting from Ca^{2+} entry is a requirement for the potentiation effect between cyclic AMP and Ca^{2+} pathways in mucous-secreting cells. This conclusion is consistent with and extends previous findings showing that carbachol's potentiation of isoprenaline-induced secretion of [^3H]glucosamine-labelled high-molecular-mass glycoprotein from rat submandibular glands required extracellular Ca^{2+} [28,29].

Finally, aside from being a useful model for studying the exocytotic response of mucous cells to several secretagogues in relation to the way they mobilize Ca^{2+} , the Cl.16E cell line provides a powerful approach by which to examine cross-talk between intracellular signalling pathways.

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