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ATP-stimulated Electrolyte and Mucin Secretion in the Human Intestinal Goblet Cell Line HT29-Cl.16E

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Abstract. The response of confluent monolayers of HT29-Cl.16E cells to stimulation by extracellular ATP and ATP analogues was investigated in terms of mucin and electrolyte secretion. Mucin secretion was measured as release of glucosamine-labeled macromolecules trapped at the stacking/running gel interface of polyacrylamide gels and electrolyte secretion as short-circuit current (I_{sc}). Luminal ATP stimulated a transient increase in the release of mucins and of I_{sc} corresponding to a secretory Cl^- current. Both secretions peaked at 3 to 5 min after addition of ATP. Maximal ATP-stimulated mucin secretion over 15 min was up to 18-fold above control with an apparent ED_{50} of approximately 40 μM. Maximal peak I_{sc} after stimulation with ATP was approximately 35 μA/cm^2 with an apparent ED_{50} of about 0.4 mM. ATP-dependent I_{sc} was at least in part due to Cl^- secretion since removal of Cl^- from the medium reduced the peak I_{sc} by 40% and the I_{sc} integrated over 40 min by 80%. The secretory responses were not associated with cell damage as assessed by failure of ethidium bromide to enter into the cells, absence of release of lactate dehydrogenase, maintenance of monolayer conductance, viability, and responses to repeated applications of ATP. The order of efficacy of nucleotide agonists was similar for both processes with ATP > ADP > AMP ≥ adenosine. Luminal ATP was much more effective than basolateral addition of this compound. These results suggest involvement of a luminal P_2-type receptor which can initiate signaling pathways for granule fusion and mucin release as well as for activation of Cl^- channels. P_2-receptor-stimulated mucin and I_{sc} release was strongly inhibited by a 30 min preincubation with the classical K^+ channel blockers quinine (1 mM), quinidine (1 mM), and Ba^{2+} (3 mM). Experiments with amphotericin B to measure separately the conductance changes of either luminal or basolateral plasma membrane revealed that quinidine did not directly block the ATP-induced basolateral K^+ or the luminal anion channels. The quinidine inhibition after preincubation is therefore most easily explained by interference with granule fusion and location of anion channels in granule membranes. Luminal P_2 receptors may play a role in intestinal defense mechanisms with both fluid and mucin secretion aiding in the removal of noxious agents from the mucosal surface.

Key words: Chloride secretion — Exocytosis — P_2 receptor — Quinine — Quinidine

Introduction

Goblet cells form part of the normal mucosal lining in the intestine and other epithelia. They are characterized by an abundance of exocrine granules filled predominantly with mucins. Exocytosis and mucin secretion have been studied in considerable detail, mainly by morphological techniques. Functional studies have been limited because they require preparations of relatively homogeneous cell populations so that functional observations can be related to specific cell types, and such preparations have not been available. From the morphological studies, it is evident that mucin secretion by goblet cells is a regulated process. For example, cholinergic agents have been identified as one of the secretagogues of goblet cells in vivo (Neutra & Forstner, 1987).

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The establishment of cell lines that differentiate to goblet cells (Augeron & Laboisse, 1984; Phillips et al., 1988; Kreusel et al., 1991) has been a major advance for functional studies. Cultured HT29-Cl.16E produce and, after appropriate stimulation, secrete massive amounts of mucins that are visible to the unaided eye. The carbohydrate chains of the secreted mucins have recently been characterized (Capon et al., 1992). HT29-Cl.16E cells have the particular advantage that they form confluent monolayers and differentiate in normal, glucose-containing growth medium. Thus, this cell line provides a very convenient model for studying the regulation of secretory processes.

Recent reports demonstrate that extracellular ATP is a very potent secretagogue of Cl^- in many epithelial cells. It is also a major stimulus for mucin secretion in goblet cells from the respiratory tract (Davis et al., 1992). These findings raised the possibility that ATP could stimulate both types of secretions in HT29-Cl.16E and thus provide an opportunity to study whether mucin and Cl^- secretion are coordinated or interrelated processes.

Materials and Methods

Cell Culture

HT29-Cl.16E cells were propagated in Falcon culture flasks (25 cm^2) in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. The cells were fed every day with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 4 mM L-glutamine. At monthly intervals, cultures were tested for mycoplasm (Chen, 1977) and found to be free of contamination. The passage numbers for the reported experiments were between 20 and 44.

Unless indicated otherwise, cell monolayers that were used for measurements of electrical properties were grown on Vitrogen-coated, Millicell-CM (Millipore, Bedford, MA, size 12 mm) and those for mucin release on nitro-cellulose (Millipore, HAHY) filters. No differences were apparent between cells grown on either filter type. HT29-Cl.16E cells were seeded at a relatively high density of about 1.2×10^6/filter (0.6 to 1.2 cm^2). Cells became visually confluent after seven days and were used for secretion studies between days 8 and 14.

Measurement of Cell Viability and Permeability

Cell viability was determined by measuring lactate dehydrogenase (LDH) activity released into the supernatant (Madara & Staffort, 1989) with an enzyme kit (Enzyline LDH kit, Biomerieux, Dardilly, France). Release from filter-grown, control and ATP-stimulated cells were compared. Cell permeability was assessed with ethidium bromide (Gomperts, 1983). The penetration of ethidium bromide into cells was measured by monitoring cellular fluorescence with a microscope. Excitation and emission wavelengths were 510 and 615 nm, respectively, and the fluorescence was quantified by an intensified video camera connected to image acquisition hard- and software. Cells were grown on round glass coverslips and mounted in a Sykes-Moore chamber for continuous perfusion with a bicarbonate-free Ringer solution containing 50 μM ethidium bromide. The bicarbonate-free Ringer solution had an identical composition to the bicarbonate-Ringer described below, except that Na-HEPES, pH 7.4, replaced $NaHCO_3^-$. After an initial control perfusion of 10 min, the solution was switched to one containing also 3 mM ATP and, after an additional 10 min, to one containing 5 mg/ml digitonin. The digitonin perfusion served as positive control as this compound is known to permeabilize cell membranes.

Morphology

Cell monolayers on Millipore filters were fixed and embedded in situ according to the method of Phillips et al. (1988). Thin sections (80 nm) were cut normal to the filter. They were stained with uranyl acetate and lead citrate and examined with a Jeol 100 CX transmission electronic microscope.

Electrophysiology

Transepithelial electrophysiologic measurements were performed in a modified Ussing-type chamber constructed to accept filters with an outer diameter of 1.2 cm (Analytical Bioinstrumentation, Cleveland, OH). The chamber was equipped with a conventional 4-electrode system for measuring I_{sc}, transepithelial potential, and conductance. These parameters were measured with a voltage-clamp module (Model 558-C-5, Bioengineering, University of Iowa, Iowa City, IA) and continuously recorded on a strip-chart recorder and, with the aid of an A/D converter, also on a microcomputer. Positive currents correspond to anion secretion/cation absorption, i.e., a lumen negative potential under open-circuit conditions. The Ussing chamber and all solutions were maintained at 37°C and 5% CO_2 in a Plexiglass incubator. Unless otherwise indicated, the perfusion solution consisted of DMEM and Ham's F12 (1:1).

Ion Conductances of Plasma Membranes

To evaluate the ion conductances separately for luminal and for basolateral plasma membranes of confluent monolayers, the ionophore amphotericin B was added at a concentration of 20 μM to the perfusion solution of either the basolateral or the apical side (Kirk & Dawson, 1983; Wong et al., 1990). The plasma membrane facing the amphotericin-containing solution takes up this ionophore, thereby artificially increasing its ion conductances. As a result, the other plasma membrane becomes rate limiting for overall, transepithelial ion transport, and hormone-dependent changes of its ion conductances can be assessed either as I_{sc} due to ion gradients or as transepithelial conductance changes. In these experiments, the basic perfusion was carried out with bicarbonate Ringer solution containing in (mM) KCl (4), NaCl (114), $CaCl_2$ (1.25), $MgCl_2$ (1), $NaHCO_3^-$ (23), and D-glucose (25).

To measure hormone-dependent changes in basolateral K^+ conductance, luminal NaCl and KCl were both replaced by K^+ gluconate, while basolateral NaCl was substituted by Na^+ gluconate and basolateral KCl by K^+ gluconate, thus generating a luminal-to-basolateral K^+ gradient. For similar questions concerning apical Cl^- conductance, luminal NaCl was replaced by Na^+ gluconate.

Mucin Measurements

Secreted mucins were quantified using a previously described electrophoretic assay (Maoret et al., 1990; Augeron et al., 1992). Briefly,

confluent monolayers on filters (diameter 1.2 cm) were labeled with ^{3}H-glucosamine (usually 4 μCi/ml) in DMEM plus 10% FBS over 24 hr. The labeled filters were washed three times with serum-free medium of a 1:1 mixture of DMEM and Ham's F12 medium plus 0.01% bovine serum albumin (BSA-F12-DMEM). The cells were then exposed at 37°C to secretagogues in BSA-F12-DMEM. At appropriate intervals, the medium was removed and the monolayer rinsed with additional BSA-F12-DMEM to remove adherent mucins. The mucin-containing medium was dialyzed at 4°C for 36 to 48 hr against several changes of deionized water and subsequently lyophilized. The secretory glycoproteins were separated by SDS-PAGE on 3% 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.

Data Analysis

Except where indicated otherwise, results are expressed as mean ± SEM of at least three experiments.

Materials

DMEM, Ham's F12, and FBS were bought from GIBCO (Grand Island, NY). Amphotericin B, ATP purified from natural sources, synthetic ATP, ATPγS, ADP, AMP, adenosine, bumetanide, carbamylcholine chloride (carbachol), quinine hydrochloride, quinidine, ethidium bromide, 4,4′-di-isothiocyanostilbene-2,2′-disulfonate (DIDS), 4-acemido, 4′-isothiocyanostilbene-2,2′-disulfonate (SITS), and *N*-methyl-glucamine were obtained from Sigma Chemical (St. Louis, MO). Forskolin (7β-desacetyl-7β-[γ-(N-methylpiperazino)-butyryl]) was from Calbiochem, San Diego, CA. Dimethylsulfoxide and barium hydroxide were purchased from Fisher Scientific (Pittsburgh, PA). Chemicals for SDS-PAGE were purchased from Bio-Rad, Hercules, CA, or Touzart et Mattignon, Paris, France. Vitrogen is a product of Celtrix Lab, Palo Alto, CA. Soluene 350 was from Packard Instruments, Downers Grove, IL. D-[6-^{3}H]-glucosamine hydrochloride at 20–40 Ci/mmol (TRK 398) was purchased from Amersham, Les Ulis, France. di-Cl-DPC was a gift of Dr. H.L.J. Lang, Hoechst AG, Frankfurt, Germany.

Abbreviations

di-Cl-DPC = di-chloro-diphenylamine-2-carboxyate; DMEM = Dulbecco's Modified Eagle's Medium; ED_{50} = half-maximal effective dose; FBS = fetal bovine serum; I_{sc} = short-circuit current; PAGE = polyacrylamide gel electrophoresis; SDS = sodium dodecyl sulfate; VIP = vasoactive intestinal peptide.

Results

Tightness of Monolayers

HT29-Cl.16E cells gradually developed confluent and tight monolayers when cultured on permeable filter support for eight days. This change is observed visually, but is also evident in terms of electrical conductance of the monolayer (Fig. 1). After formation, the monolayers remained tight for at least a week as evidenced by their low conductance. For subsequent experiments, cells were cultured for 8 to 14 days after their passage and possessed an average conductance of 6.4 ± 2.6 mS/cm^2 (n = 35).

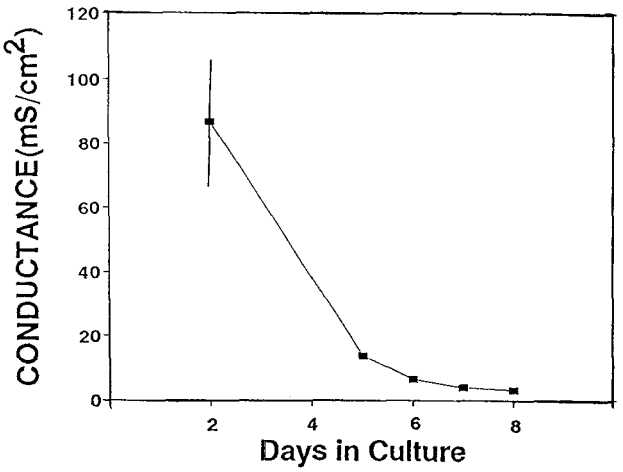

Fig. 1. Development of confluence of HT29-Cl.16E on Vitrogen-coated Millicell-CM filters. Data are mean ± SEM of 20 to 30 filters. Data points without error bars indicate SEM values less than the drawn point size.

Morphology

Electron microscopy of unstimulated, confluent (low-conductance) HT29-Cl.16E monolayers showed the typical features of polarized, intestinal epithelial cells with apical microvilli and tight junctions (Fig. 2*A*). More than 95% of cells were differentiated to goblet-like cells as judged by the presence of mucin granules at the apical pole. The mucin granules contain small dense-staining inclusions, typical for granular type goblet cells (Cormack, 1979). In many cells, large accumulations of mucin granules were evident. Borders between adjacent granules were typically discernable in these unstimulated cells, indicating absence of fusion. Stimulation of mucin secretion with maximal doses of luminal ATP (*see below*) resulted in the fusion of granules with each other and with the luminal plasma membrane (Fig. 2*B*). Release of granular content is clearly evident (Fig. 2*C*). Since the cells are tall and columnar, rather than goblet shaped, the compound exocytosis results in formation of intracellular canaliculi.

ATP as Secretagogue for Mucin Release

The addition of ATP to Cl.16E cells grown on filters elicited a rapid and strong stimulation of mucus release which can be seen with the unaided eye or quantified by measuring the release of mucins metabolically labeled with ^{3}H-glucosamine. At 1 mM ATP, an effect was seen as early as 1 min (Fig. 3*A*). Because the cumulative re-

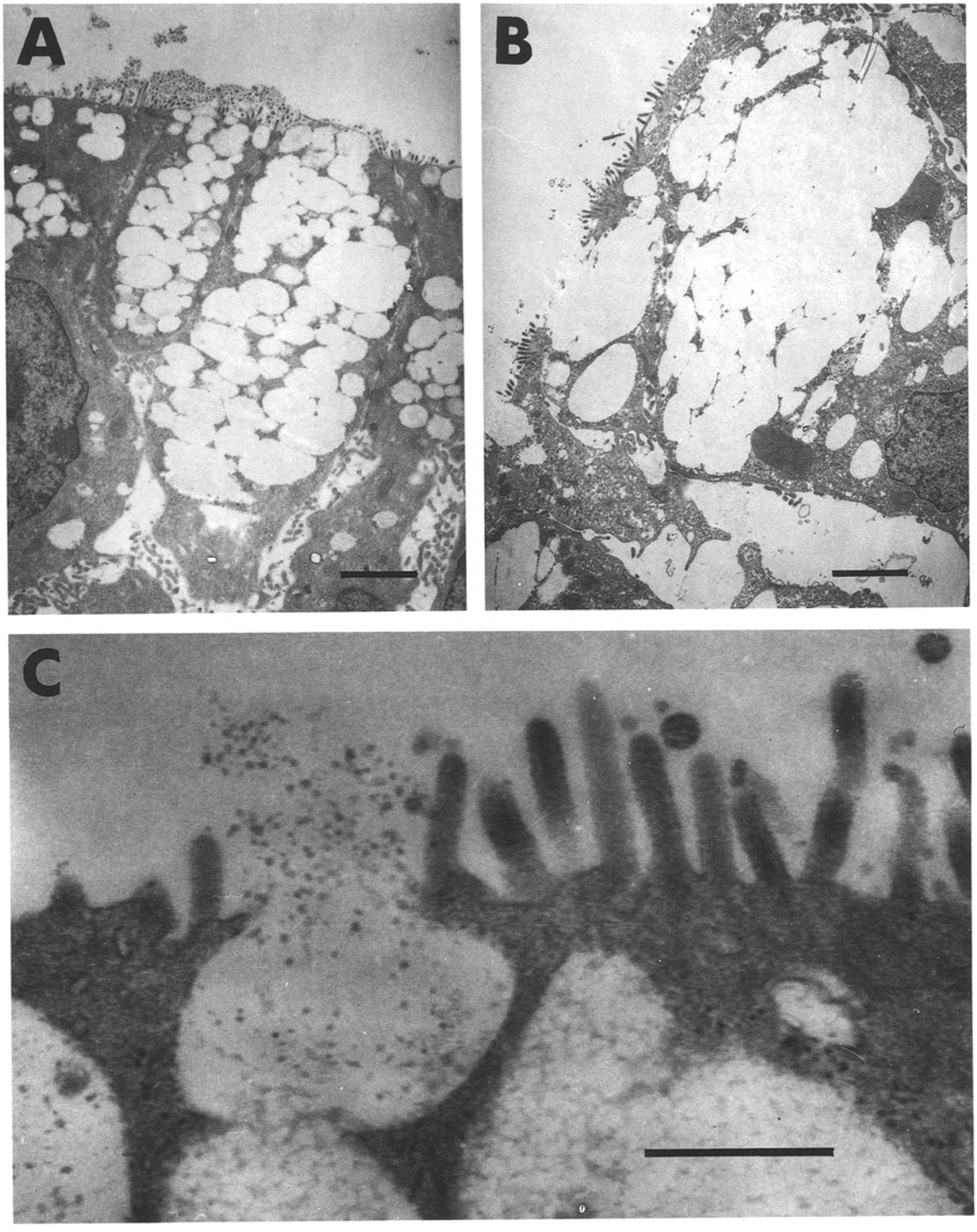

Fig. 2. Electron micrographs of HT29-Cl.16E cells grown to confluence (nine days) on Millicell-CM filters. (*A*) Untreated cells. Bar = 2 μm; (*B*) cells treated with 1 mM luminal ATP for 5 min. Bar = 2 μm; (*C*) same condition as in *B*, but with greater magnification. Bar = 0.5 μm.

lease was maximal by 15 min, this time point was used in further experiments to characterize this process. The effect of ATP on mucin secretion was dose dependent with a maximal increase of 18-fold relative to unstimulated controls (*see* Fig. 8*A*) and an apparent ED_{50} of approximately 40 μM (Fig. 4). The sequence of efficacy of different analogues tested at 1 mM was: ATP > ADP >> adenosine ≥ AMP, whereby adenosine and AMP

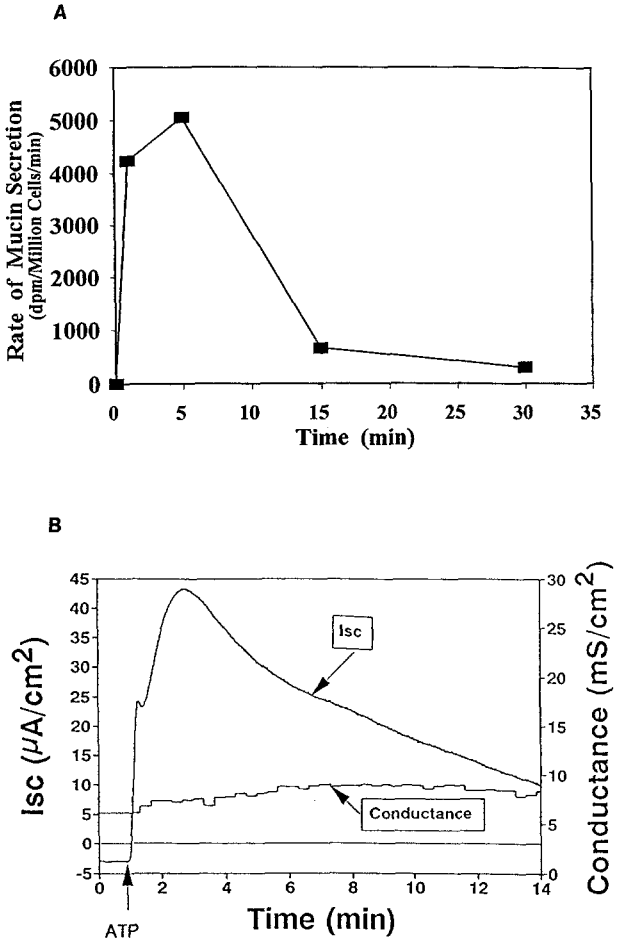

Fig. 3. Time dependence of extracellular ATP-stimulated secretion of (*A*) mucin and (*B*) anions in confluent HT29-Cl.16E cells. (*A*) Mucin secretion after addition of 1 mM ATP to apical and basal medium of confluent monolayers grown on filters; (*B*) I_{sc} and conductance changes after addition of 3 mM luminal ATP to confluent monolayers grown on filters. For mucin secretion, cells were prelabled with ^{3}H-glucosamine for 24 hr and mucin release was quantitated as described in Materials and Methods. I_{sc} and conductance tracings are from one experiment representative for at least three filters.

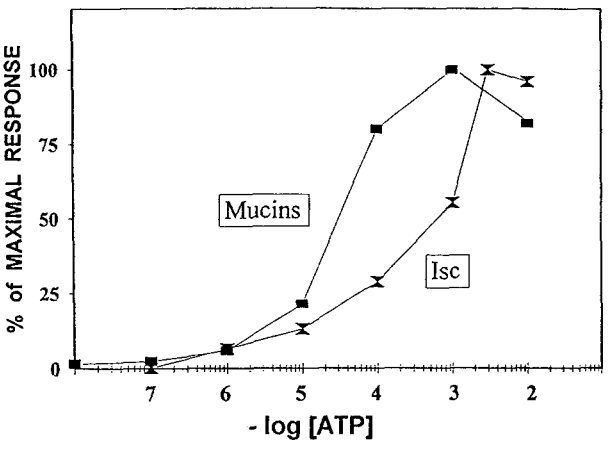

Fig. 4. Dose dependence of extracellular ATP-stimulated secretion of mucin and anion secretion. Mucins were labeled with ^{3}H-glucosamine by incubation for 24 hr prior to the secretion studies. Secreted ^{3}H-mucins were collected over 15 min after stimulation by ATP and quantitated as described in Materials and Methods. I_{sc} was monitored continuously and peak responses measured after stimulation with ATP. The cumulative response to successively higher ATP concentrations was recorded for nine different filters and averaged after normalization of each filter to its maximum. The results are expressed as percent of the maximal effect of ATP at 1 and 3 mM for mucin and anion secretion, respectively. Mean ΔI_{sc} = 100% = 35 μA/cm^2.

were essentially without any effect (Fig. 5*A*). The common ATP preparations are isolated from natural sources and could potentially contain a trace compound that is responsible for the observed cellular response. To eliminate this possibility, synthetic ATP was tested at the same concentration as ATP purified from natural sources. It had the same potency, suggesting that indeed ATP and not a contaminant was responsible for the cellular effect. Furthermore, the poorly hydrolyzable analogue ATPγS at 1 mM was as effective as ATP in stimulating mucin secretion. The nucleotide specificity and efficacy for mucin secretion argue for the involvement of a P_2-type receptor on the plasma membrane in ATP-stimulated mucin secretion.

The sidedness of these receptors on the plasma membrane was determined with cells grown on Millicell-CM filter inserts. Mucin secretion was measured with 1 mM ATP added to the solution on either the apical or basolateral side. Apical ATP (1 mM) was four times more efficacious than basolateral ATP in promoting mucin release (mucin release for apical *vs.* basolateral ATP: 1,185 ± 112% *vs.* 302 ± 20% of control without ATP). Therefore, the relevant P_2 receptors are located in the apical plasma membrane.

ATP Stimulates Electrolyte Secretion

To evaluate electrolyte secretion by Cl.16E, the short-circuit current (I_{sc}) in confluent monolayers on Millicell-CM filters was measured. As shown in Fig. 3*B*, addition of 3 mM ATP to the apical compartment caused an immediate increase in I_{sc} corresponding to a secretory anion current (or absorptive cation current). This I_{sc} reached a peak within a few minutes and declined slowly to the baseline over the next 25 min. Maximal doses of ATP stimulated changes in I_{sc} with a peak change of about 35 μA/cm^2 at 3 mM ATP. The ATP effect was dose dependent with an apparent ED_{50} of 0.4 mM (Fig.4). This measured ED_{50} has to be considered an upper limit because of the possibility that the unstirred mucus layer reduces the ATP concentration at the surface (*see* Discussion). Indeed, when in paired experiments, the contribution of the mucus layer was reduced by

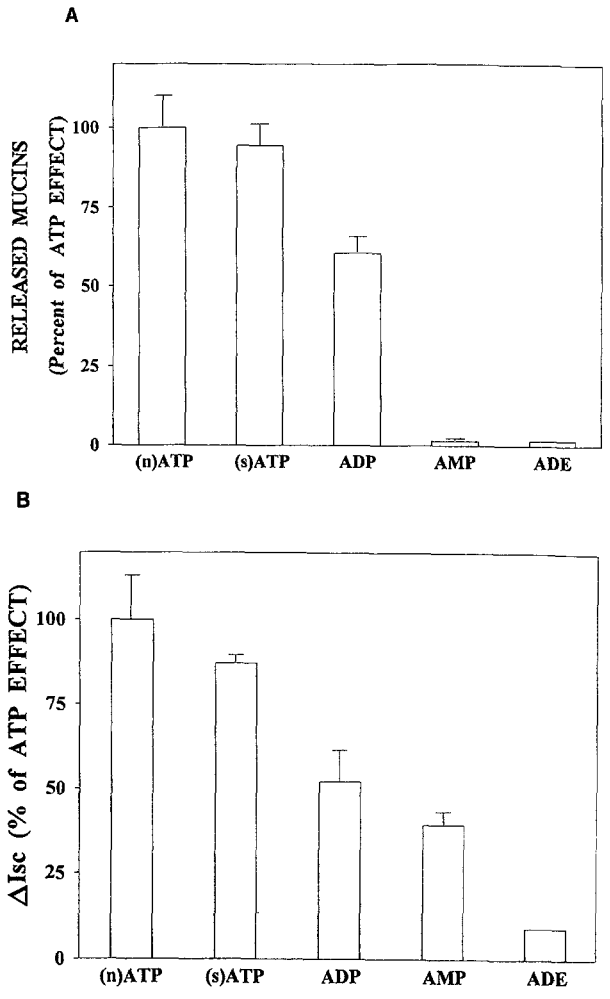

Fig. 5. Potency of ATP analogues on secretion of (*A*) mucins and (*B*) anions. Mucin release and anion secretion are expressed relative to values obtained after (n)ATP stimulation. Mucin release was quantitated as secreted ^{3}H-glucosamine-labeled mucin 15 min after stimulation by 1 mM adenosine or adenine nucleotides. Anion secretion was measured as peak I_{sc} changes after stimulation by 3 mM adenosine or adenine nucleotides ΔI_{sc} after (n)ATP = 100% 27.2 μA/cm^2). *(n)ATP:* natural ATP; *(s)ATP:* synthetic ATP; *ADE:* adenosine. Each bar represents mean ± SEM for three monolayers.

thorough rinsing of the apical surface, ATP was much more potent and efficacious in the rinsed monolayers (e.g., the I_{sc} increased by 100% at 10 μM, and 25% at 3 mM).

Repetitive stimulation with relatively high concentrations of luminal ATP (1 to 3 mM) resulted in successively smaller responses; the decrease was about 40% each time, consistent with receptor desensitization or depletion of granules that are required for the I_{sc} response. The secretory effect of ATP was restricted to the apical side since perfusion of the basolateral side with similar ATP concentrations had only a small effect on I_{sc} (Δpeak I_{sc} of three experiments with 1 mM ATP on apical *vs.* basolateral side: 23.3 ± 0.5 *vs.* 2.8 ± 1.4 μA). One could argue that basolateral ATP did not reach the cells because of slow diffusion through the filter. However, other compounds, such as VIP and carbachol (*see e.g.,* Fig. 8*B*) stimulated Cl^- secretion from the basolateral side.

To better define the nature of the receptor mediating the I_{sc} change, synthetic ATP, ATPγS, and different analogues were tested at 1 to 3 mM. As with mucin secretion, synthetic ATP and the poorly hydrolyzable ATPγS were as effective as natural ATP, indicating that ATP itself, and not a contaminant or hydrolysis product, was the real agonist. The order of response to ATP analogues at 3 mM was ATP > ADP >> AMP ≥ adenosine (Fig. 5*B*). This order is almost the same as for mucin secretion and suggests that the same or a similar P_2-type receptor is involved. The efficacy and specificity of different ATP analogues in this goblet cell line from HT29 is clearly different from the recently described P_1-type purinergic receptor in the related human T84 cells which also stimulates electrolyte secretion (Madara et al., 1993).

To establish the nature of the secretory current, several different approaches had to be used: (i) ion replacements; (ii) applications of known inhibitors of epithelial electrolyte transporters; (iii) permeabilization of either apical or basolateral plasma membrane and measurement of ion conductances in the nonpermeabilized membrane. Cl has been established as the major charge carrier for secretory currents in many epithelia. This anion is clearly responsible for the I_{sc} in the case of cAMP and carbachol-stimulated secretion because replacement of Cl^- by gluconate in the perfusion 60 min before the addition of the secretagogues forskolin and carbachol completely abolished the I_{sc} response (Table 1). However, the same treatment before ATP addition decreased the peak change of the I_{sc} by only about 40% (Table 1) and the total charge movement (integrated I_{sc} over 40 min) by 80%. This result raised the question whether another secreted anion or an absorbed cation was responsible for the residual charge movement in Cl-free medium. It is unlikely that HCO_3^- or Na^+ are involved because the additional replacement of HCO_3^- with HEPES or of Na^+ with *N*-methyl-glucamine in Cl-free medium did not change the I_{sc} response to ATP compared to Cl-free medium alone. An alternative explanation is that sufficient Cl remains in the cell even after 1 hr in Cl-free medium to support the short secretory spike seen after ATP stimulation.[1]

[1] The total amount of charge movement after ATP corresponds to maximally 22 neq/cm^2 within 1 min ($35 \times 10^{-6} * 60$ /Faraday constant (96,495 Eq/mol)). As the cell layer is about 40 μm tall, this amount of charge could be derived from intracellular anions and cations, requiring, e.g., a monovalent ion concentration change 5.5 mM/min. Therefore, a change of only about 10 mM Cl is required to give the current peak (21 μA for 3 min) in Cl-free medium.

Table 1. Effect of chloride-free medium on secretagogue-stimulated I^{sc}

Secretagogue	Concentration (mM)	+ Chloride Peak ΔI^{sc} ($\mu A/cm^2$)	− Chloride Peak ΔI^{sc} ($\mu A/cm2$)	Inhibition (%)
Forskolin	0.05	19.8 ± 0.8	1.1 ± 0.7	94
ATP	3	23.6 ± 3.5	15.2 ± 2.6	40
Carbachol	0.5	5.0 ± 0.7	0.0 ± 0.0	100

$n = 3$.

Several inhibitors of epithelial electrolyte transporters were tested at relatively high concentrations (Table 2). Only incubation with di-Cl-DPC (30 min) resulted in a large decrease (77%) of the ATP-stimulated I_{sc} (row 8). This result points to Cl as the charge carrier across the apical plasma membrane. However, in that case one would expect that bumetanide would also block the ATP-dependent increase in I_{sc} as it does for cAMP-dependent Cl secretion in epithelial cells, including T84 (Kim et al., 1988), enterocyte-like HT29 clones (Bajnath et al., 1991), and in HT29-Cl.16E (Table 2, row 4). One possible explanation for the inability of blockers of Cl influx, such as bumetanide (row 5), to inhibit the I_{sc} response is again that sufficient Cl remains stored in the cells.[1]

To test the idea that insufficient Cl depletion prevents the detection of Cl as charge carrier, the effectiveness of a blocker of Cl influx was checked after cells were depleted of Cl. Cl depletion was accomplished by stimulating adenyl cyclase with forskolin, thereby opening apical Cl channels, and simultaneously inhibiting Cl influx with bumetanide. This experimental protocol allows cytosolic Cl and K to come into electrochemical equilibrium across the plasma membrane. Without bumetanide, the secretory effects of forskolin and ATP are additive (Table 2, compare the sum of rows 1 and 2 with the value of row 3). Interestingly, after forskolin stimulation, the ATP-effect on I_{sc} is inhibited 86% by bumetanide (Table 2, row 6), indicating that, indeed, the cells contain considerable Cl concentrations that can be used for current transients.

To get more insight into the ion fluxes across the basolateral and the apical plasma membrane, the amphotericin B technique was used (Wong et al., 1990). Amphotericin partitions into membranes containing cholesterol and increases their ion conductances. Because of the requirement for cholesterol, its action remains restricted to the plasma membrane in contact with the solution containing amphotericin. Therefore, when epithelia are perfused on only one side with amphotericin, the conductance of the adjacent plasma membrane increases tremendously for small, monovalent ions and the opposite plasma membrane becomes rate limiting for overall, transepithelial electrical currents. In other words, this technique allows assessment of secretagogue-dependent conductance changes in one of the plasma membranes in an Ussing chamber setup.

To evaluate the effect of apical ATP on basolateral K^+ conductance, the epithelial monolayer was perfused on the apical side with high K^+/Cl-free-Ringer (NaCl replaced by K gluconate) and on the basolateral side with a normal K^+/Cl-free-Ringer (Cl replaced by gluconate). Amphotericin B was then added to the apical solution. The baseline currents and conductances are given in Table 3. Under these conditions, apical ATP was still able to effect changes in membrane permeability. ATP produced an acute change in I_{sc} of 25 μA (Fig. 6) which is consistent with an increase of the K^+ conductance in the basolateral plasma membrane. The conductance change was biphasic with an initial increase of 8 mS/cm^2, which lasted for about 2 min, and a sustained one of 1 mS/cm^2 (Fig. 6).

Similarly, to measure the effect of ATP on luminal ion conductances, the apical solution was switched to Cl-free Ringer (Cl^- replaced by gluconate) and amphotericin added to the basolateral solution. The baseline currents and conductances are presented in Table 4. As shown in Fig. 7, apical ATP induced an acute change in I_{sc} of 30 $\mu A/cm^2$ that declined to a sustained increase of about 10 $\mu A/cm^2$. This current change is consistent with a chloride efflux from the cytosol into the apical solution. More importantly, the conductance increased acutely by about 3.5 mS/cm^2 then declined to a sustained increase of about 1.5 mS/cm^2.

Absence of Large Pore Formation and Cytotoxicity

ATP is known to stimulate the formation of relatively large openings in the plasma membrane of some cells (El-Moatassim, Dornand & Mani, 1992). To evaluate whether such a process also occurs in HT29-Cl.16E, the general leakiness of cells was assessed by measuring release of lactate dehydrogenase. ATP had no effect on lactate dehydrogenase release (*data not shown*). Furthermore, ATP had no effect on the uptake of ethidium

Table 2. Comparison of efficacy of electrolyte transport inhibitors

Secretagogue (mM) + Inhibitor (mM)	Side of Addition	ΔI_{sc} ($\mu A/cm^2$)	n	Inhibition (%)
Forskolin (0.05)	Both	18.1 ± 5.3	24	
ATP (3)	Apical	27.6 ± 6.6	34	
Forskolin (0.05) + ATP (3)	Both/apical	43.7 ± 7.9	13	
Forskolin (0.05) + bumetanide (0.1)	Both/basolateral	1.3 ± 2.2	6	93
ATP (3) + bumetanide (0.1)	Apical/basolateral	30.4 ± 9.6	2	0
Forskolin (0.05) + bumetanide (0.1) + ATP (3)	Both/basolateral/apical	4.0 ± 2.3	4	86
ATP(3) + DIDS (1)	Apical/apical	22.0 ± 2.7	3	0
ATP (3) + di-Cl-DPC (0.1)	Apical/apical	6.3 ± 2.0	3	77
ATP (3) + DIDS (1)	Apical/basolateral	25.5 ± 4.7	4	0
ATP (3) + SITS (0.25)	Apical/basolateral	26.0	1	0
ATP (3) + SITS (0.25) + bumetanide (0.1)	Apical/basolateral/basolateral	25.8	1	0

Table 3. Effects of apical amphotericin treatment and ionic composition.

Medium	Amphotericin B	I_{sc}	Conductance
Apical/ basolateral	20 μM	$\mu A/cm^2$	mS/cm^2
NaCl-Ringer/ NaCl-Ringer	–	4.3 ± 3.3	5.0 ± 1.4
118 K-Cl-free/ 4 K-Cl-free	–	14.8 ± 4.3	2.7 ± 1.2
118 K-Cl-free/ 4 K-Cl-free	+	32.4 ± 8.2	2.9 ± 1.1

$n = 8$.

Table 4. Effects of basolateral amphotericin treatment and ionic composition.

Medium	Amphotericin B	I_{sc}	Conductance
Apical/ basolateral	20 μM	$\mu A/cm^2$	mS/cm^2
NaCl-Ringer/ NaCl-Ringer	–	2.5 ± 1.4	4.4 ± 0.6
114 Na-Cl-free/ NaCl-Ringer	–	20.3 ± 3.3	3.0 ± 0.9
114 Na-Cl-free/ NaCl-Ringer	+	20.3 ± 3.3	3.2 ± 1.0

$n = 5$.

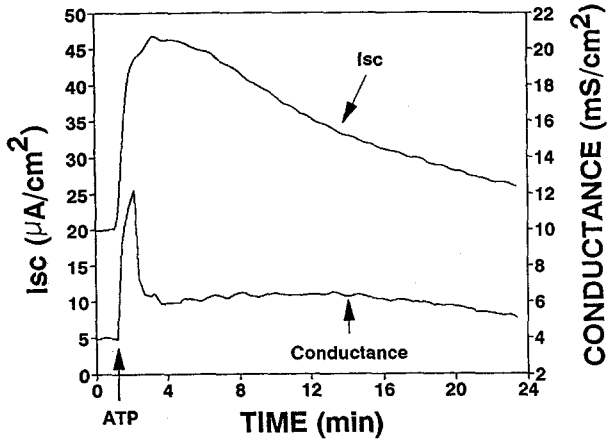

Fig. 6. Effect of luminal ATP on K^+ conductance of the basolateral plasma membrane. I_{sc} and conductance were measured simultaneously. A lumen-to-basal K^+ gradient was established across the monolayer by substituting K^+ gluconate for NaCl in the Ringer buffer bathing the luminal side and adding 20 μM amphotericin to the luminal solution. The basolateral buffer was Cl^- free. The buffers and baseline I_{sc} and conductances are given in Table 3. Twenty-five minutes after the addition of amphotericin, 1 mM ATP was added to the luminal side. The tracing is representative of results with two filters.

bromide indicating the absence of formation of pores that are permeable to solutes of MW of 400 or above. In contrast, digitonin drastically changed the permeability and induced a large influx of ethidium. The cellular fluorescence changes during perfusion with ATP were less than 1% of those induced by digitonin.

Comparison of Conditions for Mucin and Electrolyte Secretion

Cholinergic agonists and VIP acting via phosphoinositide/Ca^{2+} and cAMP, respectively, are known physiological secretagogues for Cl^- secretion (Frizzell, 1987; Hayslett et al., 1987; Cliff & Frizzell, 1990) and were previously shown to also stimulate mucin secretion in Cl.16E (Roumagnac & Laboisse, 1987; Laburthe et al., 1989; Augeron et al., 1992). The P_2-mediated secretions may involve a third signaling pathway (Tien, Laboisse & Hopfer, 1991; Merlin, Laboisse & Hopfer, 1992). To evaluate the potential importance of ATP-mediated secretions, the efficacy of the three signaling systems was compared. In preliminary experiments, maximal ef-

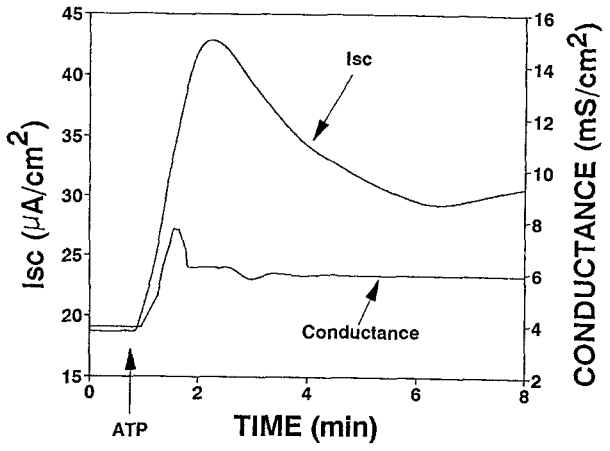

Fig. 7. Effect of luminal ATP on anion conductance of the apical plasma membrane. I_{sc} and conductance were measured simultaneously. A basolateral-to-lumen Cl^- gradient was established across the monolayer by substituting Na gluconate for NaCl in the Ringer buffer bathing the luminal side and adding 20 μM amphotericin to the basolateral solution. The basolateral buffer was a normal Ringer solution. The buffers and baseline I_{sc} and conductances are given in Table 4. ATP (3 mM) was added to the luminal side 35 min after the addition of amphotericin. The tracing is representative of results with three different filters.

fective doses for carbachol as cholinergic agonist and for forskolin as activator of adenylate cyclase were established and then used to compare the magnitude of mucin release (Fig. 8*A*) and changes in I_{sc} (Fig. 8*B*). Interestingly, among the three types of secretagogues, luminal ATP was the most efficacious for both mucin and electrolyte secretion. This comparison also holds if forskolin is replaced by the natural secretagogue VIP as maximal dose of VIP (1 μM) gave the same peak I_{sc} changes as forskolin. When carbachol and forskolin were compared, the order of efficacy for mucin secretion was different from that for Cl^- secretion: carbachol by itself had little effect on Cl^- secretion, but was very effective in terms of mucin release, while the reverse applies to forskolin.

Effects of K^+ Channel Blockers

Marcon et al. (1990) reported that the K^+ channel inhibitors quinine and Ba^{2+} block mucin release in the related T84 cells, which differentiate to a small extent to goblet cells. Based on this report and to assess the interdependence of electrolyte and mucin secretion, K^+ blockers were tested in Cl.16E in terms of effects on ATP-stimulated I_{sc} and mucin release. Interestingly, both processes were similarly reduced when cells were preincubated for 30 min with the inhibitor before addition of the secretagogue ATP: 80% by 1 mM quinine, 80% by 1 mM quinidine, 50% by 3 mM Ba^{2+} (Figs. 9*A* and *B*).

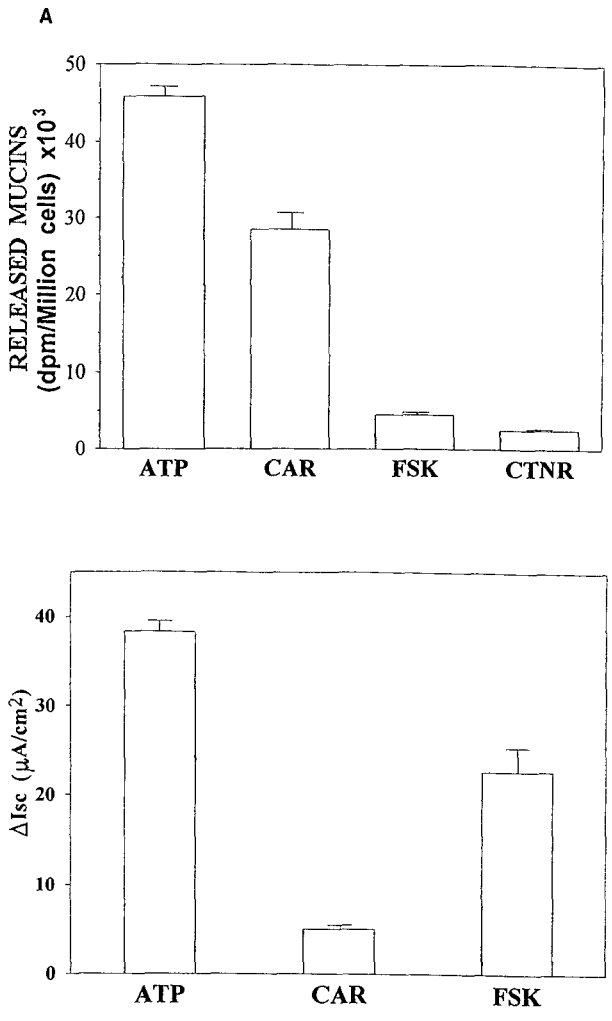

Fig. 8. Comparison of the efficacy of P_2-, cholinergic, and cAMP-mediated secretion in terms of (*A*) mucin release and (*B*) anion secretion. Mucin release is expressed in dpm/million cells, anion secretion as peak I_{sc} change after secretagogue addition. (*A*) Mucin release was quantitated as secreted ^{3}H-glucosamine-labeled mucin 15 min after ATP (1 mM), carbachol (*CAR*) (1 mM) or forskolin (*FSK*) (50 mM) stimulation. (*B*) Anion secretion was measured as peak I_{sc} changes. ATP: 3 mM luminal ATP; FSK: 50 μM forskolin; CAR: 0.5 mM carbachol. Each bar represents mean ± SEM of three monolayers.

The inhibition of the secretory anion I_{sc} by so-called "K^+ channel blockers" can, but does not necessarily involve K^+ channels. Effects on secretion can, for example, be explained by: (i) inhibition of basolateral K^+ conductance; (ii) direct inhibition of apical anion conductance; and (iii) location of apical anion conductance in granules or other intracellular vesicles and indirect inhibition of apical anion conductance by interference with fusion. The amphotericin technique allows separate testing of inhibitors in terms of effects on ion conductances in basolateral and apical plasma membranes. Thus, experiments were carried out to test the effect of quinidine on basolateral K^+ and apical anion

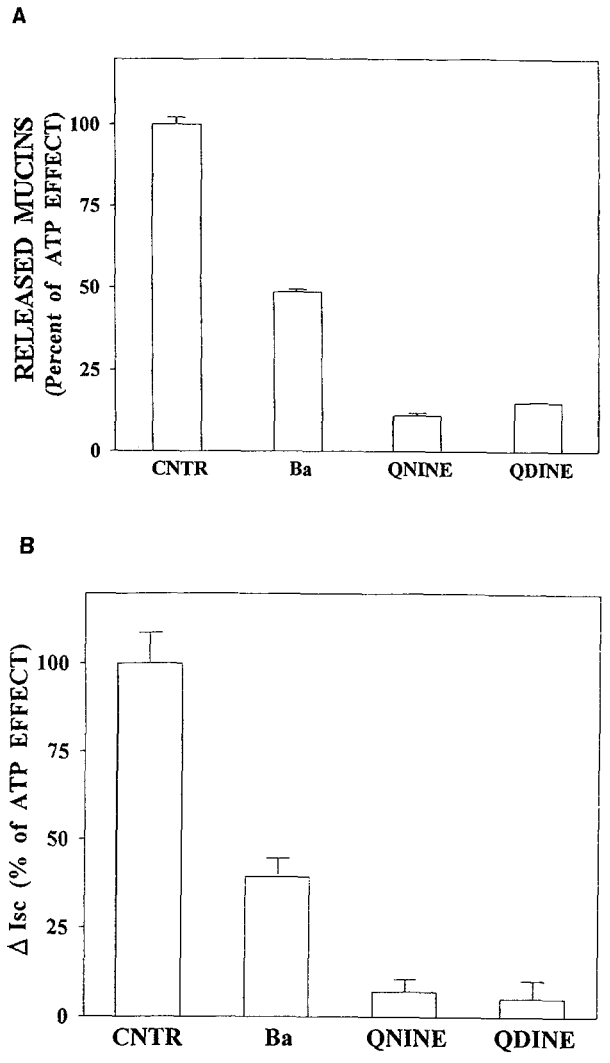

Fig. 9. Effect of K^+ blockers on ATP-stimulated secretion of (*A*) mucin and (*B*) anion secretion. Putative inhibitors were added to both sides of confluent monolayers grown on filters 30 min before addition of 1 and 3 mM ATP for experiments in (*A*) and (*B*), respectively. Mucin or anion secretion is expressed relative to control ATP stimulation in the absence of putative inhibitors. Mucin release was quantitated as secreted ^{3}H-glucosamine-labeled mucin 15 min after ATP stimulation. Anion secretion was measured as peak I_{sc} changes (ΔI_{sc} of control = 27 μA/cm^2). *CNTR:* No inhibitor; *QNINE:* 1 mM quinine; *QDINE:* 1 mM quinidine; Ba: 3 mM $BaCl_2$. Each bar represents mean ± SEM of seven monolayers for mucin secretion and three filters for electrolyte secretion.

conductance, i.e., the experiments shown in Figs. 6 and 7 were repeated in the presence of 1 mM quinidine. Quinidine was added either up to 14 min before addition of ATP or right after ATP stimulation. Interestingly, under none of these conditions, quinidine had any effect on ATP-stimulated increases in apical anion conductance (ATP-induced I_{sc} change (in μA/cm^2) without quinidine: 23 ± 2, n = 4, *vs.* with 1 mM quinidine: 25 ± 0, n = 2) or basolateral K^+ conductance (ATP-induced I_{sc} change (in μA/cm^2) without quinidine: 23 ± 2, n = 3, *vs.* with 1 mM quinidine: 22 ± 2, n = 5). These findings would rule out the first two explanations and suggest inhibition of fusion as a mechanism of action for quinidine.

Discussion

Goblet Cells Secrete Electrolytes

Electrolyte and macromolecule secretion are two important functions of the lining cells of many epithelial organs. Considerable efforts have been directed at defining cell types and cellular mechanisms involved in either type of secretion. Results have usually been interpreted with the implicit assumption that electrolyte and mucin secretion are separate processes carried out by distinct cell types. This view has been supported by observations that differentiated subclones of HT29 tend to fall into either mucin-secreting or NaCl-secreting cell types and that, among the classical secretagogues, cholinergic agonists predominantly stimulate mucin secretion, while those activating adenylate cyclase are more effective in stimulating electrolyte secretion. For example, the cell line HT29-Cl.19A forms monolayers of columnar cells that exhibit very high Cl^- secretion in response to secretagogues elevating cAMP, e.g., VIP (Rouyer-Fessard et al., 1989; Vaandrager et al., 1991). In contrast, cell lines typified by Cl.16E consist of monolayers of goblet cells that secrete a mucus gel when stimulated by cholinergic agonists, while they respond only weakly to VIP (Augeron et al., 1992).

The major finding of the present study is that extracellular ATP elicits secretion of both electrolytes and mucins in an intestinal cell through the stimulation of apically located purinergic P_2 receptors. In addition, extracellular ATP is by comparison to other agents, namely forskolin and carbachol, the most powerful secretagogue for both electrolyte and mucin secretion. Both processes must occur in the same cell for two reasons: (i) nearly all filter-grown Cl.16E cells differentiate to goblet-type cells as judged by light and electron microscopy, and (ii) I_{sc} changes are similar in magnitude to those of other cell lines that show few or no features of macromolecule secretion so that the I_{sc} changes cannot be explained by a small percentage of nongoblet, anion-secretory cells. For example, T84 cells and HT29-Cl.19A cells have only 3–5 times higher anion secretion rates when stimulated with forskolin (Cartwright et al., 1985; Bajnath et al., 1992) or ATP (Dho, Stewart & Foskett, 1992; D. Merlin, *unpublished observation*). cAMP- and ATP-dependent short-circuit currents must originate from the same cells because the inhibitory effect of bumetanide on ATP-dependent secretory I_{sc} is revealed only after cAMP levels are raised to open cAMP-dependent Cl channels.

Apical Purinergic Receptors

In terms of ATP effects, the present work complements and extends previous observations in cells from the respiratory tract and T84 cells on stimulation of mucin and electrolyte secretion (Kim & Lee, 1991; Davis et al., 1992; Dho et al., 1992). To get insight into the receptors mediating the secretory responses of Cl.16E cells to extracellular ATP, we determined the relative potency of several agonists in stimulating mucin and electrolyte secretion. The observed rank order of efficacy of several nucleotides on both mucin and anion secretion with ATP > ADP > AMP > adenosine was that of typical P_2-purinergic receptors (Fig. 5*A* and *B*). The possibility that some of the effects of extracellular ATP could be accounted for by ATP hydrolysis products, mainly adenosine acting on P_1 receptors, was discounted on the grounds that adenosine was far less effective than ATP and that ATPγS was as effective as ATP in terms of stimulating both anion and mucin secretion. In contrast, the P_1 receptor of T84 cells responds very strongly to adenosine, but not to ATPγS (Madara et al., 1993).

The location of P_2 receptors mediating the action of ATP on both mucin and Cl^- secretion was inferred from studies in which monolayers of Cl.16E cells were incubated with ATP either at their basolateral or at their apical pole. Together the results argue that P_2 receptors mediating the secretion of both Cl^- and mucins are localized at the apical surface of cultured cells. This finding is consistent with other observations showing that goblet cell degranulation was elicited by application of ATP to the apical side of isolated canine tracheal epithelium (Davis et al., 1992) and that apically applied ATP is an effective Cl^- secretagogue in the human nasal epithelium (Knowles, Clarke & Boucher, 1991; Clarke & Boucher, 1992; Stutts et al., 1992). Moreover, the recent finding that brush borders of intestinal epithelial cells possess signal transduction mechanisms similar to those normally found in the basolateral membrane fits well with the view that some G-protein coupled receptors may be located on the apical membrane of polarized epithelial cells (Tilly et al., 1991). Apart from its effects via apically located P receptors, extracellular ATP has been reported to act also via different, basolateral purinergic receptors (Davis et al., 1992; Dho et al., 1992). Our findings that basolateral application of ATP elicited a weak, but significant stimulation of mucin secretion, is consistent with the presence of basolateral receptors also in basolateral plasma membranes of Cl.16E.

Coupling of Mucin and Electrolyte Secretion

The ATP-dependent cosecretion of mucin and electrolytes raises the question of the coupling mechanism. The idea of an obligatory coupling was first proposed by DeLisle (DeLisle & Hopfer 1986). The authors sought to explain how cells solubilize the macromolecules destined for secretion and flush them out of intracellular canaliculi. Support for this idea was provided by the presence of Cl^- and K^+ conductances in the membranes of exocrine granules (DeLisle & Hopfer, 1986; Gasser, Goldsmith & Hopfer, 1990; Thévenod et al., 1992).

Coupling of mucin electrolyte secretion is clearly suggested by the almost complete inhibition of both processes by quinine or quinidine. Both are K^+ channel blockers (Findlay et al., 1985), but it is not known whether their inhibition of secretion in Cl.16E is actually based on the known property of inhibiting K^+ channels. Since quinidine had no effect on basolateral K^+ or on apical Cl^- conductance activated by ATP, it is likely that these two agents inhibit the fusion of granules to the apical plasma membrane. Since quinine and quinidine are both lipophilic, they are expected to enter cells and could affect intracellular processes. One of the consequences would clearly be inhibition of mucin release. The other would be inhibition of anion secretion if the stimulated anion channels originate in the granule membrane or their activation in the apical membrane depends on an intact fusion process. In this sense, the quinine/quinidine experiments provide support for the presence of purinergically regulated anion conductances on the granule membrane.

These conclusions are consistent with the known properties of isolated exocrine granules from salivary glands and the pancreas. For these granules, it has been shown that they possess quinine/quinidine-sensitive K^+ conductances as well as anion conductances that are modulated by increases in lipid fluidity as expected for granules undergoing membrane fusion (DeLisle & Hopfer, 1986; Gasser et al., 1990; Thévenod et al., 1992). The granular K^+ conductance may be an important initial step for swelling and fusion of exocrine granules with the plasma membrane, as originally proposed by Stanley and Ehrenstein (1985).

The inhibitory effect of Ba^{2+} on electrolyte and mucin secretion cannot be explained in terms of inhibition of fusion as this cation is unlikely to exert its effect by entering cells. However, inhibition of basolateral K^+ channels by Ba^{2+} would explain the decrease of I_{sc} and that this associated depolarization of the plasma membrane may be responsible for decreased mucin secretion.

Physiological Relevance

One of the interesting findings of this study was that extracellular ATP was the most efficacious secretagogue for both mucin and electrolyte secretion in comparison to maximal stimulation of signaling pathways acting through cAMP (forskolin) or phospholipase C (carba-

chol). However, the ED_{50} values for both mucin and electrolyte secretion with 0.04 and 0.4 mM, respectively, are relatively high in comparison to other surface purinergic receptors which have dissociation constants in the micromolar range or below (Dubyak, 1991). One explanation for the apparent high ED_{50} values may be the presence of an unstirred layer. Modeling of diffusion indicates that the effective surface concentration will be lower by at least two orders of magnitude for at least 10 sec if the unstirred layer is only 200 μm thick. Secretion is seen almost immediately after addition of ATP, well within this 10 sec time frame, and in the absence of stirring. Unstirred layer effects are likely considering that the cells are blanketed by a mucus layer and that minimal stirring was used. The conclusion is supported by the finding that thorough washing of the apical surface before ATP application substantially increased the ATP effects on I_{sc} with an apparent left-shift of the dose-response curve and an increased maximal effect of about 25%. The differences in ED_{50} values between mucin and electrolyte secretion may actually be the result of a smaller effective unstirred layer during measurements of mucin secretion because the filter sheets used for mucin secretion measurements allowed better mixing of the solution than the filter cups used in electrophysiology experiments. These considerations suggest that the actual dissociation constant of the P_2 receptor for ATP may be in the low micromolar range and play a role under physiological/pathological conditions as tissue concentrations of about 20 μM have been reported (Born & Kratzer, 1984).

The source for luminal ATP to stimulate the P_2 receptor is unclear at the moment. Two major possibilities have to be considered: (i) from inflammatory cells; or (ii) from the enterocytes themselves. Under conditions of inflammation, leukocytes migrate through the epithelium and could release ATP in the lumen. Alternatively, leukocytes and other inflammatory cells could release ATP into the interstitial solution, which then could leak through leaky junctions into the lumen. The second possibility is suggested by the recent discovery that cells expressing P-glycoprotein (i.e., the gene product of the multidrug resistance gene or MDR) release large amounts of ATP into the medium because P-glycoprotein acts as an ATP channel (Reisin et al., 1992; Abraham et al., 1993). MDR is well expressed in the intestinal epithelial cells (Arias et al., 1990) and was specifically demonstrated for the intestinal T84 and HT29 cell lines (Spoelstra et al., 1991; Bremer et al., 1992). Thus, MDR and luminal, purinergic P_2 receptors could represent part of an autocrine loop whereby ATP is released into the lumen through MDR and the extracellular ATP then stimulates purinergic receptors in the luminal membrane.

Regardless of the source for ATP, the consequences of stimulation of the P_2 receptors in situ would be massive secretion of both fluid and mucin from the epithelial surface. These secretions should have a major cleansing effect by removing noxious agents, including bacteria, from the surface and, therefore, may be part of the defense mechanisms of epithelia.

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