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Characterization of a diadenosine tetraphosphate-receptor distinct from the ATP-purinoceptor in human tracheal gland cells

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Abstract

Human submucosal tracheal glands are now believed to play a major role in the physiopathology of cystic fibrosis, a genetic disease in which ATP is used as a therapeutic agent. However, actions of ATP on tracheal gland cells are not well known. ATP binds to P2 receptors and induced secretory leucocyte protease inhibitor (SLPI) secretion through formation of cyclic adenosine monophosphate and mobilization of intracellular $[Ca^{2+}]$. Since diadenosine polyphosphates (ApnA) are also endogenous effectors of P2 receptors, we investigated their effects in a cell line (MM39) of human tracheal gland cells. Diadenosine tetraphosphates (Ap4A) induced significant stimulation ($+50 \pm 12\%$) of SLPI secretion and to a similar extent to that of ATP ($+65 \pm 10\%$). No significant effects were observed with diadenosine triphosphate (Ap3A), diadenosine pentaphosphate (Ap5A), ADP and 2-methylthio-adenosine triphosphate (2-MeS-ATP). Since Ap4A was weakly hydrolyzed ($< 2\%$ of total), and the hydrolysis product was only inosine which is ineffective on cells, this Ap4A effect was not due to Ap4A hydrolysis in ATP and adenosine monophosphate (AMP). A mixture of Ap4A and ATP elicited only partial additive effects on SLPI secretion. ADP was shown to be a potent antagonist of ATP and Ap4A receptors, with IC_{50}s of 0.8 and 2 μM, respectively. 2-MeS-ATP also showed antagonistic properties with IC_{50}s of 20 and 30 μM for ATP- and Ap4A-receptors, respectively. Single cell intracellular calcium ($[Ca^{2+}]_i$) measurements showed similar transient increases of $[Ca^{2+}]_i$ after ATP or Ap4A challenges. ATP desensitized the cell $[Ca^{2+}]_i$ responses to ATP and Ap4A, and Ap4A also desensitized the cell response to Ap4A. Nevertheless, Ap4A did not desensitize the cell $[Ca^{2+}]_i$ responses to ATP. In conclusion, both P2Y2-ATP-receptors and Ap4A-P2D-receptors seem to be present in tracheal gland cells. Ap4A may only bind to P2D-receptors whilst ATP may bind to both Ap4A- and ATP-receptors.

Keywords: Nucleotide; Ap4A; P2D receptor; Airway

1. Introduction

The P2 purinoceptors, which bind the potent secretagogue ATP, have been classified in $P2X_{1-7}$ and $P2Y_{1-11}$ receptors according to their sequence homologies (Ralevic and Burnstock, 1998). The P2X receptors are receptor-operated cationic channels recognized by α,β-methylene ATP. From the pharmacological properties deduced after expression of cDNAs in cells deprived of P2 receptors, three classes of P2Y receptors can be distinguished. The P2Y4 and P2Y6 receptors are pyrimidinoceptors specific for UTP and UDP, respectively. The ATP-receptor P2Y1 is recognized by 2-methylthio-ATP (2-Me-SATP) and the P2Y2 binds equipotently ATP and UTP. The other P2Y receptors (P2Y3, P2Y5, P2Y7, and $P2Y_{8-10}$) have not yet been identified in humans nor pharmacologically as nucleoside receptors. The recent P2Y11 receptor seems to be specific to ATP over UTP (Communi et al., 1997) but more data is still needed to confirm its pharmacology.

Diadenosine polyphosphates (ApnA) are also thought to be endogenous regulator molecules of P2 receptors (Kisselev et al., 1998). Since they are shown to be collocated with ATP and acetylcholine in the secretory granules in the nerve terminals, they are now considered as potent neurotransmitters (Miras-Portugal et al., 1998). ApnA were reported to be P2Y2 receptor agonists with a potency

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comparable to ATP and UTP (Lazarowski et al., 1995). However, increasing lines of evidence are arguing about the presence of specific $P2Y_{Ap4A}$ receptors (formerly called P2D receptors) (Pintor et al., 1993).

In vitro and clinical studies have given ATP and UTP an important role of being able to stimulate chloride secretion in respiratory epithelia from cystic fibrosis patients (Knowles et al., 1991; Mason et al., 1991). This disease is characterized by mucus overabundance as well as severe infection and inflammation of the lungs, due to mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Riordan et al., 1989). These mutations lead to a defect in cyclic AMP-dependent chloride secretion associated with the CFTR protein. Since ATP and UTP are thought to stimulate secretion through alternative mechanisms therefore bypassing the primary defect (Knowles et al., 1991), they were proposed as potent therapeutic agents.

Tracheal submucosal glands are recognized as being predominant secretory cells in the human airways. They are composed of mucous cells secreting mucins and of serous cells secreting antiproteolytic (secretory leucocyte protease inhibitor: SLPI) and bactericidal (lactoferrin, lysozyme, ...) proteins (Basbaum et al., 1990). The mucins and the latter proteins are secreted under autonomic nerve stimulation and mix into the gland lumen to form the mucus which are then evacuated through collecting ducts into the airways. Engelhardt et al. (1992) have demonstrated that CFTR is almost undetectable in the surface epithelial cells of the human bronchus whilst the serous component of the broncho-tracheal glands express CFTR at a very high level. Consequently, human tracheal gland serous (HTGS) cells of the broncho-tracheal tree are now believed to be of primary importance in the secretion of the bronchial fluids and in the antibacterial defense mechanisms of the airways. We recently developed a transformed cell line, MM39, of HTGS cells (Merten et al.,1996). As for the genuine HTGS cells (Tournier et al., 1990, Merten et al., 1993a,b), confluent MM39 cells secrete SLPI (Merten et al., 1996) and high molecular weight macromolecules (Lo-Guidice et al., 1997), and are responsive to adrenergic and cholinergic agonists as well as to ATP and UTP (Merten et al., 1998). The corresponding P2 receptors found in MM39 cells were identified as being P2Y2 and P2Y4 receptors on human tracheal gland serous cells based on binding experiments, intracellular cAMP generation, stimulation of protein secretion and identification of their respective transcripts (Merten et al., 1998). However, in this previous work, we also suggested the presence of another and as yet unidentified P2 receptor.

In the present study, we have examined ApnAs as putative secretagogues of HTGS cells and showed for the first time evidence of an Ap4A receptor distinct from the ATP receptor, providing a molecular basis for subsequent studies concerning purinergic regulation of secretion of HTGS cells.

2. Materials and methods

2.1. Materials

P^1,P^3-di(adenosine-5′)triphosphate (Ap3A), P^1,P^4-di(adenosine-5′) tetraphosphate (Ap4A), P^1,P^5-di(adenosine-5′) pentaphosphate (Ap5A), adenosine-5′-triphosphate (ATP), adenosine-5′-thiotriphosphate (ATPγS), adenosine-5′-diphosphate (ADP) and Dulbecco's modified Eagle's/Ham's F12 mixture (DMEM/F12) were obtained from Sigma (St Louis, MO, USA). Suramin was from Bayer and 2-methylthio-adenosine-5′-triphosphate (2-MeS-ATP) was from RBI. Ultroser G was from Biosepra (Villeneuve la Garenne, France). All other chemicals were of cell culture grade.

2.2. Cell culture

Culture of the SV40-transformed human tracheal gland cell line (MM39 cell line) was performed as previously described (Merten et al., 1996). Cells were cultured in a DMEM/F12 mixture supplemented with 1% Ultroser G, 0.22 g/l sodium pyruvate and 6 g/l glucose. Epinephrine (2.5 μM from a 2.5-mM stock solution made in HCl N/1000 and stored at −80°C) was routinely added to the cell culture medium in order to provide optimal growth and differentiation (Merten et al., 1993a). Cells were passaged using 0.025% trypsin (GIBCO) and 0.02% EDTA. Type 1 collagen coated, Falcon disposable tissue culture flasks and 24 well-plates were used. In these culture conditions, MM39 cells were reported to have conserved the physiological characteristics of the genuine cells such as the presence of cytokeratin, the expression of CFTR, and a purinergic regulated secretion of SLPI.

2.3. Stimulation of cell secretion

Confluent cultures of HTGS cells grown on 24 well plates were rinsed four times for 1 h with serum-free culture medium and then were exposed for 30 min to nucleosides or agents. Forty microliters of the culture medium was harvested and SLPI was directly measured by an immunoenzymatic technique (Tournier et al., 1983) with a sensitivity of 2 ng/ml and a reproducibility $\geq$ 97%. The SLPI secretory rate was expressed as percentage of SLPI secreted above control experiments. In each experiment the mean values were determined from quadruplicate assays.

2.4. Hydrolase assay

Before the enzyme assays, 15-day confluent cells were rinsed with DMEM/F12 without Ultroser G (4×1 h). Then the cells were exposed to 100 μM Ap4A in 300 μl

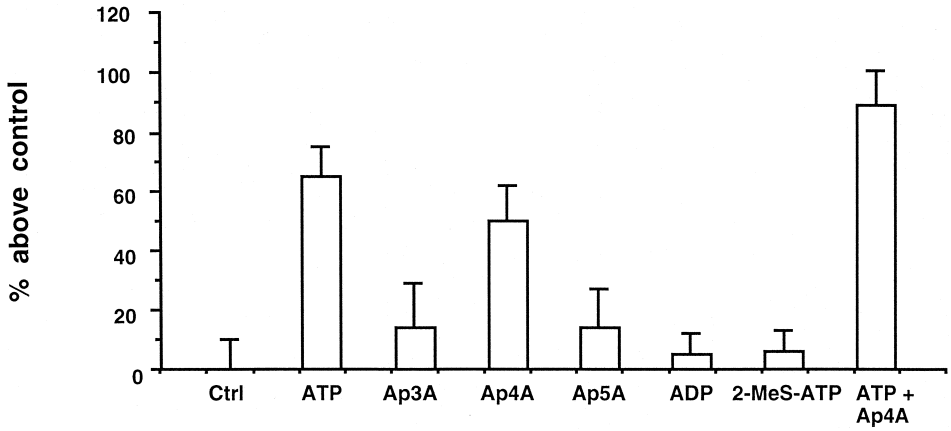

Fig. 1. Effects of nucleotides on SLPI secretion by MM39 cells. After 4×1 h washes, MM39 cells were incubated for 30 min with 100 μM of each nucleotide. SLPI was determined as described in Section 2. Only ATP and Ap4A stimulated equipotently SLPI secretion. Ap3A, Ap5A, ADP and 2-MeS-ATP did not significantly stimulate SLPI secretion. A mixture of ATP and Ap4A induced a stimulation of secretion greater than that elicited by each agonist alone, and lower than that predicted by summation of the effects of each agent added individually.

of DMEM/F12 during 0–60 min. The enzyme reaction was initiated by addition of the dinucleotide and stopped by transferring 30 μl aliquots to tubes containing 0.3 ml ice-cold water. The samples were boiled 3 min, filtered, and analyzed by reversed-phase paired-ion HPLC. The separation system consisted of a Dinamax C-18 column and a mobile phase developed with buffer A (10 mM KH_2PO_4 and 8 mM tetrabutylammonium hydrogen sulfate, pH 5.3) from 0 to 15 min, buffer B (100 mM KH_2PO_4, 8 mM tetrabutylammonium hydrogen sulfate and 10% MeOH, pH 5.3) from 15 to 35–60 min and buffer A from 35–60 to 45–75 min. Absorbance was monitored at 254 nm with an on-line Model 490 multi-wavelength detector (Shimadzu Sci. Instr., MD, USA), and radioactivity was determined on-line with a Flo-One Radiomatic β detector (Packard, Canberra, Australia) as described previously (Lazarowski et al., 1995). The assay was performed in triplicate and the data were expressed in nmol/min $\times$ 10^6 cells.

2.5. Cell $[Ca^{2+}]_i$ measurements

Cells were seeded at 10^4 cells/cm^2 onto collagen I-coated glass coverslips and cultured for 48 h in complete culture medium. After 4×1 h washes in serum-free medium, cells were incubated for 30 min in the dark at 37°C in a 10-mM HEPES buffer containing 135 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$ and 5 μM Fluo-3/AM (from a 0.1-mM, 3% Pluronic F127 stock solution prepared in 20% dimethylsulfoxide). 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 $\times 40$ objective lens and equipped for epifluorescence and photometry with a Lhesa 4015 SIT video camera as described in detail elsewhere (André et al., 1990). When the baseline level of fluorescence had stabilized, agents were added to the cell preparation.

2.6. Statistics

All results were expressed as means ± standard deviation (S.D.) made in quadruplicates. The significance between the effects of the concentrations of agents or between the effects of the agonists was determined by analysis of variance (ANOVA). The difference between the agents or between the concentrations of agents was isolated by the Scheffé's multiple comparison tests.

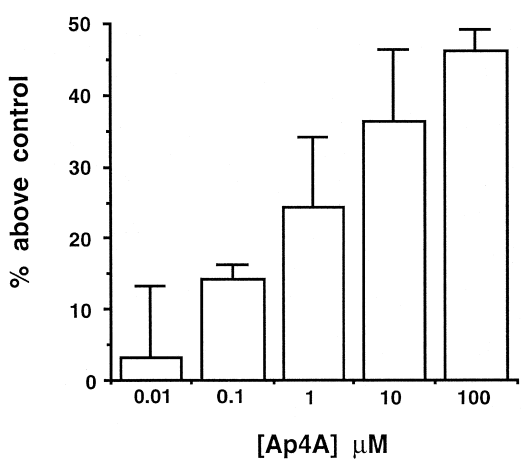

Fig. 2. Dose dependency of the effects of Ap4A on stimulation of SLPI secretion by MM39 cells. MM39 cells were grown until confluency and were then washed and exposed for 30 min to increasing concentrations of Ap4A. Each point represents means ± S.D. for four experiments ($n = 12$).

3. Results

3.1. Effects of ApnA on MM39 cells

MM39 cells are of secretory glandular nature and were previously shown to be responsive to ATP by an increase in SLPI secretion. We therefore looked for the possible effects of ApnAs on biological responses by MM39 cells. Fig. 1 shows the action of nucleotides on SLPI secretion. Ap4A significantly induced stimulation of SLPI secretion, ($+50 \pm 12\%$, compared with control experiment, ANOVA: $p < 0.001$, Scheffé test: $p < 0.05$), and to a similar extent to that elicited by ATP ($+65 \pm 10\%$). No significant stimulation was observed with Ap3A, Ap5A, ADP and 2-MeS-ATP. We also looked at whether the combination of Ap4A and ATP was able to trigger additive effects on SLPI secretion. Increased stimulation was observed by using a mixture of Ap4A and ATP (each at 100 μM). This stimulation was significantly higher than those elicited by agonists alone (ANOVA: $p < 0.001$, Scheffé test: $p < 0.05$). However, the responses were less than that predicted by summation of the effects of each agent added individually. The responses to ATP plus Ap4A were: $+89 \pm 12\%$ ($p < 0.01$). The Ap4A-induced increase in SLPI secretion was dependent on the concentration (Fig. 2).The minimal concentration of Ap4A which give significant stimulation of SLPI secretion was 0.1 μM.

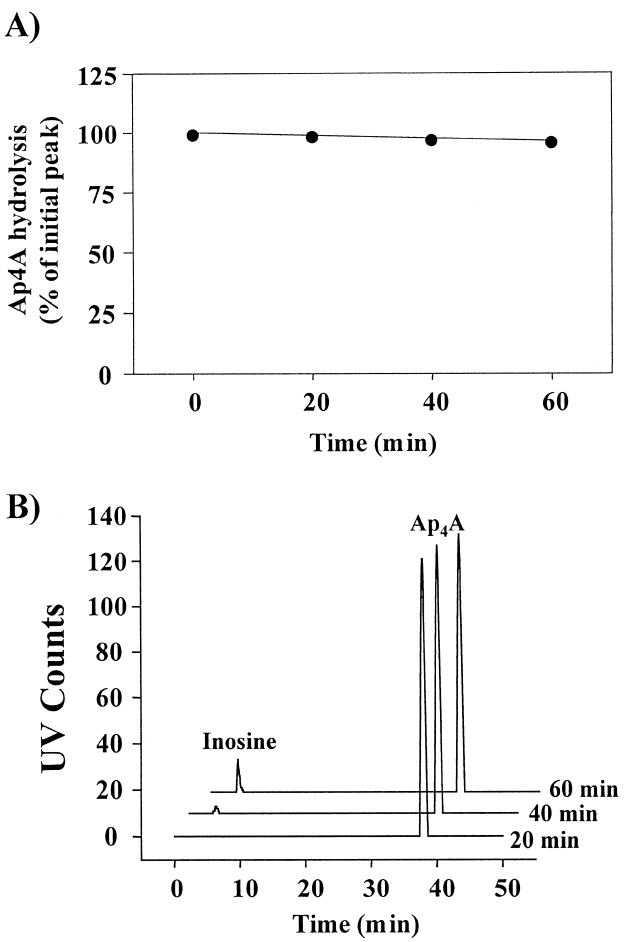

Fig. 3. Metabolism of Ap4A by human tracheal gland cells. (A) The enzyme assay was started with the addition of 100 μM Ap4A, and stopped by transferring 30 μl aliquots every 20 min to tubes containing 0.3 ml ice-cold water. The samples were boiled 3 min, filtered, and their nucleotide content analyzed by reversed-phase paired-ion HPLC. The assay was performed in triplicate and expressed as percentage of Ap4A hydrolysis over time. About 98% of the dinucleotide remained after 30 min of incubation on the cells. (B) HPLC traces of samples collected after 20, 40 and 60 min after the addition of Ap4A. The only product of Ap4A hydrolysis that accumulated over time was inosine.

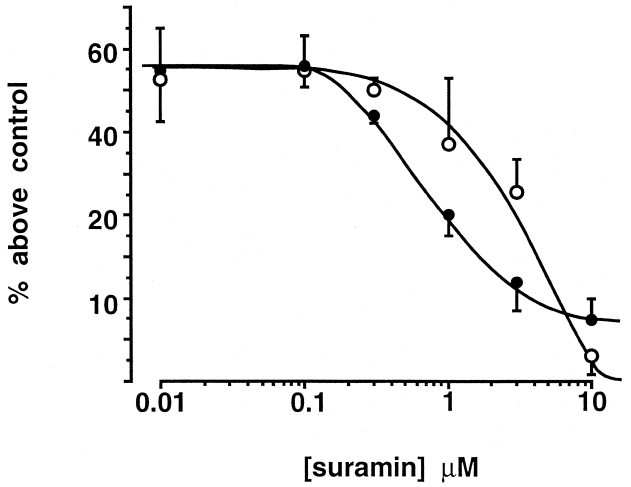

Fig. 4. Concentration-dependent blockade of the ATP-induced (○) and Ap4A-induced (●) stimulation of SLPI secretion by suramin. MM39 cells were grown until confluency and were then washed and exposed for 30 min to increasing concentrations of antagonists in the presence of 30 μM of Ap4A and ATP. Each point represents means ± S.D. for four experiments ($n = 12$). Calculations of means ± S.D. of EC_{50}s gave the values of 4 and 1 μM for ATP-induced and Ap4A-induced stimulation, respectively.

3.2. Hydrolysis of Ap4A by MM39 cells

Diphosphohydrolase is a membrane bound enzyme that is able to actively catalyze Ap4A in ATP + AMP, or ApnA in A($n-1$)P + AMP. Since high activities of diphosphohydrolase have been demonstrated on membranes of lung epithelial cells (Sevigny et al., 1997), we checked whether this enzymatic activity was present in MM39 cells. Fig. 3A shows the time-course of 100 μM Ap4A hydrolysis by MM39 cells. More than 95% of the dinucleotide remained after 60 min of incubation. The rate of Ap4A hydrolysis was 0.061%/min (±0.005), or 10.3 pmol/min cm^2. Fig. 3B shows HPLC traces of samples collected after 20, 40 and 60 min of incubation. Although Ap4A was slowly degraded by MM39 cells, the presence of ATP, ADP, AMP or adenosine could not be detected. The accumulation of inosine suggests that the products of the Ap4A hydrolysis were dephosphorylated and deaminated by ectonucleotidases reported on airway epithelial cells. Similar results were obtained for Ap2A and Ap5A.

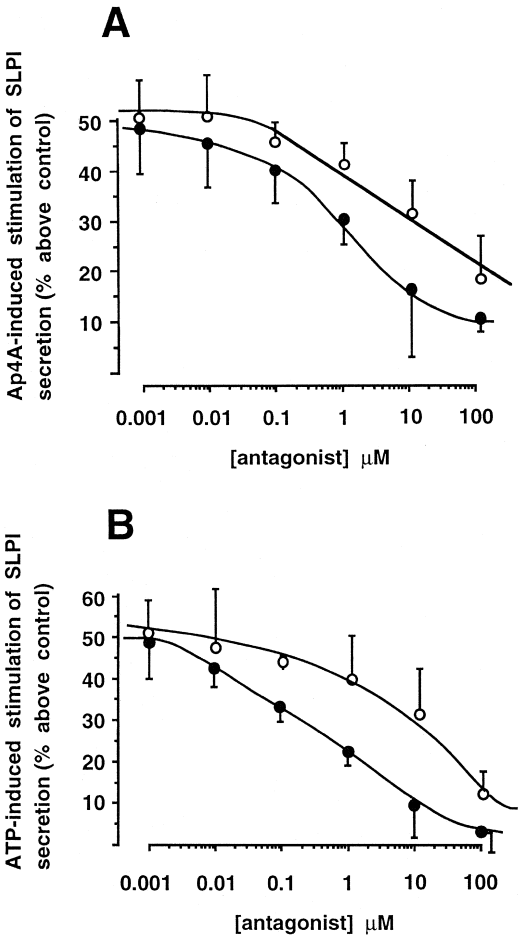

Fig. 5. Concentration-dependent blockade of the Ap4A-induced (A) and ATP-induced (B) stimulation of SLPI secretion by ADP (●) and 2-MeS-ATP (○). MM39 cells were grown until confluency and were then washed and exposed for 30 min to increasing concentrations of antagonists in the presence of 30 μM of Ap4A or ATP. Each point represents means ± S.D. for four experiments ($n = 12$). For Ap4A as agonist, calculations of means ± S.D. of IC_{50}s gave the values of 2 and 30 μM for ADP and 2-MeS-ATP, respectively. For ATP as agonist, calculations of means ± S.D. of IC_{50}s gave the values of 0.8 and 20 μM for ADP and 2-MeS-ATP, respectively.

These results indicate that the receptor-mediated responses were induced by Ap4A, and not by nucleotides resulting from ectoAp4A hydrolase activity.

3.3. Effects of suramin, ADP and 2-MeS-ATP

Suramin is a potent P2 antagonist and dose dependently inhibited both Ap4A- and ATP-induced stimulation of SLPI secretion (Fig. 4). The calculated IC_{50}s were 1 and 4 μM for Ap4A and ATP, respectively. ADP and 2-MeS-ATP were shown to be biologically inactive as they do not stimulate SLPI secretion whilst both agents were previously found to compete with ATPγS to its binding site (Merten et al., 1998). They, therefore, could be considered as P2 receptor antagonists. Thus, we looked for possible antagonization of ATP (Fig. 5A) and Ap4A (Fig. 5B) stimulation of SLPI secretion by 2-MeS-ATP and ADP. Both ADP and 2-MeS-ATP antagonized AP4A- and ATP-induced stimulation of SLPI secretion. ADP was the most potent with IC_{50}s being 0.8 and 2 μM for inhibition of ATP- and Ap4A-stimulated secretion, respectively. 2-MeS-ATP was less potent than ADP (one order of magnitude lower) with IC_{50}s being 20 and 30 μM for inhibition of ATP- and Ap4A-stimulated secretion, respectively.

3.4. Intracellular calcium measurements

We next carried out single cell intracellular calcium measurements ($[Ca^{2+}]_i$), which provide a convenient method for analyzing agonist-mediated changes in $[Ca^{2+}]$. After the addition of 100 μM ATP, UTP or Ap4A, $[Ca^{2+}]_i$ rose rapidly and then declined (Fig. 6). The maximal $[Ca^{2+}]_i$ levels reached were 750 ± 50 ($n = 42$), 760 ± 40 ($n = 18$) and 780 ± 60 nM ($n = 26$), for ATP, UTP and Ap4A, respectively, over a baseline $[Ca^{2+}]_i$ in unstimulated MM39 cells being 90 ± 20 nM. In addition, as shown in Fig. 6A, a first stimulation of the cells with 100 μM ATP prevented subsequent responses of the cells to Ap4A ($n = 39$). ATP-stimulated cells also failed to respond to a second challenge of ATP ($n = 16$, data not shown). In contrast, cells which were first stimulated by Ap4A re-

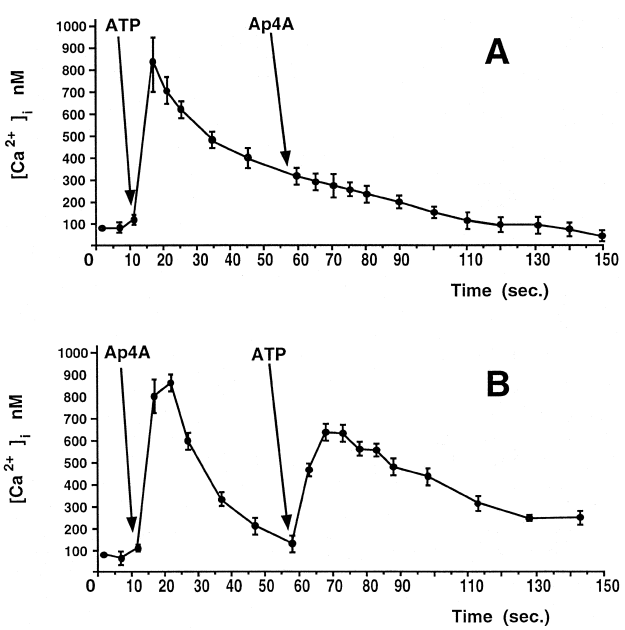

Fig. 6. Effects of Ap4A and ATP on MM39 cell secretion and $[Ca^{2+}]_i$ mobilization. $[Ca^{2+}]_i$ mobilization was measured in single cells loaded for 30 min with Fluo3/AM. ATP and Ap4A were used at 100 μM. Arrow, secretagogue addition to the cells. Data are from representative experiments. (A) Cross desensitization of actions of ATP and Ap4A. Cells were treated with ATP and Ap4A as indicated and changes in $[Ca^{2+}]_i$ were monitored. ATP elicited a transient mobilization of $[Ca^{2+}]_i$ and desensitized a further challenge with Ap4A. (B) Ap4A also induced a rapid and transient mobilization of $[Ca^{2+}]_i$. However, a second addition of ATP led to a further mobilization of $[Ca^{2+}]_i$ ($n = 19$). Data are from representative experiments.

mained able to respond ($n = 19$) to a second application of ATP (Fig. 6B) but were unable to respond to a second application of Ap4A ($n = 33$, data not shown).

We also looked for a possible cross-desensitization of UTP and Ap4A actions. We observed that a first application of Ap4A did not prevent a following UTP-induced $[Ca^{2+}]_i$ mobilization ($n = 34$) but that a first application of UTP prevented a second Ap4A-induced $[Ca^{2+}]_i$ mobilization ($n = 52$, data not shown). UTP desensitized the cells to the action of a second challenge of UTP ($n = 18$).

Thus, maximally effective concentrations of ATP, UTP or Ap4A desensitized cells to the action of a second dose of the same nucleotide. ATP and UTP desensitized the cells to the action of Ap4A. Ap4A desensitized the action of Ap4A but not that of ATP or UTP.

4. Discussion

In the human bronchus, tracheal gland serous cells express high levels of CFTR and represent an important target for the therapy of cystic fibrosis. Since ATP and/or UTP were proposed as potent therapeutic agents, it is relevant to clearly analyze and characterize the P2 receptors on these cells. We had previously given evidence for P2Y2 and P2Y4 receptors on human tracheal gland serous cells (Merten et al., 1998). We also suggested the presence of another and as yet unidentified P2 receptor. The present study was aimed at determining whether ApnA could be considered as putative secretagogues and to analyze the corresponding P2 receptor.

We herein evidenced that in MM39 cells Ap4A is able to stimulate secretion probably through $[Ca^{2+}]_i$ mobilization in a manner similar to that expected by the action on a P2Y receptor. However, whilst considering actions of nucleotides on receptors, it is important to appreciate that usually lifetime of nucleotides in extracellular medium is rapidly reduced due to ectonucleotidase activity. The ecto-phosphodiesterase (diadenosine polyphosphate hydrolase) activity present on these cells was very low (less than 2% of Ap4A was hydrolyzed in our experimental conditions) and ATP resulting from this activity was not present, indicative of a high ecto-ATPase activity. The only degradation product we found was inosine. Since inosine was not a secretagogue of MM39 cells (data not shown), the observed effects of Ap4A were assumed to be due to Ap4A itself and not to a degradation product. Moreover, due to the close structural analogy between Ap4A and ATP, it is necessary to be aware of the difficulties in clearly identifying their specific physiological receptors and to differentiate them in terms of subsequent biological responses. Ap4A have been proven to be agonist of the ionotropic P2X and the metabotropic P2Y nucleotide receptors. Since P2X were previously shown to be absent on these cells (Merten et al.,1993b), it is likely that the effects of Ap4A we observed were mediated through P2Y receptors. This assertion is further supported by different findings, (1) Ap4A induces stimulation of SLPI secretion, (2) it induces rapid $[Ca^{2+}]_i$ mobilization, and (3) it is not degraded in ATP. All these features are those expected by a receptor on which Ap4A is directly acting. This receptor seems specific for Ap4A since neither Ap3A nor Ap5A were able to induce stimulation of secretion. This rank order of effects of ApnA is similar to the one found by Lazarowski et al. (1995) but not to that of Laubinger and Reiser (1999), which found an equipotency of ApnAs ($n = 2–6$) to coupling with G proteins in rat lung. However, since their experiments were not designed to detect the physiological actions of each ApnA, it is possible that in their case the G proteins coupled with each ApnA are different and may lead to different biological responses.

Lazarowski et al. (1995) showed the action of Ap4A on the P2Y2 receptor in human astrocytoma cells with an EC_{50} of 0.72 μM, which is in the same order of magnitude as that leading to stimulation of SLPI secretion by MM39 cells (1 μM), but which is one order of magnitude lower than those found in adrenal chromaffin cells (28 μM; Castro et al., 1992), and in brain (7–18 μM; Pintor et al., 1997). These results enabled Lazarowski et al. (1995) to make a strong case for the potency of Ap4A as a full and potent agonist for the P2Y2 receptor and have termed the idea that it is possible that ApnA actions in tissues may be mediated through this receptor. However, this study was performed using a receptor cloned in a human astrocytoma cell line which does not normally express nucleotide receptors but it is not known whether the cloned receptor is functioning in these cells as it does in epithelial cells. On the other hand, many more lines of evidence favor the existence of a specific and distinct Ap4A (called P2D or $P2Y_{Ap4A}$) receptor in the nervous system (Hilderman et al., 1991; Pintor and Miras-Portugal, 1995a,b; Pintor et al., 1993).

Currently, very few data have appeared concerning the presence and characterization of ApnA receptors in lung. By using displacement studies, Laubinger and Reiser (1999) recently demonstrated the existence of an Ap4A binding site in rat lung membranes which is different from the P2X and P2Y2 receptors and showed a competition of ATP on Ap4A binding sites with an order of magnitude lower than Ap4A. This binding site is coupled to G proteins and belongs to the superfamily of P2Y receptors. Therefore, these data are in keeping with the results we obtained with human gland serous cells since these authors also evidenced two distinct ATP- and Ap4A-receptors but with an affinity of ATP to the Ap4A binding sites.

In the human tracheal gland cell line MM39, it could be assumed that ATP and Ap4A may have distinct receptors since actions of ATP and Ap4A were additive even if only partial. Furthermore, cross-desensitization experiments showed that Ap4A did not desensitize ATP actions but ATP did desensitize a further Ap4A challenge. Both experiments suggest the presence of two types of receptors: a

P2Y receptor recognized by both Ap4A and ATP, and another P2Y receptor recognized only by ATP. Thus, Ap4A seems to act on a sole receptor whilst ATP seems to be able to act on both ATP- and Ap4A-receptors. It is likely that the involved receptor leading to Ap4A-stimulated secretion by tracheal gland cells is an Ap4A-P2Y receptor. However, to insure the presence of such a receptor would need molecular data on Ap4A-specific P2Y receptors which are to date still unavailable.

A particular feature of the tracheal gland cell Ap4A-receptor is its ability to be antagonized by suramin which is not classically described as a $P2Y_{Ap4A}$ antagonist (Ralevic and Burnstock, 1998). However, it seems that pharmacological characteristics of this receptor may depend on the tissue where it is located. Pintor et al. (1997) showed an antagonism of suramin for the $P2Y_{Ap4A}$ in cortical synaptosomes but not in mid-brain synaptic terminals. Another new finding of the present work is the identification of 2-MeS-ATP and ADP as P2Y2 and Ap4A-P2Y receptor antagonists. 2-MeS-ATP is a full agonist for P2Y1 receptors and ADP a full agonist for $P2Y_{ADP}$ receptors. These clear data were found since they are based on the biological activities of the nucleotides as they were determined by analysis of stimulation of SLPI secretion. A sole analysis of effects on second messenger systems or of the binding data would not have led to that conclusion. This emphasizes the fact of how important it is to correlate binding data or second messenger system involvement to a biological response to be able to fully understand the action of effectors. We found that ADP and 2-MeS-ATP similarly antagonized ATP- and Ap4A-induced stimulation of SLPI secretion. However, the IC_{50}s for antagonistic effects of ADP and of 2-MeS-ATP were different. This probably reflects the complexity of the responses induced by ATP, which is more than a single ligand acting on a single receptor. There are two binding sites for ATP (Merten et al., 1998), one of them probably also being an Ap4A binding site.

In cystic fibrosis, nucleotides are proposed as pharmacological agents as they can stimulate Cl^- secretion through a Ca^{2+}-dependent chloride transport system different from the cAMP-dependent CFTR which is dysfunctioning in this disease. In order to avoid any possible secondary effect of the ATP-hydrolysis product adenosine in airways, the use of UTP or Ap4A was suggested since the UTP-hydrolysis product uridine seems to be innocuous and Ap4A is relatively stable. However, if gland serous cells have to be pharmacologically addressed because of their in vivo high expression of CFTR, some consideration will need to be taken into account since gland serous cells may express both P2Y4 and Ap4A-P2Y receptors. This question is particularly relevant since UTP desensitized both UTP- and Ap4A-induced calcium mobilizations whilst Ap4A desensitized only Ap4A-induced calcium mobilizations, suggesting that P2Y4 and Ap4A-P2Y receptors are distinct and that Ap4A-P2Y receptors also recognize UTP as a ligand as they do for ATP. On the other hand, even if the response of tracheal gland cells to both agonists (UTP and Ap4A) are similar in terms of SLPI secretion, more studies will have to be performed, especially concerning long term effects. For instance, UTP and Ap4A were shown to markedly influence cell proliferation (Schulze-Lohoff et al., 1995; Lemmens et al., 1996).

In summary, the present study gives evidence of the presence of a Ap4A-P2Y purinoceptor in HTG cells distinct from the P2Y2 and P2Y4 receptors. This Ap4A-P2Y receptor may recognize Ap4A, ATP and UTP. It may be coupled with mobilization of intracellular calcium and lead to stimulation of SLPI secretion. More studies are now necessary for further understanding the respective physiological role and the relevance of these evidenced nucleotide receptors in regulating secretion by human tracheal gland cells.

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