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P_2 purinoceptor regulation of mucin release by airway goblet cells in primary culture

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1 The effects of adenine analogues on mucin release by airway goblet cells have been examined in a hamster primary tracheal epithelial cell culture.

2 Adenosine, a P_1 receptor agonist, had no effect on mucin release even at 2 mM, while ATP, a P_2 receptor agonist, stimulated mucin release in a dose-dependent fashion with an apparent EC_{50} of 20 μM. The relative potency order among adenine nucleotides was ATP > ADP > AMP = adenosine.

3 $ATP_{\gamma}S$, a non-hydrolyzable analogue of ATP, was equipotent with ATP in stimulating mucin release. The potency order among some ATP analogues was ATP > 2-methylthio ATP > α,β-methylene ATP > β,γ-methylene ATP. Reactive blue 2, a putative P_{2y}-purinoceptor antagonist, did not block the ATP-induced mucin release.

4 The present results indicate that mucin release by airway goblet cells is stimulated by extracellular ATP via P_2 receptor-mediated mechanism. We suggest that this mechanism may be important in the physiological regulation of airway goblet cell mucin release *in vivo*.

Keywords: P_2-purinoceptor; airway goblet cells; mucin release

Introduction

Mucus lining the airway luminal surface plays an important role in host defence against airborne particles and chemicals. The protective function of mucus is due mainly to the physicochemical properties of constituent mucous glycoproteins (mucins). Abnormalities in either the quality or the quantity of airway mucins not only affects removal of respired particles and bacteria, but may also impair host defences leading to further pathology.

In man, airway mucins are derived from two different cell types; globlet cells of the surface epithelium and mucous cells of the submucosal gland. Although much is known about the regulation of airway mucin secretion by submucosal glands, very little is known about goblet cell mucin regulation. Release of airway goblet cell mucins can be stimulated by two classes of agents: (a) inhaled chemical 'irritants' such as sulphur dioxide (Spicer *et al.*, 1974), ammonia vapour (Gallagher *et al.*, 1986), and tobacco smoke (Jones *et al.*, 1973) and (b) various kinds of proteases released during airway inflammation (Klinger *et al.*, 1984; Adler *et al.*, 1986; Niles *et al.*, 1986; Kim *et al.*, 1987). These agents are not present in normal airways.

Recently, we have characterized a primary cell culture system derived from hamster tracheal surface epithelium (HTSE) (Kim *et al.*, 1985; Wasano *et al.*, 1988; Kim *et al.*, 1989b). Confluent cultures of HTSE cells become highly enriched (>90%) with secretory cells, resembling goblet cells in their morphology, and synthesize and secrete mucin-like glycoproteins (MLGP). Judged by their carbohydrate structure, these MLGP are indistinguishable from *in vivo* mucins. Use of tracheal surface epithelial cell cultures as an *in vitro* model for studying the regulation of goblet cell mucin release has recently been reviewed (Kim & Brody, 1989). Using this primary cell culture system, our laboratory has tested a number of agents for their possible effects on mucin release from airway goblet cells. We have identified four different conditions under which mucin release is stimulated; increases or decreases of pH of the cell culture medium (Kim *et al.*, 1989a), neutrophil proteases (Kim *et al.*, 1987; 1989a), hypo-osmolarity (Kim *et al.*, 1989a), and retraction of collagen gels on which secretory cells are maintained (Kim & Brody, 1987). Both the pH change and the presence of proteases in the airway luminal fluids are conditions which can be caused by inhalation of 'irritant gases' and during airway inflammation, respectively, and have been shown to stimulate release of goblet cell mucins *in vivo*. However, no endogenous agents which can influence mucin release from primary TSE cell cultures have been identified yet.

In this paper, we show that mucin release from cultured airway goblet cells can be stimulated by ATP via a P_2 receptor-mediated mechanism. These observations provide for the first time a secretory control mechanism for airway goblet cell mucins that could be physiological in nature and probably involves a cell surface receptor.

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Methods

Hamster primary tracheal surface epithelial (HTSE) cell culture

Tracheae were obtained from male golden syrian hamsters eight to ten weeks of age (Harlan Sprague Dawley, Indianapolis, Indiana, U.S.A.). HTSE cells were harvested and cultured as previously described (Kim *et al.*, 1989a). Dissociated cells were plated on a thick collagen gel prepared inside 24 well tissue culture dishes (Falcon) as previously described (Kim *et al.*, 1989a).

Metabolic labelling of mucins and treatment of cultures

Mucins were metabolically radiolabelled by incubating confluent cultures (24 well plates) with 0.2 ml/well of the complete growth medium containing 10 μC ml^{-1} of [^{3}H]-glucosamine for 24 h, and at the end of the labelling period the spent media (the pretreatment or pulsed sample) was collected. After collecting the spent media, the cultures were washed twice with Dulbecco's phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS) in order to remove both remaining radioactive materials and serum components. The washed cultures were then chased for 30 min in PBS containing test materials in appropriate concentrations, and the chased media (the treatment sample) collected. In the case of reactive blue 2, the drug was added 10 min prior to the 20 min treatment with ATP. Both pretreatment and treatment samples were centrifuged to remove floating cells and stored at −60°C until assayed for their ^{3}H-mucin content.

Quantitation of ^{3}H-mucins

High molecular weight glycoconjugates excluded from Sepharose CL-4B (Pharmacia) and resistant to proteoglycan-digesting enzymes were defined as mucins (Kim *et al.*, 1985). Details of the mucin assay have previously been described (Kim *et al.*, 1987). 'Mucin release' from each dish during treatment was expressed as a ratio of the amount of ^{3}H-mucin released during the treatment period to the amount of ^{3}H-mucin released during the pretreatment period in order to compensate for variations in basal release rate among dishes.

Detection of cell membrane damage

The presence or absence of cytoplasmic leak due to cell membrane damage following treatments was identified by measuring lactic acid dehydrogenase (LDH) in the culture medium with a commercial LDH assay kit (Sigma) as previously described (Kim *et al.*, 1989a). Thus, the term 'cell membrane damage' throughout the text refers to cytoplasmic leak due to cell membrane damage. Briefly, the spent medium was collected, centrifuged, and a 50 μl aliquot of the supernatant immediately added to the reaction mixture. At the end of the reaction, OD_{500} of each sample was read in a spectrophotometer. For a positive control, LDH from *Leuconostoc mesenteroidas* (Sigma) was used; the presence of 0.1 u of the enzyme in 50 μl of the medium resulted in a decrease of OD_{500} by 0.2.

Materials

The sources of chemicals used were as follows: D-[6-^{3}H]-glucosamine HCl (39.2 Ci mmol^{-1}; New England Nuclear, Boston, MA, U.S.A.); adenosine, adenosine 5'-monophosphate (AMP), adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), α,β-methylene ATP, β,γ-methylene ATP, $ATP_\gamma S$, and reactive blue 2 (60% pure) from Sigma Chemical Co. (St. Louis, MO, U.S.A.); 2-methylthio ATP from Research Biochemicals Inc. (Natick, MA, U.S.A.).

Statistics

Means of 'mucin release' values of each group were converted to percentage of the control and expressed as mean values $\pm$s.e. The difference between groups was assessed by Student's *t* test for unpaired samples. $P > 0.05$ was considered as not significantly different.

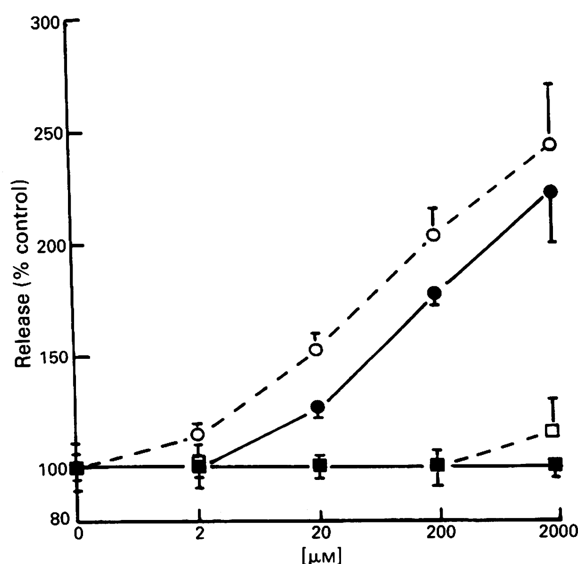

Figure 1 Effect of ATP, ADP, AMP and adenosine on ^{3}H-mucin release in hamster tracheal epithelial cell culture. Confluent cultures (16 mm) were pulsed with [^{3}H]-glucosamine for 24 h and chased for 30 min in the presence of the adenine analogues: ATP (○), ADP (●), AMP (□), and adenosine (■). Data represent means of four culture dishes with s.e. shown by vertical bars.

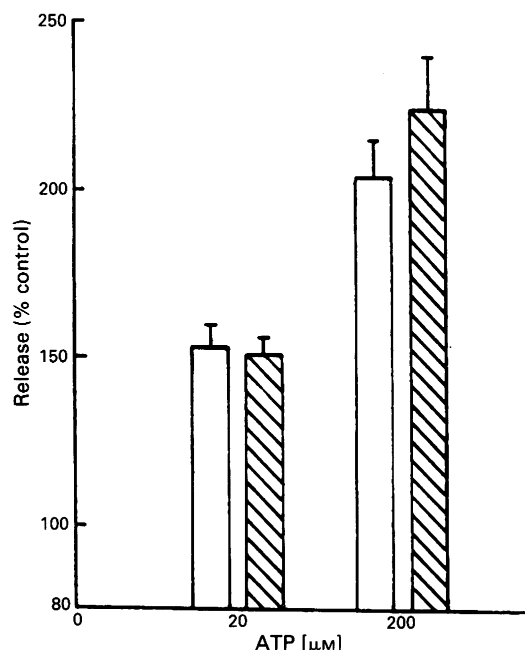

Figure 2 Effect of $ATP_\gamma S$ on ^{3}H-mucin release in hamster tracheal epithelial cell culture. Confluent cultures (16 mm) were pulsed with [^{3}H]-glucosamine for 24 h and chased for 30 min in the presence of ATP (open columns) or $ATP_\gamma S$ (hatched columns). Data represent means of four culture dishes; s.e. shown by vertical bars. There were no significant (P > 0.05) differences between ATP- and $ATP_\gamma S$-treated groups.

Results

Effect of adenine nucleotides on mucin release

ATP caused a concentration-dependent increase of mucin release with an apparent EC_{50} of 20 μM (Figure 1), whereas neither adenosine nor AMP influenced the rate of mucin release even at 2 mM concentration. Mucin release was stimulated also by ADP in a dose-dependent fashion; 28% by 20 μM and 78% by 200 μM. Thus, the mucin-releasing potency among these adenine nucleotides was in the order of ATP > ADP > AMP = adenosine (Figure 1). $ATP_\gamma S$, a non-hydrolyzable analogue of ATP, was equipotent with ATP (Figure 2). Other ATP analogues tested also showed mucin releasing effects and their potency order was; ATP > 2-methylthio ATP > α,β-methylene ATP > β,γ-methylene ATP (Figure 3). No detectable cytotoxicity was observed with 2 mM ATP based on LDH release (Table 1).

Effects of reactive blue 2 and Mg^{2+} on ATP-induced mucin release

As shown in Figure 4, reactive blue 2 itself caused significant mucin release (52% by 30 μM and 79% by 100 μM). Addition of 40 μM ATP to the cultures which had been pretreated with

Table 1 Effect of ATP on lactic acid dehydrogenase (LDH) release in hamster tracheal epithelial cell culture

Treatment	*OD* (*n* = 4)
PBS	0.537 ± 0.006
ATP (2 mM)	0.550 ± 0.007

Confluent cultures (16 mm wells) were treated for a 30 min period, and at the end of the treatment period, aliquots of spent media were collected and assayed for the LDH activity as described in Methods. The OD_{500} value for the control (PBS) group corresponds to about 0.6% of the total cellular LDH activity. Each value represents a mean ± s.e. of four wells. There were no significant differences ($P > 0.05$) among the above treatment groups.

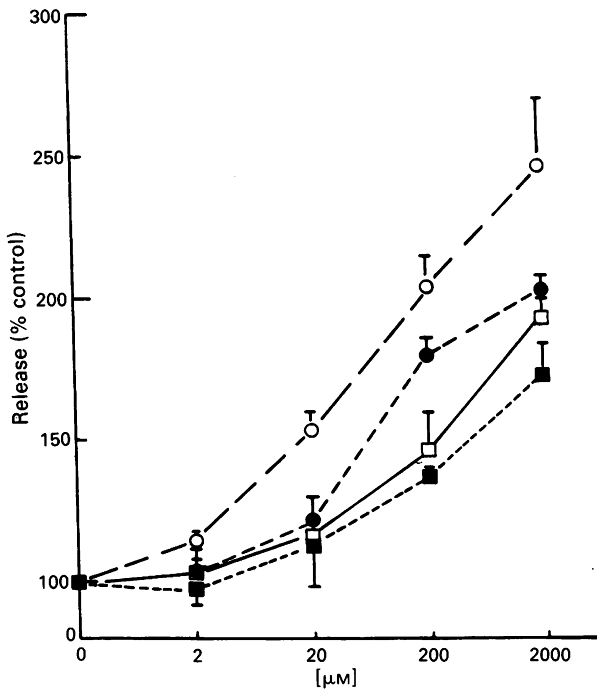

Figure 3 Effect of ATP, 2-methylthio ATP, α,β-methylene ATP, and β,γ-methylene ATP on ^{3}H-mucin release in hamster tracheal epithelial cell culture. Confluent cultures (16 mm) were pulsed with [^{3}H]-glucosamine for 24 h and chased for 30 min in the presence of the ATP analogues: ATP (○), 2-methylthio ATP (●), α,β-methylene ATP (□), and β,γ-methylene ATP (■). Data represent means of four culture dishes; vertical bars show s.e.mean.

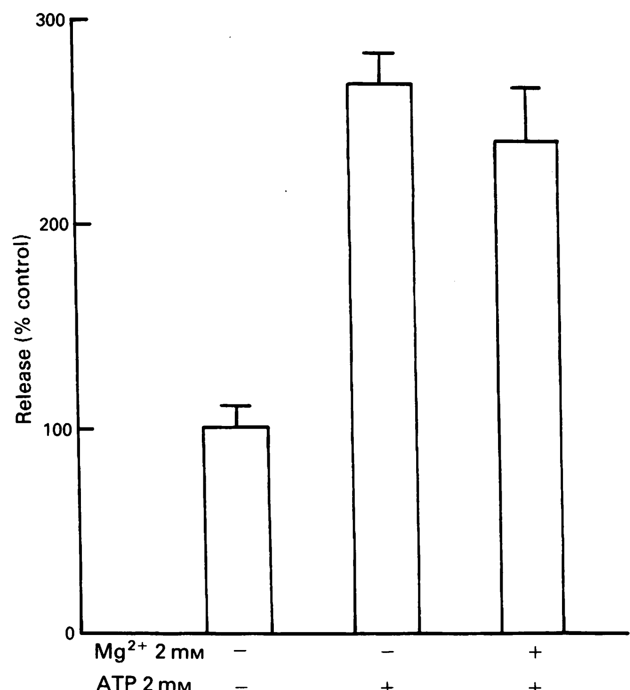

Figure 5 Effect of Mg^{2+} on ATP-induced ^{3}H-mucin release in hamster tracheal epithelial cells in culture. Confluent cultures (16 mm) were pulsed with [^{3}H]-glucosamine for 24 h and chased for 30 min in the presence of a combination of 2 mM ATP and 2 mM $MgCl_2$. ATP was prepared in Ca^{2+}, Mg^{2+} free PBS (see Methods) which was used as a chase medium. Data represent means of four culture dishes; s.e. shown by vertical bars. Mucin release by ATP was not significantly different ($P > 0.05$) in the presence or absence of Mg^{2+}.

30 μM or 100 μM reactive blue 2 resulted in greater increases in mucin release than either ATP or reactive blue 2 alone; increases in mucin release by 40 μM ATP in cultures pretreated with 0, 30 and 100 μM reactive blue 2 were 54%, 104%, and 121%, respectively. The presence or absence of 2 mM Mg^{2+} in the treatment solution did not affect the mucin releasing effect of 2 mM ATP (Figure 5).

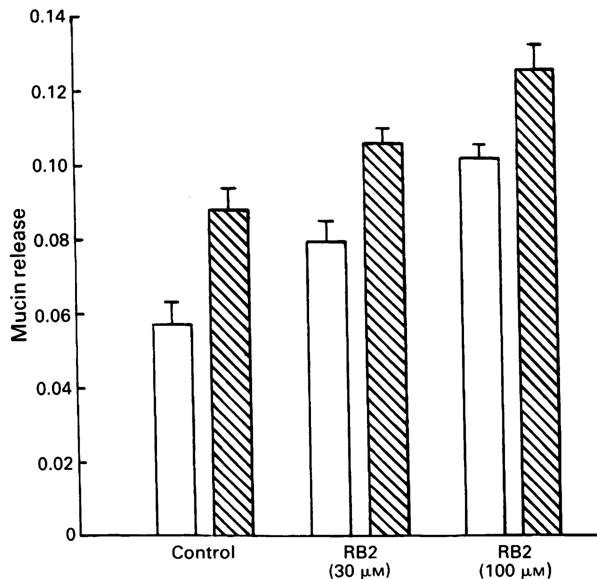

Figure 4 Effect of ATP on ^{3}H-mucin release in the presence of reactive blue 2 in hamster tracheal epithelial cell culture. Confluent cultures (16 mm) were pulsed with [^{3}H]-glucosamine for 24 h and chased for 30 min in the presence of a combination of reactive blue 2 and ATP. Reactive blue 2 was added 10 min prior to the addition of ATP. The ordinate scale (mucin release) represents the ratio of ^{3}H-mucin released during the chase period to ^{3}H-mucin released during the pulse period: without ATP (open columns) and with ATP (hatched columns). Data represent means of four culture dishes; s.e. shown by vertical bars. Addition of ATP caused significant ($P < 0.01$) increases, regardless of the presence of reactive blue 2.

Discussion

It is well known that extracellular purine nucleotides interact with their specific cell surface receptors or purinoceptors. Purinoceptors have been divided into two classes based on relative pharmacological potency order of agonists, specific antagonists, and their effects on adenosine 3' : 5'-cyclic monophosphate (cyclic AMP) levels (Burnstock, 1978): P_1 purinoceptors induce responses to adenosine > AMP > ADP > ATP, and are antagonized by the xanthines, and affect cyclic AMP levels. In contrast, P_2 purinoceptor-mediated events occur in response to ATP > ADP > AMP > adenosine, they are not antagonized by xanthines, and they do not influence cyclic AMP levels. As can be seen in Figure 1, the potency order of mucin release by these analogues indicates that mucin release by purine nucleotides involves a P_2 purinoceptor-mediated mechanism. In addition, the fact that both ATP and $ATP_\gamma S$ showed the same potency (Figure 2) seems to suggest that the ATP-induced mucin release may not involve the hydrolysis of ATP. Recently, P_2 purinoceptors were subdivided into at least two subtypes based purely on potency order (Burnstock & Kennedy, 1985): P_{2x} purinoceptors respond to α,β-methylene ATP = β,γ-methylene ATP > ATP = 2-methylthio ATP, while P_{2y} purinoceptors are stimulated by 2-methylthio ATP ≫ ATP > α,β-methylene ATP = β,γ-methylene ATP. Figure 3 shows that the mucin-releasing potency among these four analogues is in the order of ATP > 2-methylthio ATP > α,β-methylene ATP > β,γ-methylene ATP. Thus, interestingly, the P_2 purinoceptor responsible for mucin release does not appear to belong to

either P_{2x} or P_{2y}. It is important to note that the original classification of these P_2 purinoceptors was based on the potency order of these ATP analogues on smooth muscle contractility (Burnstock & Kennedy, 1985), but there are considerable variations among tissues and cell types in their relative potency order (Gordon, 1986).
1986).

Recently, the type II pneumocyte, another population of airway epithelial secretory cells, has been shown to release phospholipids in response to ATP presumably via a P_{2y} receptor-mediated mechanism, again purely based on the pharmacological potency order (Rice & Singleton, 1986). The ATP-induced phospholipid release by cultured type II pneumocytes was blocked by reactive blue 2 in a dose-dependent manner (Rice & Singleton, 1989). Reactive blue 2 was previously found to be a specific P_{2y} receptor blocker in smooth muscle preparations (Burnstock & Warland, 1987). In the present HTSE cell culture system, the concentrations of reactive blue 2 which caused phospholipid release in type II pneumocytes did not block the ATP-induced mucin release, but rather increased mucin release by itself (Figure 4). Therefore, it appears that the P_2 purinoceptor on HTSE cells is different from the one on type II pneumocytes. However, the difference may not be the receptor itself but the different micromilieu on the cell surface; we have recently shown that the cell surface of HTSE cells contains mucins tightly bound to the external plasma membrane (Kim *et al.*, 1987). It is possible that these cell surface mucins or the mucin layer serve as a barrier to these ligands, thus altering their effective concentrations at the receptor site.

Lastly, some evidence exists for the presence of another purinoceptor on the cell membrane, specific only for ATP^{-4} (a tetravalent form of ATP) (Tatham *et al.*, 1988). Stimulation of this receptor causes the permeabilization of the cell membrane (Cockcroft & Gomperts, 1979) and the subsequent Ca^{2+}-dependent release of histamine from mast cells (Bennett *et al.*, 1981). However, in HTSE cells, ATP-induced mucin release does not seem to involve such a mechanism judging from the following data: (1) ATP induced mucin release even in a Ca^{2+}-free PBS solution, and (2) addition of a high concentration of Mg^{2+} which should dramatically reduce the actual concentration of ATP^{-4} did not significantly ($P > 0.05$) influence the effect of ATP (Figure 5).

We conclude that mucin release from cultured airway goblet cells can be stimulated by ATP and other purine nucleotides via a P_2 receptor-mediated mechanism. This may be a physiological mechanism involved in the regulation of mucin release by airway goblet cells *in vivo*. Understanding of the pharmacology of the P_2-purinoceptor-induced mucin release may provide useful strategies for development of new drugs controlling airway mucin secretion. We are currently working on the biochemical mechanisms involved in the ATP-mediated mucin release.

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