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# Evidence for, and characterization of, a lipopolysaccharide-inducible adenosine A2 receptor in human tracheal gland serous cells

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**Abstract Human tracheal glands are considered as the principle secretory structures in the bronchotracheal tree. In earlier studies, we successfully performed primary cultures of human tracheal gland (HTG) serous cells and noted that these cells were responsive to many secretagogues including purinergic agonists but not to the inflammatory mediator adenosine. In this study, we demonstrate that adenosine was capable of inducing stimulation of protein secretion by HTG serous cells which had previously been cultured in pro-inflammatory conditions (induced by lipopolysaccharide (LPS)). This stimulation was inhibited by 8-phenyltheophylline but not by dipyridamole, which is indicative of a P1 purinoceptor. This inducible receptor is of the adenosine A2 subtype [rank potency order: 5′-(*N*-ethyl)-carboxamidoadenosine (NECA) > adenosine > $N^6$-(phenylisopropyl)-adenosine (PIA); and stimulation of adenylyl cyclase]. The adenosine-induced protein secretion was concentration-dependent, however, increased intracellular cyclic adenosine monophosphate (cAMP) was not dependent on the concentration of adenosine. The adenosine-induced secretion and the ATP-induced secretion were shown to be additive. This study concludes that there is evidence of a LPS-inducible adenosine A2 receptor in human tracheal gland serous cells.**

*Key words:* Airways; Human trachea; Submucosa; Secretion; Cell culture

## 1. Introduction

Human tracheal glands are considered as the major secretory cells in the bronchotracheal tree [1]. They are composed of mucous cells, producing mucins, and serous cells which are responsible for the secretion of antibacterial and antiproteolytic proteins [2]. We successfully performed primary cultures of human tracheal gland cells [3,4]. In culture, these cells were shown to be of the serous type [3] and to have retained most of their in vivo epithelial and secretory characteristics, such as secretion of the three specific serous secretory markers: bronchial inhibitor (BrI), lysozyme and lactoferrin and their ability to respond to adrenergic and cholinergic agonists. Consequently, human tracheal gland serous (HTGS) cells can be considered as a valuable tool in the study of the regulation of the human bronchial secretion at a cellular and a molecular level.

Adenosine is a potent secretagogue which has been shown to act via different mechanisms. It is recognized by purinergic receptors called P1 which are antagonized by methylxanthines [5]. A further classification of the P1 receptor into A1, A2 and A3 was achieved by the use of 5′ and $N^6$-substituted analogues of adenosine and also by positive or negative coupling of the receptor with adenylyl cyclase [6]. The A2 receptor is linked to the activation of adenylyl cyclase and is activated by adenosine analogues with the potency order: NECA ([*N*-ethyl]-carboxyamido-adenosine) > adenosine > PIA ($N^6$-[phenylisopropyl]-adenosine). The A1 receptor is characterized by an inversed potency order and its coupling to the inhibition of adenylyl cyclase. Recently, another adenosine receptor, the A3 adenosine receptor, was characterized and was also linked to the inhibition of adenylyl cyclase [7].

Adenosine metabolism in the lung has been examined mainly in the context of asthma [8], where it was shown to induce bronchoconstriction. In contrast, adenosine is virtually inactive in non-asthmatics. Moreover, it has been shown that the lung itself is able to release adenosine in sufficient concentrations to significantly induce anaphylactic broncho-constrictions [9]. There is little data however, concerning the possible action of adenosine in other pulmonary pathological situations or its effects on airway macromolecule secretion.

Cystic fibrosis (CF) is a genetic disease characterized by mucus hypersecretion, persistent bacterial infection and inflammation of the airways. The defect lies in mutations of a membrane protein called CFTR (Cystic Fibrosis Transmembrane conductance Regulator) [10], possessing a cAMP-dependent chloride channel activity [11]. A chloride channel distinct from CFTR, but which is not defective in cystic fibrosis can be activated by an incrase in intracellular calcium [12]. ATP is a potent secretagogue which binds to the nucleotide receptor P2 resulting in intracellular calcium mobilization through phospholipase C [12]. ATP 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 [13].

In a previous study, we showed that ATP is able to stimulate macromolecule secretion by HTGS cells through the mobilization of intracellular calcium [14]. Since ATP is given to patients with inflamed airways and since ATP is easily hydrolyzable into its metabolite adenosine, the aim of our present study was to determine whether or not adenosine may be active on cultured HTGS cells during a pro-inflammatory situation. This latter state was induced by the lipopolysaccharide (LPS) from *Pseudomonas aeruginosa* which is the major pathogen in CF. We illustrate hereby, that LPS induces the appearance of an adenosine A2 receptor generating a stimulation of secretion by HTGS cells.

## 2. Materials and methods

### 2.1. Chemicals and solutions

Adenosine, adenosine-5′-monophosphate (AMP), adenosine-5′-diphosphate (ADP), adenosine-5′-triphosphate (ATP), adenosine-5′-

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thiotriphosphate (ATPγS), dipyridamole, 8-phenyl-theophylline, 5'-(*N*-ethylcarboxamido)-adenosine (NECA), $N^6$-(2-phenylisopropyl)-adenosine (PIA), epinephrine, serotype 10 *Pseudomonas aeruginosa* lipopolysaccharide (LPS) and Dulbecco's modified Eagle's/Ham's F12 mixture (DMEM/F12) were obtained from Sigma (St. Louis, MO, USA). Ultroser G is from Biosepra (Villeneuve la Garenne, France). All other chemicals were of cell culture grade. Stock solutions of each secretagogue or agent were prepared as follows: adenosine, AMP, ADP, ATP and ATPγS were dissolved in the DMEM/F12 mixture at $10^{-2}$ M and then the pH was adjusted to 7.0; dipyridamole (0.1 M) was dissolved in 95% ethanol; 8-phenyl-theophylline (0.2 M) in 65% ethanol and 0.3 M NaOH; NECA (0.15 M) in dimethylsulfoxide/ethanol (50%:50%; v/v); PIA (0.15 M) in dimethylsulfoxide and LPS (at 10 mg/ml) in DMEM/F12. All stock solutions were aliquoted and stored at −20°C until required.

### 2.2. Cell culture

HTGS cells were obtained from the tracheal mucosa of young healthy adults who had died from cerebral trauma (three males aged 17, 27 and 39 years). No anatomo-pathological anomalies of the tracheal mucosae were observed. The cells were isolated by enzymatic digestion and grown in a DMEM/F12 mixture supplemented with 1% Ultroser G, 0.22 g/l sodium pyruvate and 6 g/l glucose as previously described [3]. 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 [4]. The cells were passaged using 0.025% trypsin (Gibco) and 0.02% EDTA and used at the 3rd passage, 8 days after confluency had been reached because of an extended differentiated state at that moment [3,4]. Type 1 collagen coated, Falcon disposable tissue culture flasks were used.

### 2.3. Bronchial inhibitor secretion

Confluent cultures of HTGS cells grown on 24-well plates were rinsed 4 times for 1 h with serum free culture medium and then exposed for 30 minutes to nucleosides or agents. 40 μl of the culture medium was harvested and bronchial inhibitor (BrI) was directly measured by an immunoenzymatic technique with a sensitivity of 2 ng/ml and a reproducibility ≥ 97% [15]. The polyclonal antibodies used were extremely specific and were able to recognize the molecule even complexed to mucins or to proteases. Thus, this method allowed accurate detection of the BrI in the culture medium. In order to determine the effects of LPS on the ability of the cells to respond to secretagogues, LPS was added to the cells 24 h before the pharmacological assays. We used the concentration of 50 μg/ml LPS because we have shown in a previous study that 50 μl/ml LPS gave a maximal increase in BrI secretion (unpublished data). The BrI secretory rate (BrI S.R.) was defined as the ratio of the BrI secreted in the assays to that secreted in control experiments. These were plate wells in which only vehicle solutions were added at the same times as the wells where the drugs were added. The vehicle additions were shown to be ineffective on BrI secretion by HTGS cells. In each experiment the mean BrI secretory rate was determined from quadruplicate assays.

### 2.4. Cyclic AMP measurements

Cyclic AMP was measured in ethanol extracts of cells grown on 24-well plates using a commercially available enzyme immunoassay kit (Amersham, Les Ulis, France) which allows the detection of cAMP in the range of 14–10520 pg/ml with high reproducibility (accuracy ≥ 95%). According to the manufacturer's specifications, this kit does not react with other adenine nucleosides (adenosine, AMP, ADP or ATP). The results of cAMP determinations were expressed as picomoles per million cells. Extractions were carried out either after 6 min contact with increasing concentrations of adenosine ($10^{-7}$ M to $10^{-4}$ M), or at increasing times after the addition of $10^{-4}$ M of adenosine which was shown to be the concentration giving the maximal secretory response.

### 2.5. Statistics

Each experiment was repeated at least three times on three different cultures of HTGS cells. All results were expressed as means ± standard deviation (S.D.). The significance between the effects of the concentrations of agents and the effects of the agonists was determined by analysis of variance (ANOVA). The difference between the agents and the concentrations of agents was isolated by the Scheffé's multiple comparison test. Statistics were carried out using a MacIntosh and the Software 'StatView 512+' (BrainPower Inc., Calabasas, California). The significance of potentiating interactions was determined by comparing the mean difference between the measured values for BrI secretion with two agents, and the calculated additive value of the two agents acting alone. Statistical significance: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

## 3. Results

### 3.1. Evidence of an LPS-inducible adenosine receptor

We examined the possible action of LPS incubation on the ability of the cells to respond to adenosine nucleosides. In the absence of LPS, HTGS cells responded to ATP and ADP by a stimulation of BrI secretion as previously described [14]. Fig. 1A shows that the stimulations of secretion were: + 70 ± 11%

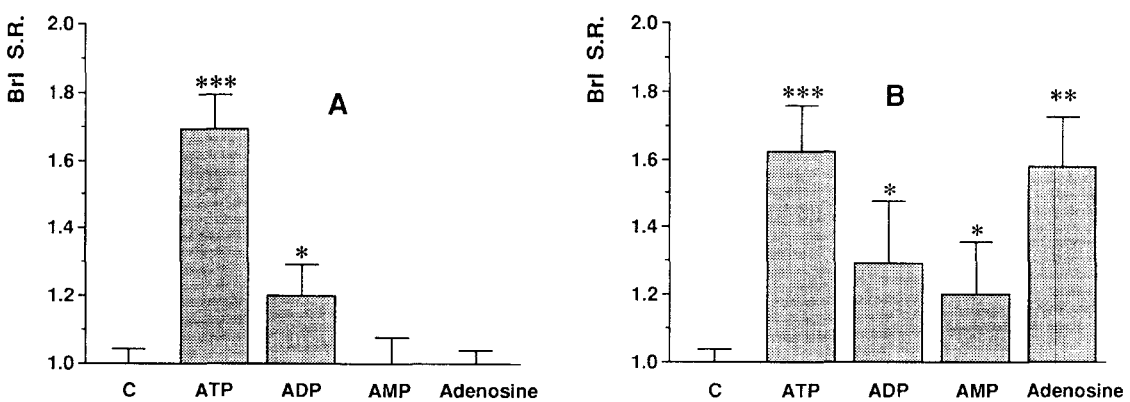


Fig. 1. Effects of adenosine nucleosides on human tracheal gland (HTGS) cell secretion. Third passage human tracheal gland cells were cultured as previously described in the text. Eight days after reaching confluency, cells were washed and exposed for 30 min to $10^{-4}$ M of each agonist. The BrI secretory rate (BrI S.R.: ratio of BrI secreted by the assay to BrI secreted by control) was calculated and compared to control ('C') which has a value = 1. Each point represents mean ± S.D. for quadruplicates from three different experiments ($n = 4$). 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 $^{***}P < 0.001$. (A) The magnitude of the BrI secretion by HTGS cells indicates the presence of the P2-purinoceptor and the absence of the P1-purinoceptor. (B) When cells were previously incubated during 24 h with LPS, they remained responsive to ATP and ADP but acquired the ability to respond to AMP and adenosine, which is indicative of the presence of a P1 receptor. Data from representative experiments.

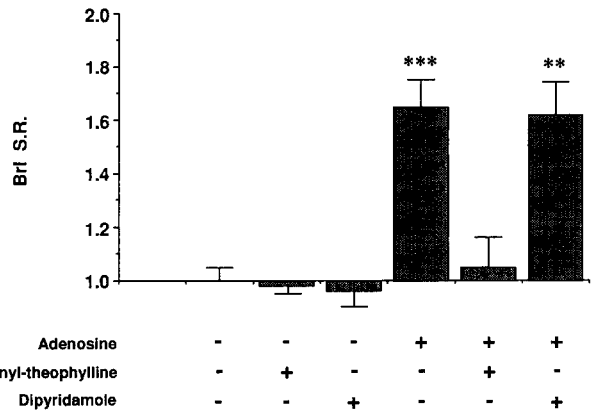


Fig. 2. Effects of 8-phenyltheophylline and dipyridamole on the adenosine induced secretion by HTGS cells. Third passage cells were grown and left for 7 days postconfluency. LPS was then added to a final concentration of 50 μg/ml and left with the cells for 24 hours. HTGS cells were then washed and exposed for 30 min with both agents in the presence or absence of $10^{-4}$ M of adenosine. All agents were added to the cells simultaneously. The BrI secretory rate (S.R.) and statistical significances as for Fig. 1. Values are given as mean ± S.D. of three expeirments ($n = 4$). Where no agent was added only the vehicle was added to the cells. Note that 8-phenyltheophylline (10 μM) antagonized the adenosine induced stimulation of BrI secretion whereas dipyridamole (10 μM) caused no effect.

($P < 0.001$) and + 21 ± 10% ($P < 0.05$) for both agents, respectively. No stimulation was observed with AMP and adenosine. Following incubation of the cells for a period of 24 h with 50 μg/ml of LPS, we observed that the stimulation of BrI secretion induced by ATP and ADP remained unchanged. In addition, AMP and adenosine elicited a significant stimulation of the secretion of BrI (Fig. 1B). The magnitudes of secretion were: + 18 ± 14% ($P < 0.05$) and + 58 ± 15% ($P < 0.01$) for AMP and adenosine, respectively. This increase in secretion induced by adenosine was antagonized by the P1-purinoceptor selective antagonist 8-phenyltheophylline (10 μM) (Fig. 2). This concentration of 8-phenyltheophylline was proved to be ineffective per se on BrI secretion when added in the absence of adenosine. Furthermore, the response of the cells to adenosine was unaffected by the adenosine uptake inhibitor dipyridamole [16,17].

### 3.2. Characterization of the LPS-inducible adenosine receptor

In order to determine which type of adenosine subclass is expressed after HTGS cells have been cultured with LPS, the effects of adenosine analogues on BrI secretion were examined on LPS-pretreated HTGS cells. The effect of adenosine was reproduced by its analogues with a rank order of potency: NECA > adenosine > PIA, suggesting the involvement of an adenosine A2 receptor (Fig. 3). A $10^{-4}$ M concentration of each analogue gave maximal stimulation of secretion with the following respective values: + 100 ± 15% ($P < 0.001$), + 63 ± 8% ($P < 0.01$) and + 51 ± 6% ($P < 0.02$). The effective concentrations producing 50% of the maximal responses ($EC_{50}$) were 1.8 ± 0.1 μM, 2.9 ± 0.3 μM and 7.2 ± 0.6 μM, respectively. None of these analogues were shown to be effective if cells had not initially been incubated for 24 h with LPS. 8-Phenyltheophylline completely reversed the increase in secretion induced by each of the adenosine analogues in the presence of LPS (data not shown).

Since cAMP is a second messenger known to be induced by a number of agonists in HTGS cells, we examined the ability of adenosine to induce changes in cAMP levels. Moreover, the activation of the adenosine A2 receptor has been described as being linked to a generation of intracellular cyclic AMP. As shown in Fig. 4A stimulation of LPS-pretreated HTGS cells with maximal doses of adenosine led to rapid increases in intracellular cAMP. We noted, however, a discrepancy between the concentration-dependent effects of adenosine on BrI secretion and cAMP generation (Fig. 4B). All the concentrations of adenosine tested gave maximal production of cAMP. Lower concentrations produced only minimal stimulation of secretion but produced submaximal increases in cAMP.

### 3.3. Additive actions of ATP and adenosine

It is known that secretagogues, the effects of which are mediated by cAMP, can act in addition to other secretagogues acting through calcium mobilization [18]. We therefore examined the effect of adenosine on BrI secretion when added with ATP using HTGS cells which had been pretreated for 24 h with LPS. The combination of adenosine and ATP showed additive effects on BrI secretion. The responses were similar to that predicted by summation of the effects of each agent added independently (Fig. 5). The responses to ATP, adenosine and ATP plus adenosine were: 67 ± 10% ($P < 0.01$), 56 ± 8% ($P < 0.01$) and 125 ± 3% ($P < 0.001$), respectively. ATPγS, a non-hydrolysable analogue of ATP produced similar responses to those elicited by ATP when added either alone (62 ± 8%, $P < 0.01$) or in a combination with adenosine (118 ± 6%, $P < 0.001$).

## 4. Discussion

The aim of the present study was to determine the possible effects of a pro-inflammatory environment on the ability of human tracheal gland serous cells to respond to adenosine, which is known to be an inflammatory mediator and also a

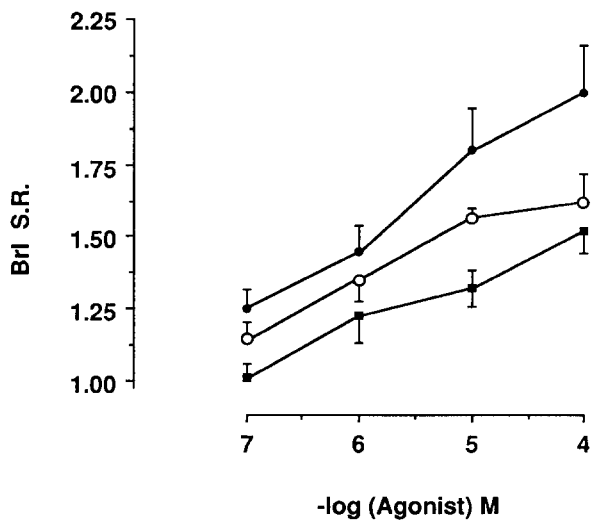


Fig. 3. Dose dependency of the effects of adenosine and its analogues on stimulation of BrI secretion by HTGS cells. Third passage HTGS cells were grown and left for 7 days postconfluency. LPS was then added to a final concentration of 50 μg/ml and left for 24 h with the cells. HTGS cells were then washed and exposed for 30 min to increasing concentrations of NECA (●), adenosine (○) or PIA (■). Each point represents means ± S.D. for four experiments ($n = 4$).

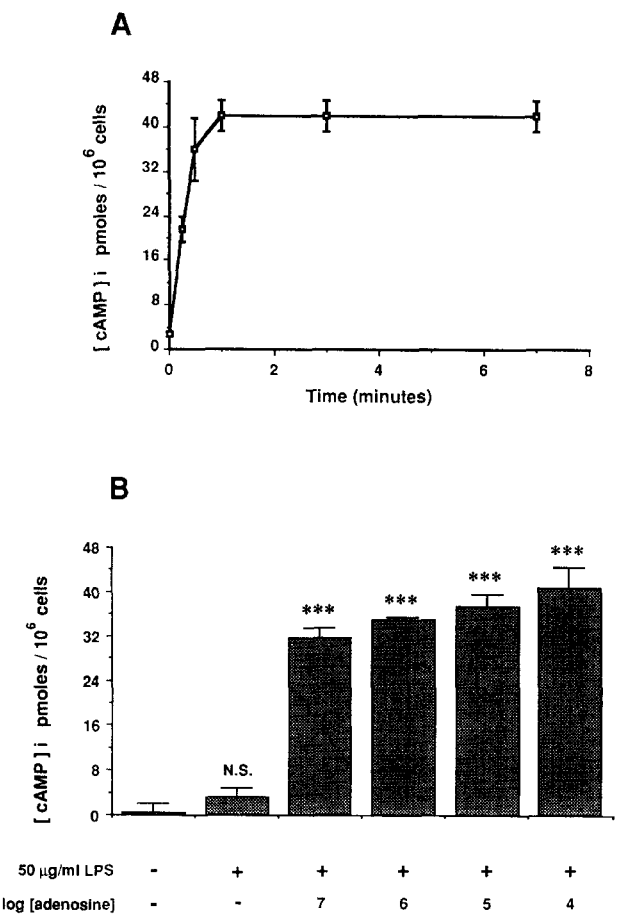


Fig. 4. Effects of adenosine on the generation of cyclic AMP by HTGS cells. Eight day confluent third passage HTGS cells were washed and exposed to adenosine. Cyclic AMP was then extracted and measured at increasing times or concentrations of adenosine as described in the text. Each point represents means ± S.D. for duplicates from three different experiments. Statistical significances as for Fig. 1, N.S. = not significant. (A) Adenosine (at $10^{-4}$ M) provokes a rapid generation of cyclic AMP within the HTGS cells. Basal levels of cyclic AMP in unstimulated cells were 4.0 ± 0.4 picomol/$10^6$ cells. (B) Dose dependency of the effects of adenosine on the cAMP accumulation in HTGS cells. Cyclic AMP measurements were carried out 6 min after adenosine addition to HTGS cells.

metabolite of the therapeutic agent ATP. We show here that HTGS cells which do not respond to adenosine are however responsive to this nucleoside when pro-inflammatory conditions are induced by LPS. In this case, adenosine led to a stimulation of secretion comparable to that induced on control cells by other well known secretagogues such as carbachol, isoproterenol or ATP [3,4,14]. It is unlikely that the observed effects were artefactual since 8-phenyltheophylline, an antagonist of the P1 adenosine receptor, totally inhibited the stimulation induced by adenosine. Furthermore, dipyridamole did not act on the adenosine-induced stimulation of secretion indicating that the observed effects were not as a result of a possible adenosine uptake by the cells.

There are some disparities in the literature regarding the presence of adenosine receptors on airways cells. Whilst examining different airway epithelial cells, some authors describe the presence of an active adenosine A2 receptor [19,20], but others detected little or no action of adenosine [12,21]. In contrast to the above data, we found the presence of this receptor in HTGS cells only when submitted to pro-inflammatory conditions. Our observations lead us to the conclusion that HTGS cells may have the ability to respond differently to physiological agents depending on the pathological context. It is worth noting that Ali et al. described an adenosine A1 receptor as being inducible in the asthmatic airways [22].

The action of adenosine on ionic transport and bronchoconstriction has previously been described, but it is our understanding that this work represents the first demonstration of an effect of adenosine on macromolecule secretion. The ability of HTGS cells to respond to adenosine by increasing protein secretion may provide a new insight into the role of adenosine which is found in high concentrations during inflammation, HTGS cells being responsive to this inflammatory mediator during an 'infection-like situation'. Indeed, the emergence of this ability of HTGS cells to be stimulated by adenosine in response to bacterial LPS may also underlie some physiological implications. It seems justifiable to hypothesize that cells specialized in the defense of the bronchotracheal tree may reorientate the aptitude of their antibacterial/antiproteolytic system to be stimulated when respiratory infection occurs. However, if adenosine also provokes mucus secretion by mucus secretory cells (gland mucous cells or goblet cells) when LPS is present, then this may not be so beneficial in bronchial hypersecretory diseases such as cystic fibrosis or acute exacerbations of asthma or COPD associated with respiratory infection. An analysis of adenosine-induced mucin secretion under a pro-inflammatory situation would therefore be of considerable interest.

Both the potency order of adenosine analogues inducing secretion and the increase in cAMP generation provoked by adenosine are characteristic of those generally observed for adenosine A2 receptor agonists. They also differed completely from the characteristics of A1 and A3 adenosine receptors [6,7]. Adenosine and its secretagogues provoked protein secretion which was similar to the induced chloride secretion by the same agents on other epithelial cells [20]. Adenosine-induced chloride

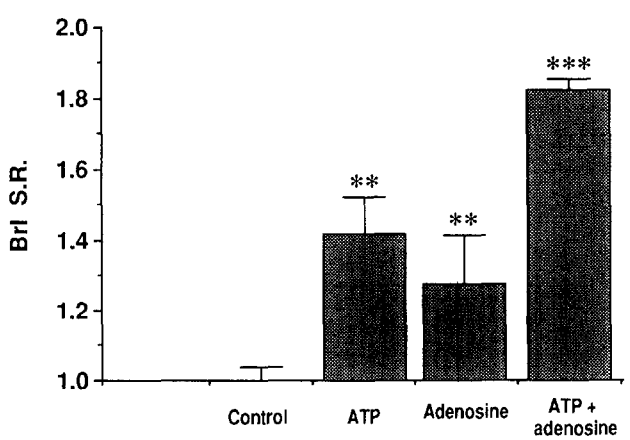


Fig. 5. Effects of ATP, adenosine or the combination of ATP plus adenosine on Brl secretion by HTGS cells. Third passage cells were grown and left for 7 days postconfluency. LPS was then added to a final concentration of 50 μg/ml and left for 24 h with the cells. HTGS cells were then washed and exposed for 30 min with ATP, adenosine or ATP + adenosine (all at $10^{-4}$ M). Brl secretory rate (S.R.) and the statistical significance as for Fig. 1. Means ± S.D. are shown ($n$ = 4). Responses to the combined agents were identical to those calculated by summation of the actions of each agent independently added.

secretion is mediated by an A2 adenosine receptor in several epithelia and in the T84 cell line [20].

The effects of the combination of adenosine and ATP were additive which leads us to the conclusion that both agents may act through different intracellular mechanisms. This may be a relevant observation since ATP is a component easily hydrolysable in adenosine. As a consequence, it is possible that the therapeutic agent ATP may also act through its metabolite adenosine resulting in unexpected effects when the airways are inflamed. It would be interesting, therefore, to investigate the actions of adenosine in airway cells provided by CF patients.

This study demonstrates that adenosine stimulated protein secretion by a mechanism linked to an accumulation of cyclic AMP. However, adenosine continued to stimulate cyclic AMP production in concentrations which were not sufficient to provoke secretion. The reasons for this discrepancy are not clear. It can be suggested that more than one second messenger is mobilized by the A2 receptor, and that the secretion is effective only when a optimal balance in the generation of these second messengers is achieved. It can also be hypothesized that adenosine may have different physiological effects depending upon its concentration. In addition to protein secretion, HTGS cells have the capacity to participate in the ionic balance of mucus since they notably express the CFTR-chloride channel [23,24]. Our results are in contrast to those described in the T84 cell line in which an adenosine analogue was shown to induce a maximal short circuit current at concentrations where no cyclic AMP was detectable [20]. In other studies, adenosine was shown to be unrelated to adenylyl cyclase involvement [25]. It has now been demonstrated that the adenosine receptor is a typical G protein-coupled receptor, which may be linked to second messengers other than adenylyl cyclase such as phospholipase A2 or C, or guanylyl cyclase [26]. Thus, it is difficult to relate adenylyl cyclase stimulation to its consequent biological response. However, in our case, both adenylyl cyclase stimulation and the potency order of analogues are clearly indicative of the A2 subtype nature of this adenosine receptor, and now a subsequent adenosine receptor classification into the A2A or the A2B subtype has to be performed preferably based on the order affinity of specific antagonists [5,27].

In conclusion, these results show that LPS from *Pseudomonas aeruginosa* induces the expression of a P1 purinoceptor of the A2 adenosine subtype which leads to the stimulation of protein secretion. However, the mechanism of induction of this purinoceptor is not yet understood.

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