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Defective ATP-dependent mucin secretion by cystic fibrosis pancreatic epithelial cells

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Abstract The response of confluent monolayers of normal and cystic fibrosis (CF) pancreatic epithelial cells to stimulation by extracellular ATP and ATP analogues was investigated in terms of mucin secretion. Mucin secretion was measured as release of M1 antigens by a direct sandwich enzyme immunoassay. Extracellular ATP provoked rapid (≤15 min) and strong mucin secretion (+480 ± 35%) by the normal pancreatic cell lines but was not able to induce mucin secretion by the CF cell lines. The order of efficacy of nucleotide agonists with ATP > ADP > AMP > adenosine was that of typical P_2-purinergic receptors. ATP induced a rapid and transient intracellular $[Ca^{2+}]$ mobilization in both normal and CF pancreatic epithelial cells. This work demonstrated that CFTR seemed to mediate ATP-dependent mucin secretion.

Key words: Nucleotides; CAPAN; CFPAC; M1 antigens; CFTR

1. Introduction

Cystic fibrosis is a genetic disease characterized by mucus hypersecretion, persistent bacterial infection and inflammation of the airways. The defect lies in mutations of the membrane protein called CFTR [1], possessing a cAMP-dependent chloride channel activity [2], located in the apical membrane of many secretory epithelial cells [3].

Extracellular triphosphate nucleotides are increasingly recognized as potentially important regulators of epithelial ion transport [4]. Exogenous ATP and UTP can bind to a P_2-purinoceptor located in the apical cell membrane of airway epithelia [4], therefore inducing mobilization of intracellular calcium through phospholipase C, which consequently activates a Ca^{2+}-dependent Cl^- conductance. ATP, reported to be a potent secretagogue of electrolyte, has been consequently proposed for use as a therapeutic agent for cystic fibrosis in order to bypass the defective function of CFTR, and to restore chloride secretion in CF patients [5]. Recent reports demonstrate that extracellular ATP is also a very potent secretagogue of mucin secretion in goblet cells from the respiratory tract [6], via a P_2-receptor-mediated mechanism [7,8], and of SLPI (secretory leucoprotease inhibitor) in human tracheal gland cells [9].

Several gastrointestinal cell lines have been found to be mucin secreting and used to study the regulation of mucin secretion [10,11]. Particularly mucin and electrolyte secretion in response to ATP has been observed in the human intestinal goblet cell line HT29-Cl.16E [10]. No work has been reported about ATP-dependent mucin secretion in normal and cystic fibrosis pancreatic epithelial cells in culture: it therefore seemed interesting to us to study the differential ATP-dependent mucin secretion and the role of Ca^{2+} as a second messenger.

In a previous study, we found secretion of mucin M1 antigens by a pancreatic cell line derived from a patient with cystic fibrosis: the CFPAC-1 cell line [12]. Mucin M1 antigens were initially found in gastric mucosae and in human ovarian mucinous cystic fluids [13]. These antigens are borne by high-molecular-weight components, which exhibit blood-group-related antigens and a density of 1.4 by CsCl density-gradient ultracentrifugation; they are consequently associated with mucins [14]. M1 antigens were subsequently shown to be also present and secreted by a mucin producing cell line: CAPAN-1 [12,15,16], by the CFPAC-1 cells that were stably transfected with a retroviral vector containing the full-length cDNA encoding wild-type CFTR (CFPAC-PLC-CFTR6) and by the CFPAC cells that were mock-transfected with vector alone (CFPAC-PLJ6) (unpublished data). We therefore took into account the property of the CFPAC cell lines to produce mucin M1 antigens to compare the ATP-induced mucin secretion in CF and reverted CF cells. CAPAN-1 cells were used as control cells.

Our results have shown that ATP is not able to induce mucin secretion by the CF and mock-transfected cell lines whereas it provokes rapid and strong mucin secretion by the reverted cell lines and the control cells. However, ATP may be bound in all the different cells to a P_2-purinoceptor as shown by the induction of a rapid and transient intracellular calcium mobilization. These observations provide for the first time the evidence of an alteration in the secretory control mechanism of CF pancreatic cell mucins and that this alteration is distinct from the second messenger system.

2. Materials and methods

2.1. Chemicals and solutions

Adenosine, adenosine-5′-monophosphate (AMP), adenosine-5′-diphosphate (ADP), adenosine-5′-triphosphate (ATP), adenosine-5′-thiotriphosphate (ATPγS), uridine-5′-triphosphate (UTP), dimethyl sulfoxide (DMSO), forskolin, carbachol, vasoactive intestinal peptide (VIP) and RPMI-1640 medium were obtained from Sigma (St. Louis, MO, USA). Stock solutions of each secretagogue or agent were prepared as follows: adenosine, AMP, ADP, ATP, ATPγS, UTP were dissolved in the RPMI-1640 medium at 10^{-2} M. Forskolin was dissolved in ethanol at 10^{-2} M to a final concentration of 10^{-5} M. Carbachol was diluted in the culture medium and used at 10^{-3} M. VIP was dissolved in phosphate buffer pH 7.2 at 10^{-3} M to a final concentration of 10^{-6} M, in the culture medium.

2.2. Cell lines

The CAPAN-1 cell line used in this study was established by Fögh et al. [17] from a liver metastasis resulting from a pancreatic adeno-

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carcinoma in a 40-year-old patient. CFPAC-1 is a pancreatic adenocarcinoma cell line derived from a homozygous patient for phenylalanine deletion at position 508 (ΔF 508) and expresses the appropriate CF gene product [18]. CFPAC-1 cells were stably transfected with a retroviral vector (PLJ) that contained the full-length cDNA encoding wild type CFTR: CFPAC-PLJ-CFTR6 [19]. CFPAC-PLJ6 were CFPAC-1 cells exposed to control retrovirus alone (PLJ) [19]. Those three CFPAC cell lines were generous gifts from Professor R.A. Frizzell (University of Alabama in Birmingham, USA). The presence of normal and mutated CFTR mRNA in CFPAC-PLJ-CFTR6 cells was assessed in our laboratory using cDNA-PCR amplification and polyacrylamide gel electrophoresis (data not shown).

2.3. Cell culture

All cell lines were routinely grown in RPMI-1640 medium supplemented with 15% FCS for the CAPAN-1 cell line as described by Levrat et al. [20]; with 10% FCS for the CFPAC cell lines. Media were supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml) and fungizone (0.25 mg/ml). Cells were maintained at 37°C in a 5% CO_2/95% air atmosphere. They were regularly harvested with a 0.05% trypsin solution, in 0.53 mmol/l EDTA after 9 and 14 days of culture. Cells were seeded onto Falcon 6-well cell culture plates, each well containing about 5×10^5 cells in 2 ml of culture medium, for the different stimulations. Culture supernatants of confluent cultures were used for secretion studies between days 8 and 14.

2.4. M1 antigens measurement

Confluent cultures of the different cells grown on 6-well plates were rinsed two times for 2 h with serum-free culture medium and then exposed either for 5, 15 and 30 min to 0.1 mM and 1 mM ATP or for 30 min to ATP (from 10^{-6} M to 10^{-3} M) to study cumulative dose-response curve or for 30 min to fixed concentrations (10^{-3} M) of different nucleotides or agents. The culture medium was harvested and M1 antigens were measured by a direct sandwich enzyme immunoassay, using two monoclonal antibodies (MAbs): 1–13 M1 and 9–13 M1 [12]. These antibodies were shown to recognize the *a* and *d* epitopes present on the same peptide core of high-molecular-weight macromolecules characterized as M1 antigens [14]. The detection limit of the assay optimized with streptavidin-peroxidase was 1.6 ng/ml. This ELISA was highly specific, sensitive, reproducible, raised to 520 ng of protein/ml and quickly performed. The M1 antigen secretory rate (M1 Ag S.R.) was defined as the ratio of M1 Ag secreted by

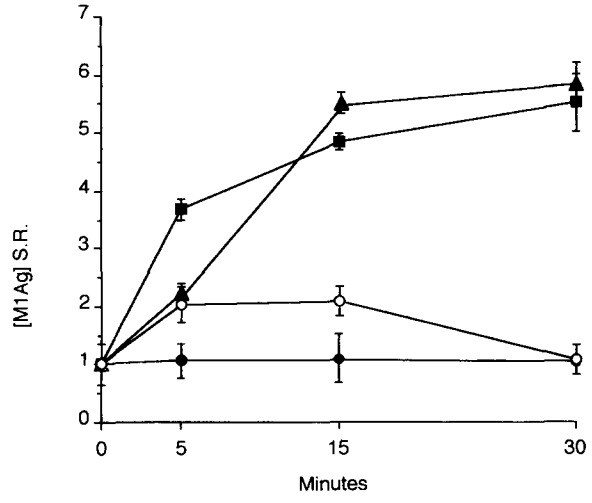

Fig. 1. Time dependence of extracellular ATP-stimulated secretion of mucin in confluent CAPAN-1 cells (■), CFPAC-1 cells (●), CFPAC-PLJ-CFTR6 cells (▲) and CFPAC-PLJ6 cells (○). Mucin secretion after addition of 1 mM ATP to culture medium. The M1 Ag secretory rate (M1 Ag S.R.: ratio of M1 Ag secreted by the assay to M1 Ag secreted by control) was calculated and compared to control which has a value of 1. Each point represents mean ± S.D. for triplicates from two different experiments ($n=6$). Statistical significances between the response to agents and the control in this and the following figures are indicated by *: $P<0.05$, **: $P<0.01$ and ***: $P<0.001$.

cells upon stimulation with different agents to M1 Ag secreted by the same unstimulated cells which has a value of 1. In each experiment, the mean M1 Ag secretory rate was determined from at least 6 assays.

2.5. Single cell [Ca^{2+}] measurement

Cells were seeded at 10^4 cells/cm^2 onto glass coverslips and cultured for 48 h in complete culture medium. After four 1 h washes in serum-free medium, cells were incubated for 30 min in the dark at 37°C in a 10 mM Tris/HCl buffer containing 135 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$ and 1 μM Fluo 3/AM (from a 0.1 mM, 3% Pluronic F127 stock solution prepared in 20% DMSO). Changes in fluorescence were monitored at 37°C using the same incubation buffer and a fluorescence microscopy imaging system consisting of an Olympus IMT2 inverted phase-contrast microscope with a ×40 objective lens and equipped for epifluorescence and photometry with a Lhesa 4015 SIT video camera as described in detail previously [9]. When the baseline level of fluorescence had stabilized, 0.1 mM of ATP was added to the cell preparation. This experiment was performed three times and 3–6 single cells of each cell line were analyzed per experiment. Results within an experiment and between the experiments were found to be highly reproducible.

2.6. Statistics

Each experiment was repeated at least six times. All results are expressed as means ± standard deviation (SD). Differences between means were analyzed by paired or unpaired *t*-tests as appropriate: thus, a paired or unpaired Student's *t*-test was used to show statistical significance. A *P* value of <0.05 was considered significant. Statistics were carried out using the Software Statwork 1.2.

3. Results

3.1. Constitutive secretion of M1 antigens by the pancreatic cell lines

Repeated measurements of M1 antigens by the supernatants of the different cells at confluence during unstimulated basal periods were similar and remarkably reproducible, showing a continuous and constant release of 0.8 ± 0.16 ng/10^5 cells for CFPAC-1; 1.28 ± 0.08 ng/10^5 cells for CFPAC-PLJ-CFTR6; 1.04 ± 0.064 ng/10^5 cells for CFPAC-PLJ6 cells and 1.12 ± 0.08 ng/10^5 cells for CAPAN-1. These results were the average of 10 different assays at 1, 12 and 24 h respectively.

3.2. ATP as secretagogue for mucin release

As shown in Fig. 1, the addition of ATP to the CAPAN-1 and to the CFPAC-PLJ-CFTR6 cell lines elicited a rapid and strong stimulation of mucus release quantified by M1 antigen measurement. By contrast, the addition of ATP to the CFPAC-1 and to the CFPAC-PLJ6 cell lines did not induce any increase of M1 antigens. At 1 mM ATP, an effect was seen as early as 5 min and for the CAPAN-1 and the CFPAC-PLJ-CFTR6 cell lines the release was maximal between 15 and 30 min. Similar results were obtained at 0.1 mM ATP (data not shown). The last time (30 min) was used in further experiments to characterize this process. The dose-response curve for the action of ATP on M1 antigens secretion is shown in Fig. 2. No ATP response of the CFPAC-1 and of CFPAC-PLJ6 cells was observed at any concentration. A strong response was observed with the CFPAC-PLJ-CFTR6 cell line and the CAPAN-1 cell line: the increase of secretion was respectively +483 ± 35% ($p<0.001$) and +450 ± 51% ($P<0.001$). These results argue in favor of the presence of an ATP receptor on the CFPAC-PLJ-CFTR6 and on the CAPAN-1 cell lines. In order to determine which type of ATP receptor is expressed, the effects of other nucleotides, ATP analogues and adenosine were examined on the four

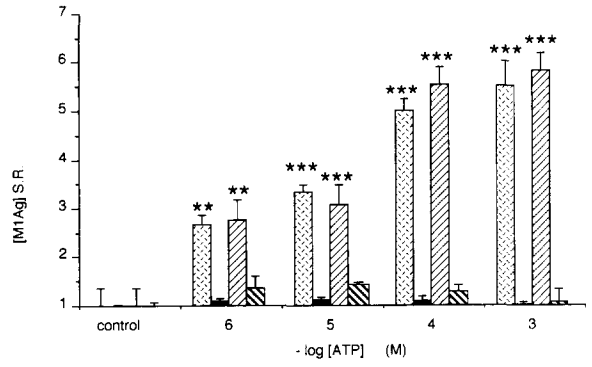

Fig. 2. Dose-response curve of extracellular ATP-stimulated secretion of mucin, by CAPAN-1 cells (first columns), CFPAC-1 cells (second columns), CFPAC-PLJ-CFTR6 cells (third columns) and CFPAC-PLJ6 cells (fourth colums). Dose response for ATP action was measured after 30 min of exposure. The M1 Ag S.R. and statistical significance as for Fig. 1.

cell lines. All drugs were used at the concentration of 1 mM. The sequence of efficacy of the different analogues tested was: ATP > ADP > AMP > adenosine > UTG (Fig. 3). Only CAPAN-1 and the CFPAC-PLJ-CFTR6 cell lines were stimulated. No response was obtained with the original and mock-transfected CFPAC-1 cell lines. Compared with the stimulation by ATP, the results from exposing the luminal surface to other selected nucleotides were modest: with CAPAN-1 and CFPAC-PLJ-CFTR6 they were respectively: $+69 \pm 24\%$ ($P < 0.05$) and $+98 \pm 8\%$ ($P < 0.05$) for ADP; $+55 \pm 35\%$ (n.s.) and $+72 \pm 17\%$ ($P < 0.05$) for AMP; $+22 \pm 27\%$ (n.s.) and $+47 \pm 17\%$ (n.s.) for adenosine; and $+10 \pm 12\%$ (n.s.) and $+17 \pm 24\%$ (n.s.) for UTP. ATPγS, the poorly hydrolyzable analogue of ATP, used at 0.1 mM concentration gave results close to those obtained with ATP, in terms of mucin secretion, indicating that ATP itself, and not a contaminant of hydrolysis product, was the real agonist.

3.3. Intracellular [Ca^{2+}] mobilization

Since ATP stimulated mucin release by CFPAC-PLJ-CFTR6 cells but not by CFPAC-1 cells nor by CFPAC-PLJ6 cells, we checked the presence of a functional P_2 receptor on these cells by analyzing [Ca^{2+}]i mobilization by ATP. Fig. 4 shows representative experiments of a single cell of each cell line. The addition of 0.1 mM ATP induced a rapid [Ca^{2+}]i increase which then declined. The transient [Ca^{2+}]i mobilization by ATP of the three cell lines was similar in amplitude and duration which was between 100 and 160 s. The maximal fluorescence ratios were 2.4 ± 0.2 ($n = 13$), 2.6 ± 0.15 ($n = 16$), and 2.5 ± 0.4 ($n = 9$), for CFPAC-PLJ6, CFPAC-1 and CFPAC-PLJ-CFTR6 cells, respectively. No difference was observed when the cells were investigated in the presence of extracellular [Ca^{2+}] (1.8 mM) or in a Ca^{2+}-free (< 50 nM) medium indicating that this observed [Ca^{2+}]i mobilization derived from internal stores.

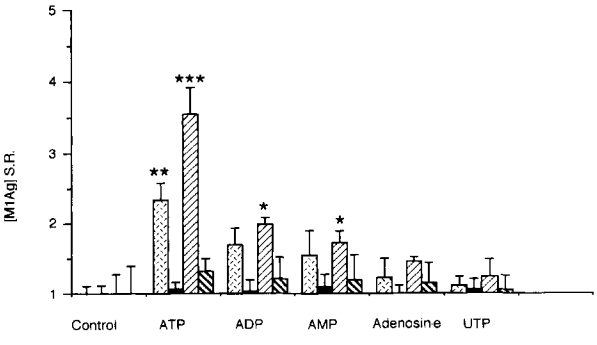

Fig. 3. Potency of ATP analogues on mucin secretion of CAPAN-1 cells (first columns), CFPAC-1 cells (second columns), CFPAC-PLJ-CFTR6 cells (third columns) and CFPAC-PLJ6 cells (fourth columns). Cells were exposed for 30 min to 10^{-3} M of each agonist. The M1 Ag S.R. and statistical significance as for Fig. 1.

3.4. Cholinergic agonist, forskolin, VIP and mucin secretion

To evaluate the potential importance of ATP-mediated mucin secretion, the efficacy of the different signalling systems was compared. We carried out a series of experiments comparing the mucin M1 secretion induced by carbachol, a cholinergic agonist, forskolin, an activator of adenylate cyclase, the natural secretagogue VIP and ATP (Fig. 5). The strongest responses were obtained with the CAPAN-1 and the CFPAC-PLJ-CFTR6 cell lines with all the above mentioned secretagogues. The increase of secretion was, with the CAPAN-1 and the CFPAC-PLJ-CFTR6 cell lines respectively: for carbachol: $+55 \pm 5\%$ (n.s.) and $+88 \pm 35\%$ ($P < 0.02$); for VIP: $+70 \pm 40\%$ (n.s.) and $+166 \pm 50\%$ ($P < 0.01$); for forskolin: $+90 \pm 35\%$ ($P < 0.01$) and $+170 \pm 46\%$ ($P < 0.01$). The compared increase of secretion for ATP with the CAPAN-1 and the CFPAC-PLJ-CFTR6 cell lines was respectively: $+250 \pm 25\%$ ($P < 0.001$) and $+375 \pm 24\%$ ($P < 0.001$). No significant response was observed with the CFPAC-1 and with the CFPAC-PLJ6 cell lines. The increase of secretion stimulated by ATP was about double the amplification of that of the other secretagogues, used however at their maximal effective dose determined in preliminary experiments. Carbachol, forskolin and VIP have some efficacy in the mucin release by the CFPAC-PLJ-CFTR6 and the CAPAN-1 cell lines, but little or no effect on the CFPAC-1 and the CFPAC PLJ 6 cell lines.

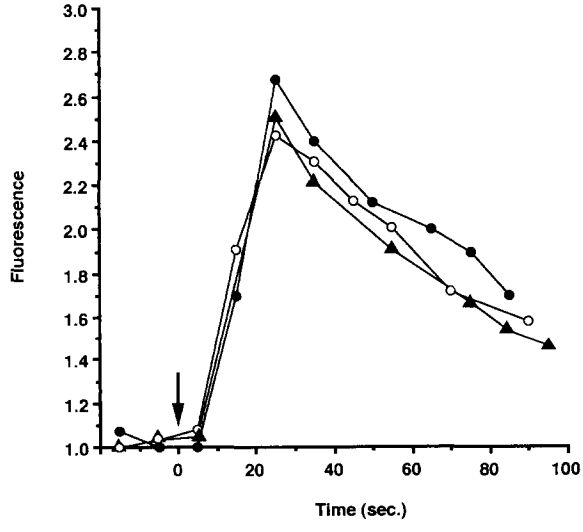

Fig. 4. Actions of 0.1 mM of ATP on intracellular Ca^{2+} concentration ([Ca^{2+}]i) in CFPAC-1 cells (●), CFPAC-PLJ-6 cells (○), and CFPAC-PLJ-CFTR-6 cells (▲). Intracellular Ca^{2+} mobilization was detected in single cells loaded with Fluo 3/AM. Arrow, ATP addition to the cells. Data are from representative experiments.

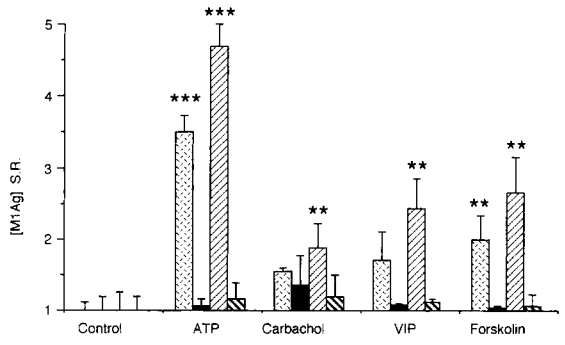

Fig. 5. Comparison of the efficacy of ATP, carbachol, forskolin and VIP on mucin secretion in CAPAN-1 cells (first columns), CFPAC-1 cells (second columns), CFPAC-PLJ-CFTR6 cells (third columns) and CFPAC-PLJ6 cells (fourth columns). Cells were exposed for 30 min to 10^{-3} M ATP, 10^{-3} M carbachol, 10^{-5} M forskolin or 10^{-6} M VIP. The M1 Ag S.R. and statistical significance as for Fig. 1.

4. Discussion

The aim of the present study was to determine the effect of ATP and its analogues on mucin secretion and $[Ca^{2+}]i$ mobilization in CF and CF reverted pancreatic epithelial cells in culture. It is well known that via a purinergic receptor in the apical cell membrane, ATP may stimulate Cl^- secretion in both CF and normal epithelia when applied in micromolar concentrations [4,21]. However, the ATP-induced mucin release in both CF and CF reverted epithelial cells has not yet been studied. Since ATP is proposed to be a therapeutic agent in CF, the most deleterious syndrome being mucus hypersecretion, it seemed to us very important to examine ATP-induced mucin secretion in both CF and CF reverted pancreatic epithelial cells in culture. Our findings showed a rapid and strong effect of ATP on mucin secretion by control epithelial cells and the reverted CF cells but no effect on the CFPAC-1 cells (original and mock-transfected). It has already been shown that the retrovirus-mediated transfection of cDNA for wild type CFTR into CFPAC-1 cells conferred cAMP-dependent regulation of Cl^- conductance [19], alleviated the alteration of sulfate transport via an anion exchange mechanism [22] and corrected the defect in intracellular trafficking of the CF antigen [23].

Our present data show that transfection of these pancreatic CF cells with normal cDNA was also able to restore the defective ATP-induced mucin secretion observed in the CF cells. Our results are in keeping with the data from Lloyd Mills et al. [24] which directly show that the incorporation of anti-CFTR antibodies into submandibular cells inhibited β-adrenergic-stimulated mucin secretion. In addition, observations were recently reported showing that adenovirus-mediated gene transfer of CFTR to immortalized CF human tracheal epithelial cells restored defective cAMP-dependent secretion not only of chloride but also of glycoconjugates [25]. The presence of a functional CFTR protein seems therefore to be necessary for the regulation of glycoconjugate secretion, however the mechanism of CFTR contribution to this process still remains unclear.

For a better insight into the receptors mediating the secretory responses of normal pancreatic epithelial cells and of pancreatic epithelial CF cells to extracellular ATP, we determined the relative efficacy of several agonists in stimulating mucin secretion. The observed rank order of efficiency of several nucleotides tested at 1 mM concentration on mucin secretion with ATP > ADP > AMP > adenosine was that of typical P_2-purinergic receptors (Fig. 3). 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. We did not find any secretory effect induced by UTP while UTP and ATP are known to be equipotent to stimulate cells via P_2-purinoceptor [26]. However, recent data demonstrate the occurrence of a pyrimidinergic receptor with high affinity to UTP [27,28] which is distinct from the purinergic receptor specific for ATP. It is therefore appealing to explain the lack of response to UTP by the absence of this pyrimidinoceptor. This point will need more investigation, however.

The lack of responsiveness to ATP by the CF cells is unlikely to derive from an absence of the receptor on the pathological cells or from an alteration in transduction mechanisms since normal and CF cells respond similarly to ATP by transient intracellular calcium mobilization. McPherson et al. demonstrated an alteration of β-adrenergic stimulation of mucin secretion by CF submandibular cells while β-agonists were able to generate intracellular cyclic AMP in the same manner in normal and CF cells [29]. The authors postulated an alteration in a regulatory mechanism distal to the second messenger system. We here demonstrate that in addition to an alteration in β-adrenoceptor-induced secretion, there is also an alteration in purinoceptor-induced secretion which also appears distal to the second messenger system. However, the alteration of adrenoceptor stimulation in CF cells concerns both chloride and mucin secretion. Since it has been shown that ATP induced chloride conductance in CF cells, the occurrence of no ATP-induced mucin secretion can have interesting therapeutic consequences. ATP is used as a therapeutic agent in the CF lung because of its action on fluid and electrolyte transport. However, no data were given about its possible action on mucin secretion. A stimulation of mucin secretion by ATP would be very deleterious in CF patients. Thus, if it is confirmed, our observation on mucin-secreting cells of the CF airways would be of relevance for the use of ATP in the disease.

One interesting finding of this study was that extracellular ATP was a more efficient secretagogue for mucin secretion in comparison to maximal stimulation of signalling pathways acting through cAMP (forskolin, VIP) or phospholipase C (carbachol) in the normal epithelial cell line CAPAN-1 and in the transfected CF epithelial cell line CFPAC-PLJ-CFTR6. No significant response by CFPAC-1 cells and by CFPAC-PLJ6 cells was observed with any secretagogue we used. Carbachol and VIP acting via phosphoinositide/Ca^{2+} and cAMP respectively, known to be physiological secretagogues for Cl^- secretion [30,31], were shown to also stimulate mucin secretion in the T84 human colonic adenocarcinoma cell line [11] and in the human intestinal goblet cell line HT29-Cl.16E [10]. Forskolin, which raised intracellular cAMP, stimulated Cl^- secretion by increasing the magnitude of CFTR-mediated Cl^- conductance in the apical membrane [32], by a PKA-dependent stimulus, was capable of stimulating mucin secretion in a Ca^{2+}-independent manner [33]. In addition, the above mentioned results of McPherson and Dormer [29] who have shown a defect in β-adrenergic stimulation of mucin

and serous protein secretion from submandibular salivary tissues from CF patients and those of Bradbury et al. [34] demonstrating the correction by transfection of wild type CFTR, of the defect in cAMP-mediated endocytosis and exocytosis in CFPAC-1 cells also correlated closely with our findings.

In summary, we showed in this study that ATP is a potent secretagogue for mucin secretion by epithelial pancreatic cells, via a P_2-purinoceptor, and that CF cells did not respond to ATP by stimulation of mucin secretion. If this phenomenon also occurs in the bronchotracheal tree or in the gastrointestinal tract of CF patients it would have relevance in the use of ATP as therapeutic agent.

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