ELSEVIER

Biochemical Pharmacology 61 (2001) 623–629

Biochemical Pharmacology

Physiological evidence for a P2Y receptor responsive to diadenosine polyphosphates in human lung via Ca^{2+} release studies in bronchial epithelial cells

Werner Laubinger, Gundula Streubel, Georg Reiser*

Institute of Neurobiochemistry, Otto-von-Guericke University of Magdeburg, Faculty of Medicine, Leipziger Strasse 44, D-39120 Magdeburg, Germany

Received 5 June 2000; accepted 9 August 2000

Abstract

$P2Y_2$ receptors that are activated by the extracellular nucleotides ATP or UTP mediate Cl^- secretion via an increase in $[Ca^{2+}]_i$ (intracellular calcium concentration). Therefore, in the lung of patients suffering from cystic fibrosis, inhalation of aerosolized UTP offers a way to circumvent the defect in Cl^- secretion by the cystic fibrosis transmembrane conductance regulator. A possible alternative for the relatively unstable UTP in inhalation therapy is the more resistant diadenosine tetraphosphate (Ap_4A). In human and rat lung membranes, Ap_4A binds to P2 receptor sites coupled to G proteins. Here, we showed that Ap_4A caused an increase in $[Ca^{2+}]_i$ with an EC_{50} of 17 μM in human bronchial epithelial cells (HBE1). The $[Ca^{2+}]_i$ rise evoked by ATP and UTP was completely, but that induced by Ap_4A only partially, caused by release of Ca^{2+} from internal stores. Moreover, the potency of Ap_4A to mobilize Ca^{2+} was lower than that of ATP and UTP (EC_{50} 1.5 and 1.8 μM, respectively), and the maximal increase in $[Ca^{2+}]_i$ was considerably smaller than that after ATP or UTP. In accordance with our previous results providing evidence for a common binding site for various diadenosine polyphosphates in lung membranes, all Ap_nA analogues tested (n = 3 to 6) caused a comparable $[Ca^{2+}]_i$ increase. Homologous or heterologous prestimulation largely diminished the increase in $[Ca^{2+}]_i$ found after a second pulse of either UTP or Ap_4A. Although specific binding characteristics and functional responses of Ap_4A on lung cells are in favor of a distinct receptor for Ap_4A, the cross-talk between UTP and Ap_4A in HBE1 cells and the only slight differences in Ca^{2+} mobilization by ATP or UTP and Ap_4A render it impossible at this point to state unequivocally whether there exists a distinct P2Y receptor specific for diadenosine polyphosphates in lung epithelia or whether Ap_4A activates one of the nucleotide receptors already described.

Keywords: Diadenosine tetraphosphate; P2 receptors; Human bronchial epithelial cells; Cystic fibrosis; Calcium mobilization

1. Introduction

In airway tissue, Cl^- channels are activated by different signaling pathways: (a) CFTR Cl^- channels are a cyclic adenosine 5′-monophosphate-stimulated Cl^- transport system, and (b) other Cl^- channels are coupled to the increase in $[Ca^{2+}]_i$ that appears after activation of G protein-coupled P2Y receptors by extracellular nucleotides such as ATP or UTP [1]. Controlled Cl^- transport through airway epithelia plays a vital role in lung physiology, e.g. in the regulation of mucus hydration, periciliary fluid homeostasis, secretion of mucin, and optimal mucociliary lung clearance [2,3]. As CFTR Cl^- channels are impaired in cystic fibrosis due to a mutation in the *CFTR* gene, Cl^- channels controlled by P2Y receptor subtypes (particularly $P2Y_2$) are of prominent interest as an alternative route to induce Cl^- ion secretion [4]. In CF patients, Ca^{2+}-activating agonists such as UTP were successfully applied to stimulate this pathway of Cl^- secretion [5]. However, for an effective treatment of CF, a comprehensive characterization of these nucleotide receptors in lung and their physiological role in the cell is indispensible.

Recently, we have identified P2-type receptors in rat and human lung with high affinity for $[^3H]Ap_4A$ [6]. Ap_4A belongs to the group of diadenosine polyphosphates (Ap_nA, with n being the number of phosphate groups between the

* Corresponding author. Tel.: +49-391-671-3088; fax: +49-391-671-3097.

E-mail address: georg.reiser@medizin.uni-magdeburg.de (G. Reiser).

Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; $[Ca^{2+}]_i$, intracellular calcium concentration; CF, cystic fibrosis; CPA, cyclopiazonic acid; HBE cells, human bronchial epithelial cells; and Ap_4A, diadenosine tetraphosphate.

0006-2952/01/$ – see front matter
PII: S0006-2952(00)00590-6

adenosine moieties), a novel class of signaling molecules [7,8]. [^{3}H]Ap_4A binding sites in lung showed comparable affinities for several Ap_nA (n = 2 to 6). As ATP, UTP, and α,β-methylene adenosine 5′-triphosphate interfered with considerably lower affinity or practically not at all, these binding sites are assumed to be different from $P2Y_2$ and P2X receptor binding sites [9]. Determination of ligand-induced stimulation of [^{35}S]GTPγS binding revealed that the Ap_4A receptor in lung membranes is coupled to G proteins [10].

Ap_4A is a much more stable nucleotide than UTP, which is readily degraded by the numerous hydrolyzing enzymes present in CF lung [11]. Thus, if activation of P2Y receptors by Ap_4A is an alternative way to stimulate Cl^- secretion in lung, long-lived Ap_4A or synthetic Ap_4A analogues could replace the rather unstable UTP in CF therapy [12]. Therapeutic applications of Ap_4A in the regulation of blood pressure have already been investigated [13,14]. However, Ap_4A has not yet been used for experimental studies in the lung.

To explore the considerable therapeutic value of Ap_4A in the treatment of lung diseases like CF, investigations of the functional effects of extracellular Ap_4A on signal transduction in lung cells are still necessary. Therefore, continuing our studies on the effect of diadenosine polyphosphates on P2Y receptors in lung, in this work we used a physiological approach. In the past, cell lines and cell cultures derived from the respiratory system were already used to study P2 receptors. In a human tracheal gland serous cell line (MM 39), both extracellular ATP and UTP increased $[Ca^{2+}]_i$, and two distinct P2Y receptors were characterized in these cells [15]. In a bronchial epithelial cell line (16HBE14o$^-$), a $P2Y_2$ receptor activated a transient K^+ conductance [16]. In primary cultures of HBE, it was shown that UTP, in addition to activating Cl^- secretion, also regulated Na^+ absorption, which is disturbed in CF patients [17]. So far, there is evidence for the existence of $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors in HBE cell lines, whereas a $P2Y_1$ receptor could not be detected [18].

At present, however, nothing is known about the existence of a receptor specific for Ap_4A in bronchial epithelial cells. As 16HBE14o$^-$ cell lines were used to investigate the regulation of chloride ion transport in human airway epithelium [19], HBE1 cells seemed to be a suitable model to study physiological responses caused by nucleotide or dinucleotide receptor activation. G protein-coupled P2Y receptors activate phospholipase C, which leads to the formation of inositol 1,4,5-trisphosphate and the mobilization of intracellular Ca^{2+} [20]. Thus, by measuring $[Ca^{2+}]_i$, we showed herein the response to extracellular Ap_4A in HBE1 cells in comparison with that induced by ATP and UTP. In conclusion, diadenosine polyphosphates were able to evoke an increase in $[Ca^{2+}]_i$ in HBE cells either as an agonist of the $P2Y_2$ receptor, being less potent than ATP or UTP, or as an agonist of another P2Y receptor specific for diadenosine polyphosphates, whose existence still has to be confirmed by further studies.

2. Materials and methods

2.1. Materials

ATP, Ap_3A, Ap_4A, Ap_5A, Ap_6A, and UTP were from Sigma. Fura-2 acetoxymethylester (Fura-2/AM) and CPA were from Alexis.

2.2. Cell culture and calcium measurement

The HBE1 cell line derived from human bronchial epithelial cells is described in detail in [21]. Cells were cultured with 5% CO_2 at 37° in a medium containing Dulbecco's modified Eagle's medium/NUT MIX F12 (1:1), 5% fetal calf serum, and the following supplements: gentamycin (50 μg/mL), kanamycin (50 μg/mL), insulin–transferrin–selenium supplement (10 μg/mL), hydrocortisone (1 μM), pituitary extract (3.75 mg/mL), epidermal growth factor (25 μg/mL), T3 (30 nM), and cholera toxin (10 ng/mL). For cultivation, cells were grown to 70–90% confluency and then subcultured.

HBE1 cells were seeded onto glass coverslips and cultured for 5 to 6 days. Cells with 40–60% confluency were used for single-cell measurement of $[Ca^{2+}]_i$. Before measurement, cells were preincubated with 2 μM Fura-2/acetoxymethylester in Na-HBS (HEPES-buffered saline: 145 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM glucose, 20 mM HEPES/Tris pH 7.4) for 30 min at 37°. For experiments in nominally Ca^{2+}-free incubation medium, $CaCl_2$ was omitted from Na-HBS, without supplement with EGTA. Fluorescence was recorded at 340-nm/380-nm excitation and 520-nm emission. Changes were monitored at 37° in a perfusion chamber on a fluorescence imaging system from TILL Photonics GmbH with an X40 immersion objective and a flow rate of 2 mL/min as described earlier [22]. Test pulses of nucleotides usually lasted for 60 sec. Images were stored on a personal computer and data were analyzed with the Fuchal 5.12.C software program.

2.3. HPLC analysis

The stability of Ap_4A in the presence of HBE1 cells was analyzed by HPLC. HBE1 cells were washed with HBS and 10 mL of fresh HBS then applied. After incubation of HBE1 cells in the presence of Ap_4A (100 μM), 200-μL samples of the supernatant were removed at different intervals and treated for HPLC application as described earlier [9]. Nucleotides were separated by HPLC (Kontron) using a NUCLEOGEL SAX 1000-8 column (50 × 4.6 mm) from Macherey-Nagel. The column buffer was 10 mM Tris/HCl, pH 8.0. Samples (50 μL) were injected onto the column and

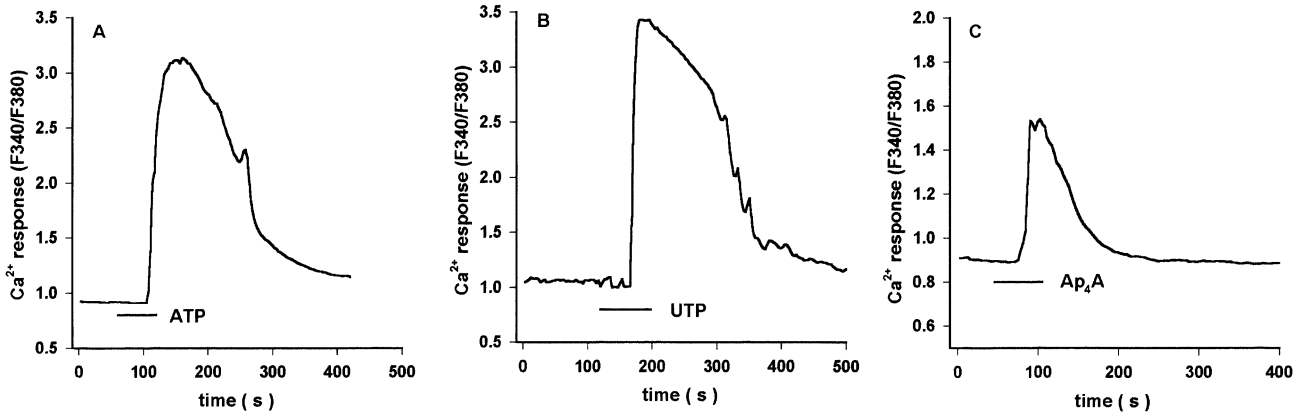

Fig. 1. Elevation of intracellular Ca^{2+} by ATP, UTP, or Ap_4A in HBE1 cells. HBE1 cells loaded with 2 μM Fura-2 were stimulated with 100 μM ATP (A), 100 μM UTP (B), or 400 μM Ap_4A in Ca^{2+}-containing solution at the time periods indicated by the bars. The data represent the means of a typical experiment with at least 22 single-cell measurements.

eluted using a linear gradient of NaCl (0–500 mM) in 10 mM Tris/HCl pH 8.0. Elution profiles were analyzed by monitoring absorption at 260 nm. Authentic compounds (50 μL of a 100-μM solution in water) were used as reference to identify the resulting elution peaks.

3. Results

In human and rat lung membranes, we earlier found binding sites for [^{3}H]Ap_4A that are coupled to G proteins [6,9,10]. For further characterization, we established a system where we could study physiological responses caused by extracellular Ap_4A. We found that HBE1 cells also possess [^{3}H]Ap_4A binding sites. However, the limited amount of cell membrane material available allowed only a partial characterization of [^{3}H]Ap_4A binding sites in HBE1 cells. [^{3}H]Ap_4A was displaced by Ap_2A or Ap_5A in a similar fashion as it was displaced by unlabeled Ap_4A. ATP was a less potent displacer and UTP was unable to displace [^{3}H]Ap_4A from this binding site (data not shown). As the results obtained were comparable to our detailed previous analysis found for [^{3}H]Ap_4A binding sites in human and rat lung tissue, HBE1 cells proved to be a suitable system to study functional effects evoked by extracellular Ap_4A.

As extracellular Ap_4A might be hydrolyzed by membrane-bound ectoenzymes [23] probably also present on HBE1 cells, we investigated whether Ap_4A was degraded to ATP or ADP during exposure to HBE1 cells. For this reason, samples of the cell incubation medium were taken 1, 2, 5, 10, and 15 min after application of Ap_4A to the cells, and the nucleotides present in the medium were analyzed by HPLC using anion exchange chromatography. By HPLC analysis, Ap_4A can be separated from the possible degradation products by a different retention time (R_t). Up to 15 min after addition of Ap_4A to the cells, Ap_4A (R_t = 12.9 min) was the only nucleotide found in the incubation medium, and no trace amounts of ATP (R_t = 12.1 min) or ADP (R_t = 10.7 min) could be detected. Taking into account that the exposure time in the experiments was usually short, it is evident that the effects described here resulted from the intact ligand Ap_4A and not from the mononucleotides ATP or ADP, degradation products that potently activate P2 receptors.

It is well known that HBE1 cells possess $P2Y_2$ receptors [16–18]. As this P2 receptor subtype is activated by ATP and UTP with equal potency, we determined the effect of these nucleotides on the rise in $[Ca^{2+}]_i$ in single HBE1 cells by measuring the $[Ca^{2+}]_i$ signal derived from the fluorescence of Fura-2 and comparing it to the $[Ca^{2+}]_i$ signal evoked by Ap_4A, as exemplified by the sample traces in Fig. 1. The concentration–response curves (Fig. 2) showed that HBE1 cells were sensitive to ATP or UTP in a similar manner, whereas they were less sensitive to Ap_4A. The maximal Ca^{2+} response (ΔF340/F380; mean values of 5 independent experiments ± SE) was 1.9 ± 0.06 and 1.8 ± 0.06 for ATP and UTP (100 μM each), respectively, whereas it was only 1.2 ± 0.05 for Ap_4A at a maximal concentration of 500 μM. Data analysis revealed similar EC_{50} values (mean values of 4 independent experiments ± SE) for ATP (1.5 ± 0.05 μM) and UTP (1.8 ± 0.05 μM), whereas the EC_{50} value for Ap_4A was one order of magnitude higher (17 ± 0.04 μM). Thus, $P2Y_2$ receptors have either a lower affinity for Ap_4A or HBE1 cells possess distinct receptors specific for Ap_4A whose signaling processes are also coupled to a rise in $[Ca^{2+}]_i$. Stimulation of HBE1 cells with the P1-specific agonist adenosine (10 μM) gave only a weak increase in $[Ca^{2+}]_i$ (<20% of the UTP response), whereas the $P2Y_1$-specific agonist 2-methylthioadenosine 5′-diphosphate and the P2X-specific agonist α,β-methylene adenosine 5′-triphosphate induced practically no stimulation of $[Ca^{2+}]_i$ (data not shown).

Recently, we demonstrated that various diadenosine polyphosphates evoked comparable stimulation of [^{35}S]GTPγS binding to human and rat lung membranes [10]. To test

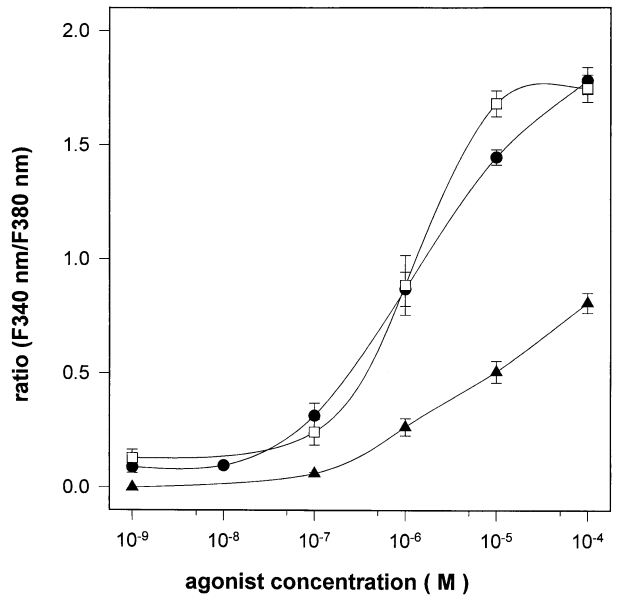

Fig. 2. Concentration–response curves for the increase in $[Ca^{2+}]_i$ elicited by purinergic agonists in HBE1 cells. HBE1 cells were loaded with 2 μM Fura-2 and then stimulated with ATP (●), UTP (□), or Ap_4A (▲). Data represent average maximal $[Ca^{2+}]_i$ changes evoked by increasing concentrations of the agonists in Ca^{2+}-containing saline. Each point is the mean ± SE of at least 58 individual cells measured.

whether different extracellular diadenosine polyphosphates also exert similar $[Ca^{2+}]_i$ effects, we applied increasing concentrations of Ap_3A, Ap_4A, Ap_5A, and Ap_6A to HBE1 cells. As can be seen in Fig. 3, the rise in $[Ca^{2+}]_i$ was very similar

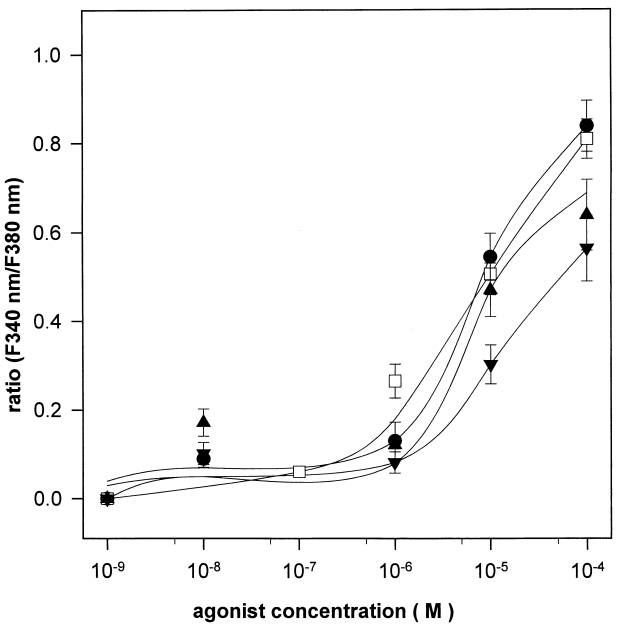

Fig. 3. Concentration–response curves for the $[Ca^{2+}]_i$ increase evoked by different diadenosine polyphosphates. Fura-2-loaded HBE1 cells were stimulated with increasing concentrations of Ap_3A (●), Ap_4A (□), Ap_5A (▲), or Ap_6A (▼) in Ca^{2+}-containing solution. Each point represents the mean ± SE of at least 43 single-cell measurements.

with each of these substances with a different length of the phosphate bridge. This result is a further indication that several diadenosine polyphosphates act on the same type of receptor.

In order to evaluate the source of Ca^{2+} for the agonist-induced $[Ca^{2+}]_i$ elevation, comparative experiments were performed in Ca^{2+}-containing and in nominally Ca^{2+}-free incubation media (Fig. 4A). As the $[Ca^{2+}]_i$ response to Ap_4A was about the same size in nominally Ca^{2+}-free medium, we may conclude that the rise in $[Ca^{2+}]_i$ evoked by UTP was only due to Ca^{2+} release from internal stores. In the case of Ap_4A, the $[Ca^{2+}]_i$ response was diminished ($P < 0.05$) in the absence of extracellular Ca^{2+}. Thus, a small part of the Ap_4A-induced $[Ca^{2+}]_i$ increase presumably results from Ca^{2+} flowing through the plasma membrane (Fig. 4A). The suggestion that intracellular Ca^{2+} stores were the main source in both cases was confirmed by the finding that after depletion of internal Ca^{2+} stores with 40 μM CPA, the $[Ca^{2+}]_i$ response to UTP (Fig. 4B) or Ap_4A (Fig. 4C) was completely abolished.

To investigate whether UTP and Ap_4A interact in HBE1 cells, we stimulated HBE1 cells twice, where either the homologous or the heterologous nucleotide was given for the second pulse (Fig. 5). When the homologous agonist was used for the second stimulation, the magnitude of the $[Ca^{2+}]_i$ signal was diminished to 65% for UTP and 47% for Ap_4A. Desensitization was also found when the heterologous agonist was used for the second stimulation. Thus, after Ap_4A stimulation, the $[Ca^{2+}]_i$ increase evoked by UTP was reduced to 43% of the maximal effect inducible by UTP while after UTP stimulation, the Ap_4A-evoked $[Ca^{2+}]_i$ increase was 39% compared to the maximal effect inducible by Ap_4A in the control experiment. Thus, when cells had been prestimulated with UTP and Ap_4A, we observed an almost identical $[Ca^{2+}]_i$ response to Ap_4A. However, for UTP the reduction in the $[Ca^{2+}]_i$ increase was significantly higher when cells had been prestimulated with Ap_4A (57%) than with UTP (35%).

4. Discussion

Despite intensive work in the past, evidence for the existence of a separate P2 receptor specific for Ap_4A is still very ambiguous. Ap_4A has been reported to be merely another potent agonist for the human $P2Y_2$ receptor when analyzed in a heterologous expression system [11]. However, previous results from us and others provide evidence for the existence of a distinct P2 receptor highly specific for Ap_4A and related diadenosine polyphosphates [9,10,24,25]. Both in rat and human lung, we recently demonstrated high-affinity P2Y receptor-like binding sites for $[^3H]Ap_4A$ with pharmacological characteristics different from $P2Y_2$ receptors [9] and coupling to G proteins [10]. However, nothing is yet known about the physiological function of extracellular Ap_4A in lung. Thus, the observations presented here show for the first time the functional effects of Ap_4A in lung epithelial cells.

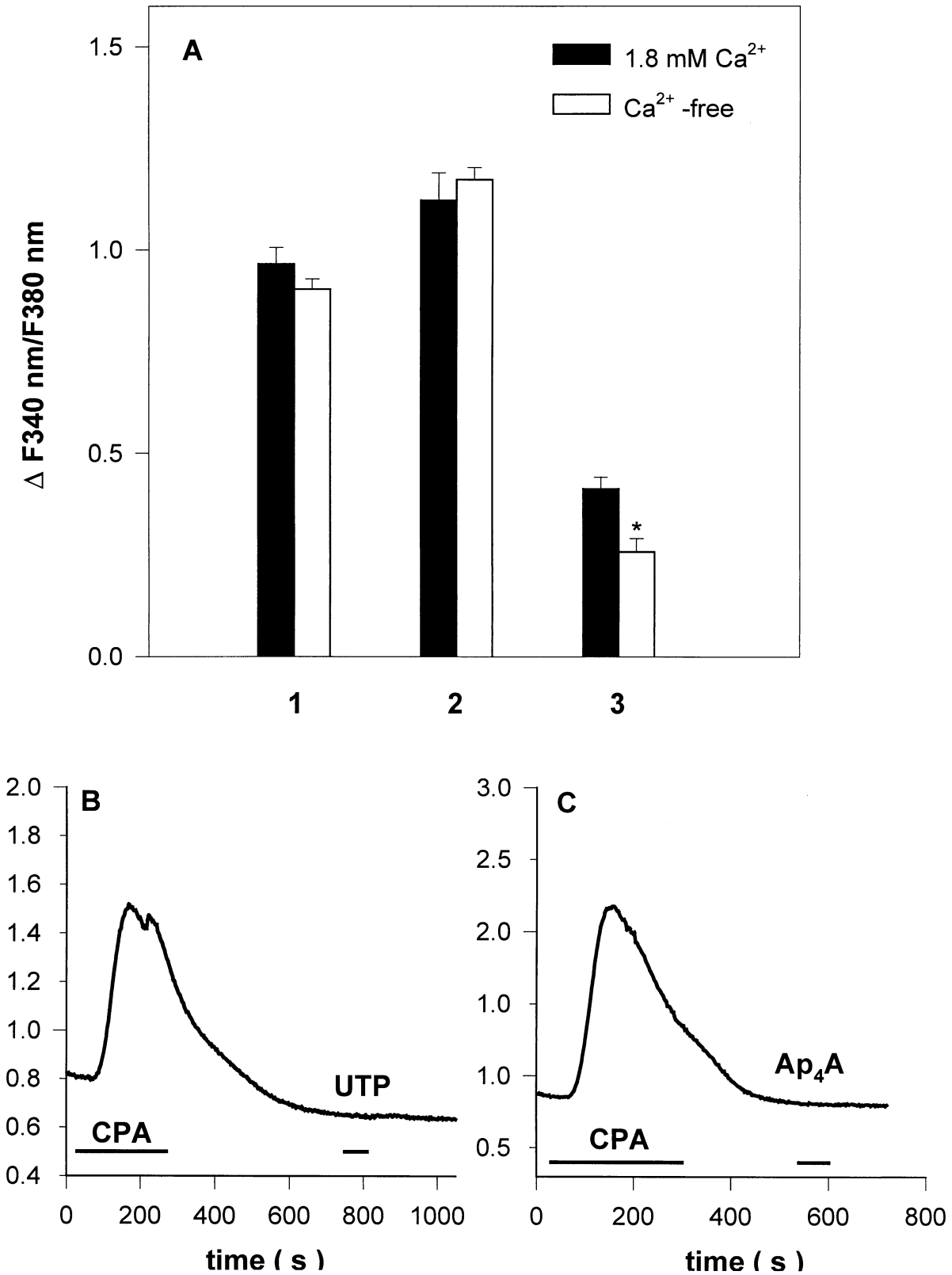

Fig. 4. Source of Ca^{2+} for agonist-evoked $[Ca^{2+}]_i$ increase. (A) Influence of extracellular Ca^{2+} on the rise in agonist-induced $[Ca^{2+}]_i$. Experiments were carried out in the presence of 1.8 mM $CaCl_2$ or in nominally Ca^{2+}-free incubation medium. $[Ca^{2+}]_i$ was recorded after addition of 10 μM UTP (1), 10 μM ATP (2), or 100 μM Ap_4A (3), as described under Materials and Methods. The results represent the means ± SE of at least 86 single-cell measurements (*$P < 0.05$ by unpaired t-test analysis). (B and C) Calcium responses to UTP (B) and Ap_4A (C) after depletion of internal Ca^{2+} stores. HBE1 cells were incubated with 40 μM CPA before stimulation with UTP (10 μM) or Ap_4A (100 μM) at the times indicated by bars.

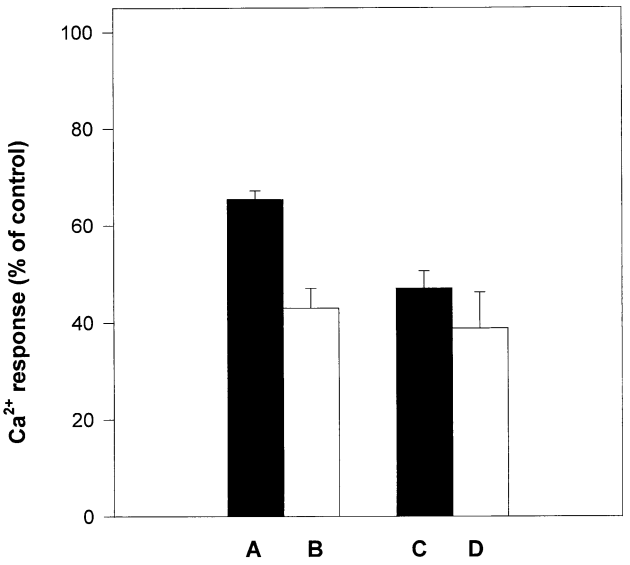

Fig. 5. Desensitization of agonist-stimulated $[Ca^{2+}]_i$ increase in HBE cells. HBE1 cells were stimulated for 3 min with 10 μM UTP (A and D) or with 100 μM Ap_4A (B and C). Cells were washed with incubation medium and 5 min after the first stimulation were stimulated for a second time (1 min) with the homologous (A and C) or the heterologous nucleotide (B and D). Bars represent the percentage of the maximal Ca^{2+} response ± SE of the first stimulation. For each experiment at least 57 single-cell measurements were made. The difference was significant ($P < 0.05$ by unpaired *t*-test analysis).

In the present study, nucleotide receptors in HBE1 cells were characterized by studying single-cell Ca^{2+} responses induced by various nucleotides. Extracellular ATP and UTP caused a strong increase in $[Ca^{2+}]_i$ in HBE1 cells. The almost identical EC_{50} values (1.5 and 1.8 μM for ATP and UTP, respectively) are an indication that both nucleotides activate the same receptor subtype, most probably the $P2Y_2$ receptor, found previously in HBE1 cells by other workers [16,18]. As $P2Y_4$ and $P2Y_6$ receptors are also present in HBE1 cells [18], UTP might also stimulate these receptor subtypes. Extracellular Ap_4A also evoked an increase in $[Ca^{2+}]_i$, albeit with a lesser affinity. The EC_{50} value of 17 μM for Ap_4A is about 10 times higher than the values for ATP or UTP, but is comparable to the EC_{50} value of 28 μM for the $[Ca^{2+}]_i$ increase evoked by Ap_4A in adrenal chromaffin cells [24]. Due to inherent difficulties, absolute values for the concentration of calcium derived from calibration of Fura-2 signals [26] are open to misinterpretation. However, the physiological consequences of the $[Ca^{2+}]_i$ increase evoked by Ap_4A stimulation can be reliably assessed by comparison with the half-maximal rise in $[Ca^{2+}]_i$ induced by ATP or UTP. Thus, we may conclude that the $[Ca^{2+}]_i$ increase evoked by Ap_4A is sufficiently high to activate calcium-dependent Cl^- channels in lung epithelia.

As other diadenosine polyphosphates (Ap_3A, Ap_5A, and Ap_6A) caused a similar increase in $[Ca^{2+}]_i$ to that induced by Ap_4A, it is very likely that all these polyphosphates activate the same receptor. This is in contrast to findings in adrenal chromaffin cells, where Ap_5A was less potent than Ap_4A in inducing $[Ca^{2+}]_i$ increase [24], but is in agreement with our previous findings with rat and human lung membranes, where $[^3H]Ap_4A$ binding sites showed equal affinities for several Ap_nA (n = 2 to 6) [9]. Thus, both biochemical and functional data underpin the assumption of a common receptor binding site for several diadenosine polyphosphates. Furthermore, we found that stimulation of $[^{35}S]GTP\gamma S$ binding to lung membranes caused by different Ap_nA (n = 2 to 6) was almost identical [9].

Experiments in nominally Ca^{2+}-free medium showed that the main source of Ca^{2+} for the increase in $[Ca^{2+}]_i$ in HBE1 cells induced by Ap_4A was intracellularly stored Ca^{2+} and that only a small part of the Ca^{2+} increase was due to transport through the plasma membrane. A similar observation was made for the Ap_4A-induced $[Ca^{2+}]_i$ increase in adrenal chromaffin cells [24]. In contrast, the rise in $[Ca^{2+}]_i$ evoked by UTP in HBE1 cells seemed to be caused only by the release from internal Ca^{2+} stores. As neither UTP nor Ap_4A was able to augment $[Ca^{2+}]_i$ after preincubation with CPA in nominally Ca^{2+}-free incubation medium, it may be concluded that CPA-sensitive Ca^{2+} stores are the main source for the $[Ca^{2+}]_i$ increase in either case. Similarly, for human erythroleukemia cells, it was reported that extracellular UTP and ATP mobilized Ca^{2+} from thapsigargin-sensitive intracellular stores via $P2Y_2$ receptors [27].

Repetitive stimulation of HBE1 cells with UTP or Ap_4A caused a substantial desensitization in either case, even when cells were first stimulated with the heterologous agonist. Slight differences in the degree of heterologous desensitization favor the idea of different receptor subtypes for ATP/UTP and Ap_4A in lung cells. However, it has yet to be investigated whether the degree of cross-desensitization between UTP and Ap_4A could also be due to activation of intracellular kinases such as protein kinase C rather than to the interaction at the same receptor [28].

Here, we showed the effect of diadenosine tetraphosphate on human bronchial epithelial cells. Although it cannot be excluded that Ap_4A is also a partial agonist of the $P2Y_2$ receptor in HBE1 cells, differences in the release of Ca^{2+} evoked either by ATP/UTP or Ap_4A support the assumption of the existence of an additional receptor for Ap_4A in these cells. In particular, the magnitude of the $[Ca^{2+}]_i$ increase was significantly different for Ap_4A and ATP or UTP. As the Ca^{2+} measurements were performed in a perfusion chamber with a permanent exchange of medium, it may be excluded that degradation products of Ap_4A such as ATP and ADP caused the Ca^{2+} response. Additionally, in human tracheal gland cells there is also evidence for the existence of P2 receptors for Ap_4A that are different from the $P2Y_2$ receptor [29].

Receptors activated by Ap_4A are involved in a signal transduction pathway coupled to G proteins and the mobilization of internal Ca^{2+}. Therefore, the possible relevance of diadenosine polynucleotides in lung physiology is of great interest. In human myocardium, the positive inotropic effect of Ap_4A was already proposed to be mediated

via G_q-coupled P2Y receptors [30]. Regardless of whether Ap_4A is an agonist of $P2Y_2$ receptors, of distinct P2Y receptors, or of both receptor subtypes, receptors for the relatively stable Ap_4A in lung epithelial cells might be promising regulatory elements for Ca^{2+}-mediated stimulation of Cl^- secretion when other Cl^- channels are deficient. This might be of therapeutic interest for the treatment of lung diseases such as CF, where the nucleotide UTP has already been applied successfully to activate Ca^{2+}-mediated Cl^- transport processes [5]. The evidence obtained thus far for the existence of distinct P2Y receptors for Ap_4A in lung should prompt further biochemical and molecular investigations to identify the targets on which diadenosine polyphosphates act in lung epithelial cells.

Acknowledgments

This work was supported by a grant from the Mukoviszidose e.V. Foundation. HBE1 cells were a generous gift from Dr. T. Meyer and Prof. L. Pott, Institut für Physiologie, Ruhr-Universität Bochum, Germany. We thank Mrs. M. Schubert for the HBE1 cell cultures, Dr. G. Zündorf for help with the HPLC analysis, and C. Vöhringer for assistance with the Ca^{2+} measurements.

References

[1] Hwang TH, Schwiebert EM, Guggino WB. Apical and basolateral ATP stimulates tracheal epithelial chloride secretion via multiple purinergic receptors. Am J Physiol 1996;270:C1611–23.

[2] Pilewski JM, Frizzell RA. How do cystic fibrosis transmembrane conductance regulator mutations produce lung disease? Curr Opin Pulm Med 1995;1:435–43.

[3] Puchelle E, de Bentzmann S, Zahm JM. Physical and functional properties of airway secretions in cystic fibrosis—therapeutic approaches. Respiration 1995;62(Suppl 1):2–12.

[4] Jiang C, Finkbeiner WE, Widdicombe JH, McCray PB, Miller SS. Altered fluid transport across airway epithelium in cystic fibrosis. Science 1993;262:424–7.

[5] Olivier KN, Bennett WD, Hohnecker KW, Zeman KL, Edwards LJ, Boucher RC, Knowles MR. Acute safety and effects on mucociliary clearance of aerosolized uridine 5′-triphosphate +/− amiloride in normal human adults. Am J Respir Crit Care Med 1996;154:217–23.

[6] Laubinger W, Reiser G. Differential characterization of binding sites for adenine and uridine nucleotides in membranes from rat lung as possible tools for studying P2 receptors in lung. Biochem Pharmacol 1998;55:687–95.

[7] Ogilvie A, Bläsius R, Schulze-Lohoff E, Sterzel RB. Adenine dinucleotides: a novel class of signalling molecules. J Auton Pharmacol 1996;16:325–8.

[8] Kisselev LL, Justesen J, Wolfson AD, Frolovas LY. Diadenosine oligophosphates (Ap_nA), a novel class of signalling molecules? FEBS Lett 1998;427:157–63.

[9] Laubinger W, Reiser G. Evidence for a G protein-coupled diadenosine-5′, 5′-5‴-P^1, P^4-tetraphosphate (Ap_4A) receptor binding site in lung membranes from rat. Eur J Pharmacol 1999;366:93–100.

[10] Laubinger W, Welte T, Streubel G, Schäfer HJ, Reiser G. In human and rat lung membranes [^{35}S]GTPγS binding is a tool for pharmacological characterization of G protein-coupled dinucleotide receptors. Life Sci 1999;65:PL183–90.

[11] Lazarowski ER, Watt WC, Stutts MJ, Boucher RC, Harden TK. Pharmacological selectivity of the cloned human P_{2U}-purinoceptor: potent activation by diadenosine tetraphosphate. Br J Pharmacol 1995;116:1619–27.

[12] Stutts MJ, Boucher RC, Lazarowski ER, Geary CA. Dinucleotides useful for the treatment of cystic fibrosis and for hydrating mucus secretions. United States Patent: Patent number US5635160, 1997.

[13] Schlüter H, Offers E, Brüggemann G, van der Giet M, Tepel M, Nordhoff E, Karas M, Spieker C, Witzel H, Zidek W. Diadenosine phosphates and the physiological control of blood pressure. Nature 1994;367:186–8.

[14] Hohage H, Reinhardt C, Borucki U, Enck G, Schlüter H, Schlatter E, Zidek W. Effects of diadenosine polyphosphates on renal function and blood pressure in anesthetized Wistar rats. J Am Soc Nephrol 1996;7:1216–22.

[15] Merten MD, Saleh A, Kammouni W, Marchand S, Figarella C. Characterization of two distinct P2Y receptors in human tracheal gland cells. Eur J Biochem 1998;251:19–24.

[16] Koslowsky T, Hug T, Ecke D, Klein P, Greger R, Grünert DC, Kunzelmann K. Ca^{2+}- and swelling-induced activation of ion conductances in bronchial epithelial cells. Pflügers Arch 1994;428:597–603.

[17] Devor DC, Pilewski JM. UTP inhibits Na^+ absorption in wild-type and ΔF508 CFTR-expressing human bronchial epithelia. Am J Physiol 1999;276:C827–37.

[18] Communi D, Paindavoine P, Place GA, Parmentier M, Boeynaems JM. Expression of P2Y receptors in cell lines derived from the human lung. Br J Pharmacol 1999;127:562–8.

[19] Cozens AL, Yezzi MJ, Kunzelmann K, Ohrui T, Chin L, Eng K, Finkbeiner WE, Widdicombe JH, Grünert DC. CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. Am J Respir Cell Mol Biol 1994;10:38–47.

[20] Ralevic V, Burnstock G. Receptors for purines and pyrimidines. Pharmacol Rev 1998;50:413–92.

[21] Yankaskas JR, Haizlip JE, Conrad M, Koval D, Lazarowski E, Paradiso AM, Rinehart CA, Sarkadi B, Schlegel R, Boucher RC. Papilloma virus immortalized tracheal epithelial cells retain a well-differentiated phenotype. Am J Physiol 1993;264:C1219–30.

[22] Ubl JJ, Vöhringer C, Reiser G, Co-existence of two types of $[Ca^{2+}]_i$-inducing protease-activated receptors (PAR-1 and PAR-2) in rat astrocytes and C6 glioma cells. Neuroscience 1998;86:597–609.

[23] Zimmermann H. Two novel families of ectonucleotidases: molecular structures, catalytic properties and a search for function. Trends Pharmacol Sci 1999;20:231–6.

[24] Castro E, Pintor J, Miras-Portugal MT. Ca^{2+}-stores mobilization by diadenosine tetraphosphate, Ap_4A, through a putative P_{2Y} purinoceptor in adrenal chromaffin cells. Br J Pharmacol 1992;106:833–7.

[25] Blouse GE, Liu G, Hilderman RH. Characterization of ATP and P_2 agonists binding to cardiac plasma membrane P^1,P^4-diadenosine 5′-tetraphosphate receptor. Biochim Biophys Acta 1998;1375:61–72.

[26] Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440–50.

[27] Baltensperger K, Porzig H. The P_{2U} purinoceptor obligatorily engages the heterotrimeric G protein G_{16} to mobilize intracellular Ca^{2+} in human erythroleukemia cells. J Biol Chem 1997;272:10151–9.

[28] Chen BC, Lin LL, Lin WW. Protein kinase C ε-dependent pathway of extracellular signal-regulated protein kinase activation by $P2Y_1$ and $P2Y_2$ purinoceptors that activate cytosolic phospholipase A_2 in endothelial cells. Eur J Pharmacol 1999;373:101–10.

[29] Saleh A, Picher M, Kammouni W, Figarella C, Merten MD. Characterization of a diadenosine tetraphosphate-receptor distinct from the ATP-purinoceptor in human tracheal gland cells. Eur J Pharmacol 1999;384:91–8.

[30] Knapp J, Boknik P, Linck B, Laer S, Müller FU, Neumann J, Vahlensieck U, Schlüter H, Zidek W, Schmitz W. Inositol-1,4,5-trisphosphate increase by diadenosine tetraphosphate in preparations from failing human myocardium. Naunyn Schmiedebergs Arch Pharmacol 1999;360:354–7.