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Adenosine induces cyclic-AMP formation and inhibits endothelin-1 production/secretion in guinea-pig tracheal epithelial cells through A_{2B} adenosine receptors

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1 The adenosine receptor subtype mediating adenosine 3′:5′-cyclic monophosphate (cyclic AMP) formation and the effect of its activation on endothelin-1 (ET-1) secretion were studied in primary cultures of tracheal epithelial cells.

2 Adenosine analogues showed the following rank order of potency (pD_2 value) and intrinsic activity on the generation of cyclic AMP by tracheal epithelial cells: 5′-N-ethylcarboxyamidoadenosine (NECA, $A_1/A_{2A}/A_{2B}$, pD_2: 5.44 ± 0.16) > adenosine (ADO, non selective, pD_2: 4.99 ± 0.09; $71 \pm 9\%$ of NECA response) $\geqslant$ 2-Cl-adenosine (2CADO, non selective, pD_2: 4.72 ± 0.14; $65 \pm 9\%$ of NECA response) > > > CGS21680 (A_{2A}; inactive at up to 100 μM).

3 Cyclic AMP formation stimulated by NECA in guinea-pig tracheal epithelial cells was inhibited by adenosine receptor antagonist with the following order of apparent affinity (pA_2 value): Xanthine amine congeners (XAC, A_{2A}/A_{2B}, 7.89 ± 0.22) > CGS15943 (A_{2A}/A_{2B}, 7.24 ± 0.26) > ZM241385 (A_{2A}, 6.69 ± 0.14) > DPCPX (A_1, 6.51 ± 0.14) > 3n-propylxanthine (weak A_{2B}, 4.30 ± 0.10). This rank order of potency is typical for A_{2B}-adenosine receptor.

4 Adenosine decreased basal and LPS-stimulated irET production in a concentration-dependent manner. Moreover, NECA but not CGS21680 inhibited LPS-induced irET production.

5 The inhibitory effect of NECA on LPS-induced irET production was reversed by XAC ($pA_2 = 8.84 \pm 0.12$) and DPCPX ($pA_2 = 8.10 \pm 0.22$).

6 These results suggested that adenosine increased cyclic AMP formation and inhibited irET production/secretion by guinea-pig tracheal epithelial cells through the activation of a functional adenosine receptor that is most likely the A_{2B} subtype. This adenosine receptor may be involved in the regulation of the level of ET-1 production/secretion by guinea-pig tracheal epithelial cells in physiological as well as in pathophysiological conditions.

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Keywords: Adenosine; adenosine receptors; adenosine 3′:5′-cyclic monophosphate (cyclic AMP); A_{2B}-adenosine receptor; endothelin-1; airway; epithelium; trachea

Abbreviations: ADA adenosine deaminase; Ado adenosine; 2CADO 2-Cl-adenosine; CCPA 2-chloro-N^6-cyclopentyladenosine; CGS15943 5-amino-9-chloro-2-(2-furyl)1,2,4-triazol[1,5-c]quinazoline; CGS21680 2-(*p*-(-carboxyethyl)-phenethylamino)-5′-N-ethylcarboxamidoadenosine; CPA N^6-cyclopenthyladenosine; cyclic AMP adenosine 3′:5′-cyclic monophosphate; DPCPX 8-cyclopentyl-1,3-diprylxanthine; ET-1 endothelin-1; irET immunoreactive endothelin; LPS lipopolysaccharide; NECA 5′-N-ethylcarboxamidoadenosine; R-PIA **R**-N^6-phenylisopropyladenosine; XAC xanthine amine congener; ZM241385 4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)phenol.

Introduction

The function of the airway epithelium is modulated by the action of multiple molecules acting through specific receptors present on epithelial cells. One of these factors is adenosine which acts through a family of receptors called P_1-purinoceptors. This family has been divided into four subclasses called A_1, A_{2A}, A_{2B} and A_3, all of which are seven-transmembrane domain proteins coupled to regulatory G-proteins that inhibit (A_1 and A_3 subtypes) or stimulate (A_{2A} and A_{2B} subtypes) adenosine 3′:5′-cyclic monophosphate (cyclic AMP) formation (Fredholm *et al*., 1994). The latter classes of receptors may be distinguished on the basis of the potency of agonists and antagonists, and by the lack of activity of the selective A_{2A}-adenosine receptor agonists and antagonists. The non-selective $A_1/A_{2A}/A_{2B}$-adenosine receptor agonist, N-ethylcarboxyamidoadenosine (NECA) and a selective A_{2A}-adenosine receptor agonist, CGS21680, have been widely used as tools to discriminate the effects mediated by the two G_s-coupled receptor (Feoktistov & Biaggioni, 1993; 1995; Alexander *et al*., 1996; Cooper *et al*., 1997). On human platelet A_{2A}-adenosine receptors, CGS21680 and NECA showed similar potency in activating cyclic AMP formation (Feoktistov & Biaggioni, 1993). In contrast, on other cell preparations which express A_{2B}-adenosine receptors, CGS21680 was inactive whereas NECA was shown to be a potent agonist (Feoktistov & Biaggioni, 1993; 1995; 1997; Alexander *et al*., 1996; Cooper *et al*., 1997). Selective A_{2A}-adenosine receptor antagonists have been used to characterize the effects mediated by A_{2A}-adenosine receptors, and to discriminate for the presence or absence of this receptor subtype. The lack of selective A_{2B}-adenosine receptor antagonist has made difficult the characterization of this subtype, but the use of a number of adenosine agonists and antagonists has allowed an acceptable characterization of this

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receptor population (Alexander *et al*., 1996, Cooper *et al*., 1997). Furthermore, the antiasthmatic drug, enprofylline (3-n-propylxanthine), a weak A_1/A_2 receptor antagonist, was also shown to block the A_{2B}-adenosine receptor (Casadó *et al*., 1992; Feoktistov & Biaggioni, 1993; 1997). Xanthine amine congener (XAC) and CGS15943 were also shown to be potent A_{2A}/A_{2B}-adenosine receptor antagonists (Fredholm *et al*., 1994; Peakman & Hill, 1994; Alexander *et al*., 1996; Cooper *et al*., 1997), whereas a selective A_{2A}-adenosine receptor antagonist, ZM241385 (Poucher *et al*., 1995), and a selective A_1-adenosine receptor antagonist, DPCPX (Poucher *et al*., 1995), appeared as poor antagonists at A_{2B}-receptors and, therefore, the affinity of these antagonists can be useful to discriminate between effects mediated by A_1, A_{2A} or A_{2B}-adenosine receptors.

The presence of adenosine receptors has been reported in various epithelia (Spinowitz & Zadunaisky, 1979; Dobbins *et al*., 1984; Pratt *et al*., 1986; Schwiebert *et al*., 1990; Barrett *et al*., 1990). In human airway epithelium, the presence of an A_2-adenosine receptor which increases cyclic AMP generation and modulates chloride secretion was reported (Lazarowski *et al*., 1992) However, the subtype mediating this effect was not well characterized. In selected cell populations, the activation of G_s-coupled receptors has been shown to modulate the production of several mediators including that of endothelin-1 (ET-1) (Sakamoto *et al*., 1992; Durieu-Trautman *et al*., 1993; Prins *et al*., 1994; Patel *et al*., 1997). A better understanding of the regulation of the production of this peptide in the pulmonary system is of interest since ET-1 has been implicated in various physiological and pathological functions (reviewed by Battistini *et al* 1993). These include (1) modulating chloride secretion by tracheal epithelial cells (Plews *et al*., 1991; Satoh *et al*., 1992), (2) promoting mitogenesis of airway smooth muscle cells (Noveral *et al*., 1992; Glassberg *et al*., 1994), (3) inducing activation of alveolar macrophages, that subsequently increase eicosanoid release (Millul *et al*., 1991), and (4) increasing superoxide production (Haller *et al*., 1991). Moreover, ET-1 is recognized as a potent constrictor of airway smooth muscle (Inui *et al*., 1994) that has been implicated in pulmonary hypertension (Uchida *et al*., 1988; Cernacek & Stewart, 1989; Horgan *et al*., 1991), asthma (Boichot *et al*., 1991) and cystic fibrosis (Plews *et al*., 1991).

In the present investigation, the effects of adenosine on basal and LPS-induced irET production/secretion by guinea-pig tracheal epithelial cells were studied. The adenosine receptors mediating cyclic AMP formation and the inhibition of irET production/secretion by these cells were also characterized.

Methods

Animals

Male Dunkin Hartley guinea-pigs (300–350 g) were obtained from Charles River Laboratory (St-Constant, Québec, Canada). The animals were killed by cervical dislocation according to the guidelines of the Canadian Council on Animal Care. The trachea was harvested under sterile conditions and dissected into Krebs-Heinseleit physiological solution.

Isolation and cell culture

Guinea-pig tracheal epithelial cells were obtained following a 1 h incubation of the trachea at 37°C with a solution of 0.15% protease type XXIV in Krebs-Heinseleit buffer, according to a previously described procedure (White *et al*., 1993). The cells were then mechanically removed from the mucosal surface of the trachea by gentle scraping with a policeman. They were centrifuged and washed twice with 5 ml of culture medium, DMEM-F12, containing 10% foetal bovine serum (FBS), penicillin (100 u·ml^{-1}), streptomycin (0,08%) and fungizone (1%). The cells were resuspended in 10 ml of medium, counted and seeded at a concentration of $4-5\times10^5$ cells·ml^{-1}·well^{-1} in a 24 wells culture plate or $1.2-1.5\times10^6$ cells··ml^{-1}··well^{-1} in 6 wells culture plate. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. The medium was renewed after 48 h with the same medium and changed every 24 h with a serum free medium until the cells reached confluency after 5 days. Confluent cells were used in all experiments.

irET production/secretion

Before the experiments, the DMEM-F12 medium was aspirated from the 24-well plates and 1 ml of fresh medium was added. The cells were incubated at 37°C with the selected pharmacological agents in a humidified atmosphere of 5% CO_2 for 24 h. At the end of the incubation period, the culture media were collected and stored at −20°C until measurement of immunoreactive-ETs (irETs). Triton X-100 (0.1%; 0.5 ml) was added to each well and, following an overnight incubation at 4°C, total proteins were assayed in the Triton X-100 (0.1%) using the Bio-Rad protein assay.

Measurement of immunoreactive-ETs by radioimmunoassay

The concentrations of ir-ETs were measured by radioimmunoassay (RIA) using a commercially available kit as previously described (Laporte *et al*., 1996). Briefly, fixed amounts of samples or standards, antiserum, and tracer (^{125}I-ET-3) were mixed together. The assay is based on the competition between unlabelled ETs (sample) and a fixed quantity of ^{125}I-ET-3 for a limited number of binding sites on an ET-specific antibody. The amount of radioactive ligand bound by the antibody is inversely proportional to the concentration of added non-radioactive ligand. The antibody-bound fraction was separated with magnetic beads. The detection limit of the assay was 0.5 fmol··tube^{-1}. The ET-1 antiserum cross-reacts with ET-1 (100%), ET-2 (144%), ET-3 (52%), and big ET-1 (0.04%). The amounts of each aliquot was expressed in picograms per milligram of total proteins.

Cyclic AMP formation

Intracellular cyclic AMP levels were determined by measuring the conversion of [^{3}H]-ATP into [^{3}H]-cyclic AMP as described by Weiss *et al*., (1985). Briefly, after 5 days of culture in 6 well plates, cells were washed and incubated for 2 h at 37°C in DMEM-F12 medium containing 2 μCi [^{3}H]-Adenine·ml-1. The cells were washed twice with Hanks Buffered salt solution (HBSS) containing 1M glucose and pre-incubated in the presence or absence of selected antagonists and inhibitors for 15 min in a solution of HBSS-Bovine serum albumin (BSA) containing 10 μM Rolipram, a selective phosphodiesterase IV inhibitor. Adenosine and congeners were then added to the incubation medium for 5 min at 37°C. The reaction was ended by the addition of 100 μl of ice-cold 50% Perchloric acid (PCA) (final concentration of 5%) and putting the 6 well plates on ice. Cells were scraped with a rubber policeman and 100 μl of ice-cold solution of ATP and cyclic AMP (5 mM of each) were added to the cellular extract. The samples were vortexed and centrifuged 15 min at 3000 r.p.m. at 4°C. The super-

natants were sequentially chromatographed on Dowex and alumina columns allowing the separation of [^{3}H]-ATP from [^{3}H]-cyclic AMP. Cyclic AMP formation was expressed as: precent of conversion of [^{3}H]-ATP to [^{3}H]-cyclic AMP and calculated using the following formula ([^{3}H]-cyclic AMP/([^{3}H]$-$ATP$+$[^{3}H]-cyclic AMP))$\times$100.

Protein assay

Guinea-pig tracheal epithelial cells in each well were disrupted overnight with 0.1% Triton detergent. A 10 μl aliquot was mixed with 200 μl of the Bio-Rad protein assay reagent and incubated for 15 min at room temperature to evaluate the concentration of cell proteins. The concentration of proteins was determined by measuring the absorbance at 590 nm, using a standard curve of bovine serum albumin (25$-$400 μg$\cdot\cdot$ml^{-1}).

Chemicals and drugs

The following chemical and drugs were used: Culture medium (DMEM-F12), fetal bovine serum (FBS) and antibiotics (Penicillin, Streptomycin and fungizone) (Gibco, New-York, U.S.A.); Protease Type XXIV, Adenosine, ATP, cyclic AMP, Bovine serum albumin, Adenosine deaminase, Imidazol and Alumina (Sigma Chemicals Co., St-Louis, MO, U.S.A.); The Bio-Rad protein assay kit, Dowex 1$\times$8 (100$-$200 mesh) (Bio-Rad, Mississauga, ON, Canada); [^{3}H]-Adenine, ETs Radioimmunoassay kits (Amersham, Oakville, ON, Canada); Rolipram, 2-Cl-adenosine (2CADO), Xanthine amine congener (XAC), CPA (N^6-cyclopenthyladenosine), CGS15943 (5-amino-9-chloro-2-(2-furyl)1,2,4-triazol[1,5-c]quinazoline), NECA (5′-N-ethylcarboxamidoadenosine), DPCPX (8-cyclopentyl-1,3-diprylxanthine), and enprofylline (3-n-propylxanthine) (RBI, Natik, CA, U.S.A.). CGS21680 (2-(*p*-(-carboxyethyl)-phenethylamino)-5′-N-ethylcarboxamidoadenosine) (generously given by Dr Arco Y. Jeng from Novartis, U.S.A.); ZM241385 (4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)phenol) (generously given by Dr Simon M. Poucher from Zeneca Pharmaceuticals., U.K.).

Adenosine receptor antagonists were dissolved to 10 mM in DMSO. NECA and CGS21680 were also dissolved in DMSO to 50 and 100 mM respectively. Adenosine and 2 CADO were dissolved in medium at 10 mM.

Data analysis and statistics

Sigmoidal curves were fitted to concentration-response data, following the substraction of the basal levels of cyclic AMP, by the use of the computer programs Microcal^{TM} (Microcal software, MA, U.S.A.) to generate estimates of EC_{50} and E_{max} values: Response$=(E_{max}\ X^n)/(EC_{50}+X^n)$, where X is the agonist concentration, E_{max} is the maximal response, n is the Hill coefficient and EC_{50} is the concentration of agonist producing half maximal stimulation. Antagonist IC_{50} values were calculated from the same equation to fit a curve for data points of increasing concentrations of antagonists in the presence of a fixed concentration of NECA (10 μM). Antagonist pA_2 values were calculated from the IC_{50} by the use of the null method described by Lazareno & Roberts (1987) and commonly used by other research groups (Alexander *et al*., 1996; Cooper *et al*., 1997): $pA_2=-\log[IC_{50}/(C/C'-1)]$, where C is the NECA concentration (i.e. 10 μM) and C′ is the NECA concentration evoking 50% of the response achieved at C, in the absence of antagonist.

The results are expressed as the mean$\pm$s.e.mean of n determinations made with different cell preparations. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by a Dunnet's multiple comparison test when comparing two or more treatments vs control or Bonferroni post test when comparing two or more treatments. P values less than 0.05 were considered significant.

Results

Effect of adenosine deaminase on the generation of cyclic AMP

To determine the effect of the endogenous adenosine on the cyclic AMP formation, the effect of adenosine deaminase (ADA; 1.5 ug$\cdot$ml^{-1}) on the generation of cyclic AMP was assayed. ADA decreased basal formation of cyclic AMP by 15% (0.19$\pm$0.01% conversion compared to 0.16$\pm$0.01% in the presence of ADA). Since a very small decrease was observed, the subsequent experiments were performed without ADA unless where indicated.

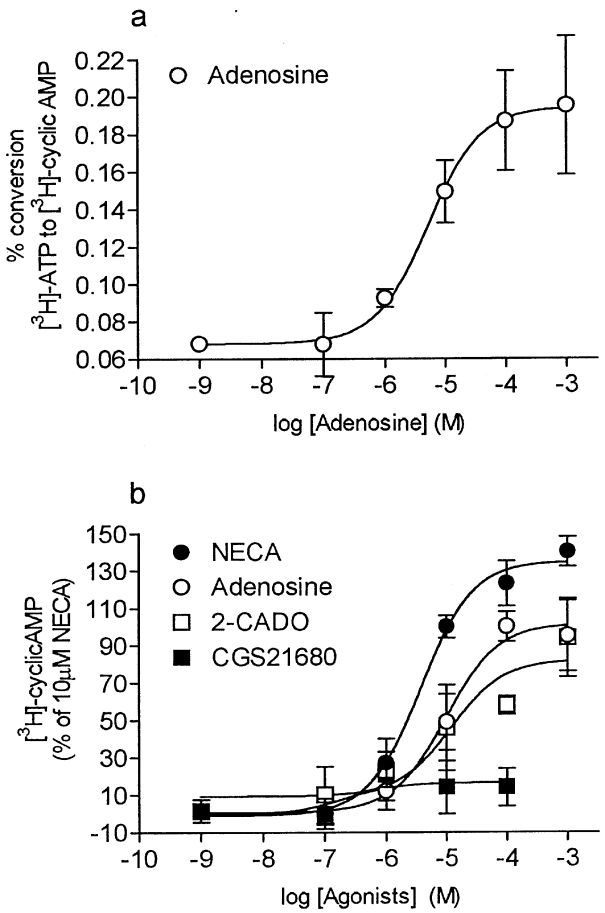

Figure 1 Cyclic AMP accumulation in guinea-pig tracheal epithelial cells in the presence of adenosine and adenosine analogues. Cultured cells were preincubated in the presence of Rolipram (10 μM) for 15 min and thereafter stimulated for 5 min with increasing concentrations of (a) adenosine. Each point represents the mean$\pm$s.e.mean of four determinations made with different cell preparations. (b) Cultured cells were preincubated in the presence of Rolipram (10 μM) for 15 min and thereafter stimulated for 5 min with increasing concentrations of NECA, 2CADO, adenosine and CGS21680. Values are the mean$\pm$s.e.mean of 4$-$8 determinations made with different cell preparations, expressed as a percentage of the response to 100 μM adenosine. See Methods for further details.

Effect of adenosine and adenosine analogues on the cyclic AMP formation

The production of cyclic AMP by tracheal epithelial cells increased in the presence of increasing concentrations of adenosine (Figure 1a). The basal generation of cyclic AMP in the presence of Rolipram (1×10^{-5} M) was $0.068\pm0.003\%$. Following stimulation of tracheal epithelial cells with adenosine at concentrations of 1×10^{-7} to 1×10^{-3} M, a concentration dependent increase was noted and the maximal effect was observed at the concentration of 1×10^{-3} M where the conversion of [^{3}H]-ATP to [^{3}H]-cyclic AMP reached $0.195\pm0.04\%$. 2CADO and NECA (1×10^{-7} to 1×10^{-3} M), two adenosine analogues, also produced concentration-dependent increases in the conversion of [^{3}H]-ATP to [^{3}H]-cyclic AMP whereas the selective A_{2A} receptor agonist, CGS 21680, was inactive at concentrations up to 100 μM (Figure 1b). The rank order of pD_2 values for these compounds on cyclic AMP formation was: NECA (5.44 ± 0.16) > adenosine (4.9 ± 0.09) $\geqslant$ 2CADO (4.72 ± 0.14) > > > CGS21680 (>100 μM) and the maximal effect of adenosine and 2CADO was $71\pm9\%$ and $65\pm9\%$ of the maximal effect produced by NECA (Figure 1b).

Effect of adenosine receptor antagonists on the production of cyclic AMP evoked by NECA

The cyclic AMP accumulation in guinea-pig tracheal epithelial cells by sub-maximal concentrations of NECA (10 μM) were inhibited in the presence of selective and non-selective antagonists of A_1, A_{2A} and A_{2B}-adenosine receptors (Figure 2). The rank order of potency based on apparent pA_2 values was XAC (7.89 ± 0.22) > CGS15943 (7.24 ± 0.26) > ZM241385 (6.69 ± 0.14) > DPCPX (6.51 ± 0.29) > 3-n-propylxanthine (4.30 ± 0.10) (Table 1). A strong correlation was found between the apparent pA_2 values obtained for these antagonists in guinea-pig tracheal epithelial cells and the pA_2 obtained in other published studies with cells expressing the A_{2B}-adenosine receptors (Table 1).

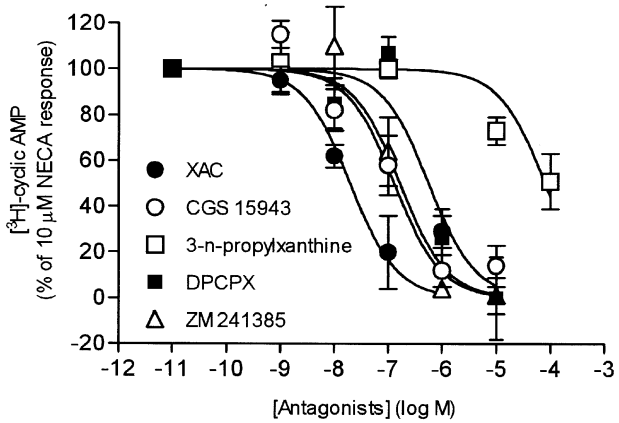

Figure 2 Effect of selective and non-selective adenosine receptor antagonists on the NECA evoked production of cyclic AMP. Cultured cells were preincubated 15 min in the presence of increasing concentrations of XAC, CGS15943, DPCPX, ZM241385 and 3-n-propylxanthine and thereafter stimulated with NECA (10 μM) for 5 min and the reaction was ended as described in Methods. Each point represents the mean ± s.e.mean of 4–8 determinations made in different cell preparations, expressed as a percentage of the response of 10 μM NECA.

Forskolin-stimulated cyclic AMP generation

Since A_1 adenosine receptors are commonly associated with inhibition of adenylyl cyclase, the possible functional linkage of this type of receptor with the regulatory G_i protein in guinea-pig tracheal epithelial cells was investigated using an adenosine analogue that selectively activates A_1 adenosine receptors (N^6-cyclopentyladenosine: CPA) and forskolin as a stimulus for cyclic AMP formation. When the cells are stimulated with 10 μM forskolin in the presence of ADA (1,5 ug·ml^{-1}), a 4 fold increase in cyclic AMP generation was observed (data not shown). In the presence of CPA (1×10^{-6} M), a further significant increase of cyclic AMP was observed in the cells ($124\pm6\%$ of forskolin responses $P<0.05$) but no effect was noted at concentration of 1×10^{-7}M (Figure 3).

Effect of adenosine and adenosine analogues on basal and LPS-induced irET production/secretion

Basal production/secretion of irET by guinea-pig tracheal epithelial cells reached 12000 pg·mg^{-1} of total proteins after a 24 h incubation period (Figure 4a). In the presence of adenosine (1×10^{-8} to 1×10^{-3} M), a concentration-dependent decrease in the production/secretion of irET was observed and the maximal effect was reached at a concentration of 1×10^{-3} M where the production/secretion was 7345 ± 366 pg·mg^{-1} of total proteins, which corresponded to a decrease of 38% of the basal level ($P<0.05$, ANOVA followed by Bonferroni post test). A strong correlation between the inhibitory effect of adenosine on irET production/secretion and the stimulatory effect of adenosine on cyclic AMP generation was noted with a slope of 0.96 and a r coefficient of 0.98 (data not shown). Basal production/secretion of irET by guinea-pig tracheal epithelial cells (12769 ± 1320 pg·mg^{-1} of total proteins) increased by 2.5 fold in the presence of LPS (10 μg·ml^{-1}) during a 24 h incubation period and reached 32973 ± 4248 pg·mg^{-1} of total proteins. In the presence of adenosine, a concentration-dependent inhibition of the LPS-stimulated irET production/secretion was observed and at the concentration of 100 μM, adenosine abolished the effect of LPS on the irET production/secretion (Figure 4b).

Table 1 Comparison of the apparent pA_2 values of selected antagonists in tracheal epithelial cells, in HEK 293 cells, in guinea-pig cerebral cortex, NIH3T3 cells and in transfected CHO.A_{2B4} cells

Antagonists	*Guinea-pig tracheal epithelial cells*	*Guinea-pig cerebral cortex**	*CHO.A_{2B} cells***	*HEK 293 cells†*	*NIH3T3 cells‡*
XAC	7.89 ± 0.22	7.46[2]	7.89	7.74	8.19
CGS15943	7.24 ± 0.26	7.33	7.75	7.79	–
DPCPX	6.51 ± 0.29	6.91[2]	7.16	7.01	7.76
3-n-propylxanthine	4.30 ± 0.10	4.59	4.63	5.13	–
ZM241385	6.69 ± 0.14	–	–	–	–

Data are means ± s.e.mean of 4–8 separate determinations. * = Alexander *et al.* (1994a); ** = Alexander *et al.* (1996); †Cooper *et al* (1997); ‡Brackett & Daly (1994).

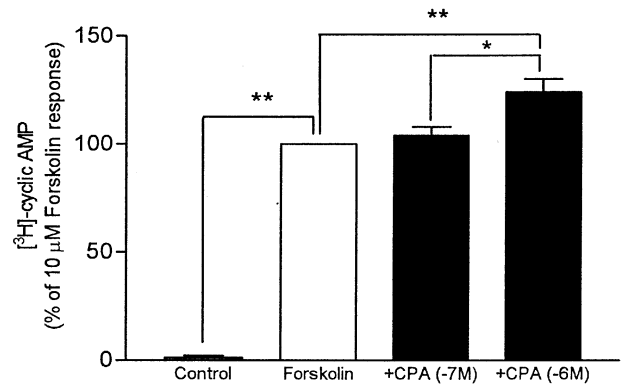

Figure 3 Effect of N^6-cyclopentyladenosine (CPA) on Forskolin-stimulated cyclic AMP accumulation in guinea-pig tracheal epithelial cells. Cells were preincubated 15 min in the presence of Rolipram (10 μM) and adenosine deaminase (1.5 u·ml^{-1}) and thereafter stimulated for 5 min in the absence (Control) or in the presence of 10 μM forskolin alone (Forskolin) or with forskolin and increasing concentrations of CPA (+CPA). Values are the mean±s.e.mean of four determinations made with different cell preparations, expressed as percentage of the response to 10 μM forskolin. *$P<0.05$ and **$P<0.01$.

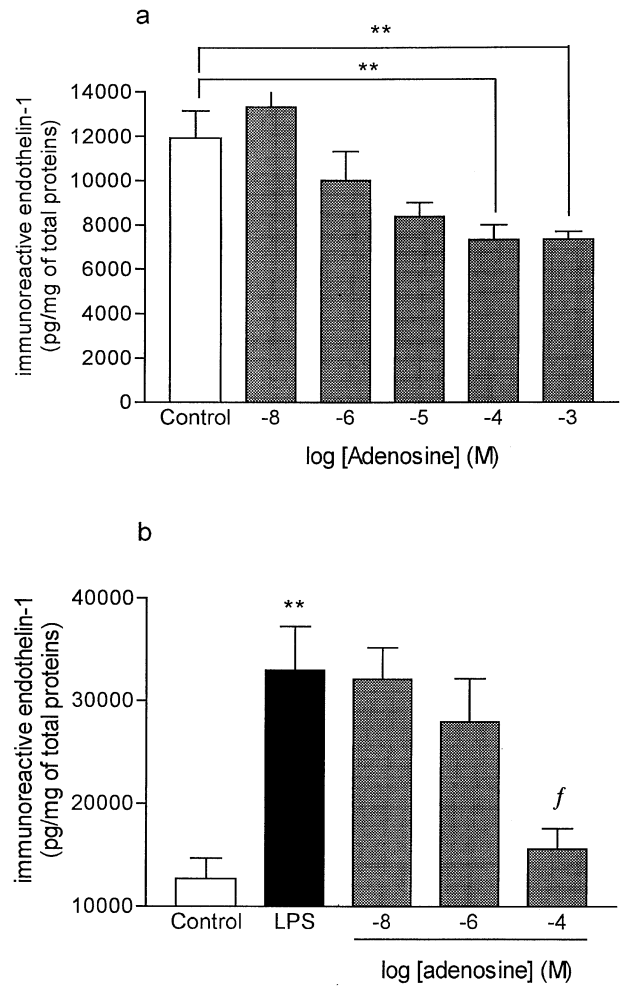

Figure 4 Effect of adenosine on basal (a) and lipopolysaccharide-induced (b) immunoreactive ET-1 production/secretion by tracheal epithelial cells. Cultured guinea-pig tracheal epithelial cells were pretreated or not (Control) with LPS (10 μg·ml^{-1}) during 30 min and therefore incubated 24 h in the presence or absence (control, LPS) of increasing concentrations of adenosine and the irET was measured as described in Methods. Values are the means±s.e.mean of four determinations made with different cell preparations. (*f* $P<0.05$ vs LPS, **$P<0.01$ vs Control).

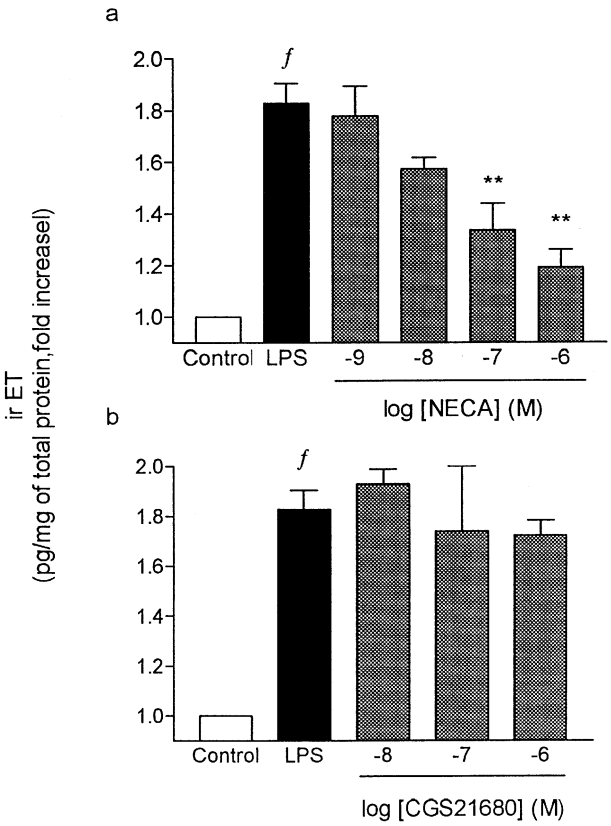

Figure 5 Effect of adenosine analogues on LPS-induced irET production/secretion by guinea-pig tracheal epithelial cells. Cultured cells were preincubated in the presence or absence of increasing concentrations of NECA (a) or CGS21680 (b) during 30 minutes and then incubated with or without LPS (10 ug·ml^{-1} LPS) for 24 h and the irET was measured as described in methods. Values are means±s.e.mean of 3–4 determinations made in different cell preparations. (**$P<0.01$ vs LPS and *f* $P<0.01$ vs Control).

NECA, but not CGS21680, inhibited LPS-stimulated irET production/secretion in a concentration-dependent manner with an IC_{50} value of 36±27 nM (Figure 5a,b). Its maximal effect was observed at a concentration of 1 μM and it abolished the LPS-induced irET production/secretion.

Effect of adenosine receptor antagonists on NECA-induced inhibition of irET production/secretion

The inhibition of LPS-induced irET production/secretion evoked by NECA was reversed by the presence of increasing concentrations of XAC and DPCPX (1×10^{-8} to 1×10^{-6} M). XAC is more potent than DPCPX with apparent pA_2 values of 8.36±0.29 and 7.58±0.39 (Figure 6a,b). CGS15943 (1×10^{-7} M) also reversed the inhibitory effect of NECA and seemed to be more potent than DPCPX (data not shown).

Discussion

In this investigation, cyclic AMP generation and inhibition of irET production/secretion by adenosine were studied in guinea-pig tracheal epithelial cells maintained in tissue culture. The results presented show that adenosine increases the cyclic AMP formation, most likely through the activation of epithelial A_{2B}-adenosine receptors. The first line of evidence was given by the observation that adenosine increases the generation of cyclic AMP in a concentration-dependent

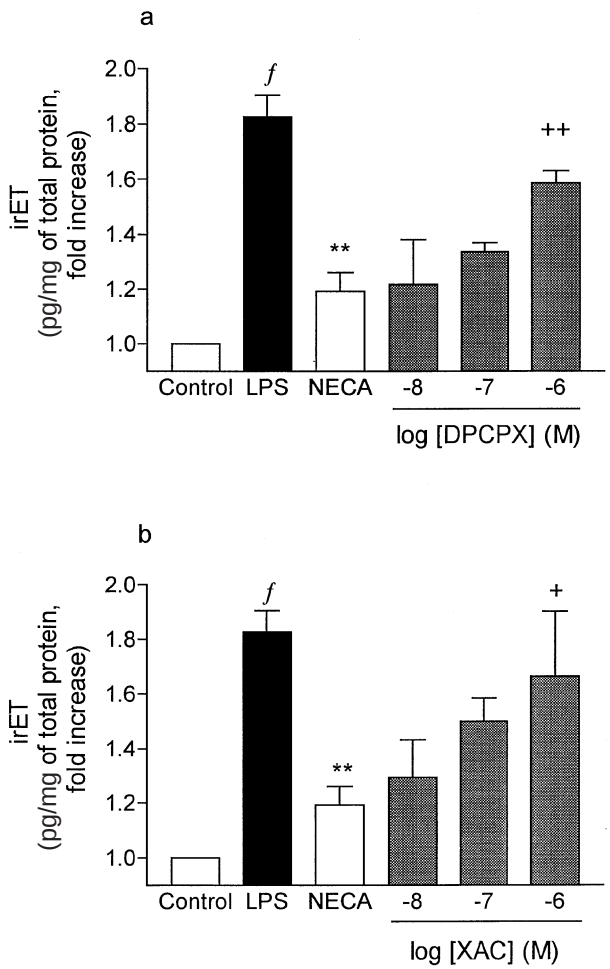

Figure 6 Effects of selective adenosine receptor antagonists on the inhibition of LPS-induced irET production/secretion evoked by NECA. Cultured guinea-pig tracheal epithelial cells were first incubated in the presence of (a) DPCPX (1×10^{-8} to 1×10^{-6} M) or (b) XAC (1×10^{-8} to 1×10^{-6} M) for 30 min. Thereafter, the cells were incubated in the presence or absence (Control, LPS) of NECA (1 μM) for another 15 min before being stimulated or not (Control) by LPS (LPS, NECA and -8, -7, -6) during 24 h. IrET was measured as described in Methods. Values are means$\pm$s.e.mean of 3–4 determinations made in different cell preparations. (f $P<0.01$ vs Control, $^{**}P<0.01$ vs LPS treated cells and $+P<0.05$ and $++P<0.01$ vs NECA).

manner and the rank order of potency of adenosine analogues in stimulating the generation of adenylyl cyclase was NECA > adenosine $\geqslant$ 2CADO > > > CGS21680 (ineffective at concentrations up to 100 μM). These findings are consistent with previous studies where the same rank order of potency was observed for the human A_{2B}-adenosine receptor that was cloned and transfected in CHO cells, the A_{2B}-receptor present in guinea-pig cerebral cortex or the one endogenously expressed in HEK293 cells (Alexander *et al*., 1996; Cooper *et al*., 1997). Furthermore, the lack of activity of the selective A_{2A} receptor agonist CGS21680 (Lupica *et al*., 1990; Feoktistov & Biaggioni, 1993) is an accepted indication of the absence of an A_{2A}-adenosine receptor which is the only other form of adenosine receptor coupled to a regulatory G_s-protein (Fredholm *et al*., 1994).

Characterization of receptors based on agonist affinity is far from ideal since the response elicited is not only dependent of the ligand binding to the receptors but also on multiple processes involved in the transduction pathway (Kenakin, 1987). The second line of evidence which suggested that adenosine acts through an A_{2B}-receptor is given by the rank order of potency of adenosine receptor antagonists on the cyclic AMP accumulation evoked by NECA. The rank order of potency found in our study i.e. XAC > CGS15943 > ZM241385 > DPCPX > > 3-n-propylxanthine is the same as those observed in previous studies characterizing the A_{2B}-adenosine receptor in other experimental models (Alexander *et al*., 1996; Cooper *et al*., 1997). The relatively high affinity of xanthine derivatives at antagonizing cyclic AMP accumulation evoked by NECA indicated that the receptors present in the tracheal epithelial cells were not related to A_3-adenosine receptors since these compounds have low affinities at A_3 receptors (Fredholm *et al*., 1994). The low affinity of the selective A_1-adenosine receptor antagonist, DPCPX (Bruns *et al*., 1987), also suggested that the receptor present in tracheal epithelial cells was not of the A_1 subtype. Furthermore, the low affinity of ZM241385, a selective A_{2A}-adenosine receptor antagonist (Poucher *et al*., 1995), also suggested that the receptors expressed in these cells were not A_{2A}-receptors. The selective but of low affinity A_{2B}-adenosine receptor antagonist, 3-n-propylxanthine (Feoktistov & Biaggioni, 1995), antagonized the NECA-evoked cyclic AMP formation with a relatively low affinity in our experimental model, which is also a characteristic feature of this receptor.

Our results showed that the non selective adenosine receptor agonist NECA had the highest potency for stimulating cyclic AMP generation by tracheal epithelial cells (pD_2 value 5.44). Similar values were reported for the A_{2B}-adenosine receptor in guinea-pig cerebral cortex and for the putative A_{2B}-adenosine receptor cloned from human brain (5.9; Alexander *et al*., 1996), on HEK293 (5.29; Cooper *et al*., 1997), in rat primary astrocytes (6.0; Peakman & Hill, 1994), in guinea-pig cerebellum (6.22; Brackett & Daly, 1994), in guinea-pig cerebral cortex (5.5; Alexander *et al*., 1994b), in NIH3T3 cells (6.34; Peakman & Hill, 1994) and finally on guinea-pig aorta (6.16; Alexander *et al*., 1994b). Comparable results were observed with the two other non selective adenosine receptor agonists, adenosine and 2CADO. The pD_2 values of these two agonists were shown to be 4.19 and 4.41 on HEK 293 endogenously expressing the A_{2B}-adenosine receptor (Cooper *et al*., 1997), 5.69 and 5.27 in CHO cells expressing the A_{2B}-adenosine receptor cloned from human brain (Alexander *et al*., 1996), 3.99 and 4.35 in guinea-pig cerebral cortex (Alexander *et al*., 1994a), 4.88 and 5.22 on guinea-pig cerebellum (Hernandez *et al*., 1993). In many experiments reported on A_{2B}-adenosine receptors, the selective A_{2A} agonist, CGS21680, was inactive (Casadó *et al*., 1992; Hernandez *et al*., 1993; Peakman & Hill, 1994; Feoktistov *et al*., 1994; Brackett & Daly, 1994; Alexander *et al*., 1994b, 1996; Strohme *et al*., 1995; Feoktistov & Biaggioni, 1995; Fiebich *et al*., 1996; Dubey *et al*., 1996; Cooper *et al*., 1997).

Linear regression of estimated apparent pA_2 values obtained from tracheal epithelial cells (Table 1) compared to the estimated apparent pA_2 of the guinea-pig cerebral cortex provided a slope of 1.14 and a r coefficient of 0.98 indicating the high degree of similarity between these two receptors. A good correlation was also observed with pA_2 apparent affinities obtained in our experiments and on the HEK293 cells endogenously expressing human A_{2B} adenosine receptor, and a slope of 1.23 and a r coefficient value of 0.98 were obtained.

The results presented herein also suggested that the A_1-adenosine receptor was not present on tracheal epithelial cells since no inhibitory effect on cyclic AMP was produced by CPA at concentrations that are reported to stimulate this receptor (Jacobson *et al*., 1992; Alexander *et al*., 1994a). Inhibition of forskolin-induced cyclic AMP generation was reversed by CPA at concentrations of 1×10^{-8} M, 1×10^{-7} and 1×10^{-6} M in guinea-pig cerebral cortex and rat primary astrocytes (Peak-

man & Hill, 1994; Alexander *et al*., 1994a). In contrast, our results suggested that the stimulatory effect of CPA at the concentration of 1×10^{-6} M was probably due to the activation of A_{2B}-adenosine receptor. Activation of cyclic AMP generation by A_1-adenosine receptor agonists was also reported in guinea-pig cerebral cortex where high concentrations of CPA, 2-chloro-N^6-cyclopentyladenosine (CCPA) and **R**-N^6-phenylisopropyladenosine (R-PIA) stimulated the cyclic AMP generation presumably through the A_{2B}-adenosine receptor (Alexander *et al*., 1994a). However, since potent and selective A_{2B}-adenosine receptor antagonists are not available, it was not possible to assess unequivocally that these effects are mediated by this receptor.

Our results also showed that adenosine inhibited in a concentration-dependent manner the basal and stimulated irET production/secretion. The non-selective adenosine receptor agonist, NECA, also inhibited the effect of LPS on irET production/secretion whereas CGS21680 failed. The lack of activity of CGS21680 is an accepted indication of the absence of an A_{2A}-adenosine receptor (Lupica *et al*., 1990; Feoktistov & Biaggioni, 1993). Furthermore, our results showed that A_1 adenosine receptor subtype is absent in our cell preparation. These pieces of evidence strongly suggest a role for the A_{2B} receptor subtype. The antagonist profile suggested similar conclusion. The non-selective A_{2A}/A_{2B} adenosine receptor antagonist, XAC, and the selective A_1-adenosine receptor antagonist, DPCPX, both reversed the inhibitory effect of NECA, but DPCPX is less potent than XAC. This is consistent with cyclic AMP assays presented in this article and other investigations of A_{2B} receptor subtypes (Brackett & Daly, 1994, Alexander *et al*., 1994b; 1996; Cooper *et al*., 1997; Peakman & Hill, 1994. In several studies, G_s-coupled receptor was also shown to decrease ET-1 production/secretion (Sakamoto *et al*., 1992; Prins *et al*., 1994; Razandi *et al*., 1996).

Finally, our findings showed that adenosine stimulated the generation of cyclic AMP through an endogenously expressed A_{2B}-adenosine receptor in guinea-pig tracheal epithelial cells. Activation of this receptor also lead to the inhibition of irET production/secretion. These pieces of evidence suggest a role for a cyclic AMP dependent pathway in the inhibition of irET production/secretion, by adenosine in guinea-pig tracheal epithelial cells. Vanio *et al*., 1996 demonstrated that adenosine increases the production/secretion of ET-1 through the activation of an A_1-adenosine receptor in FRLT-5 thyroid cells. This observation is consistent with our findings since increasing the level of cyclic AMP reduced the production/secretion of the peptide and the activation G_i-coupled receptors can be responsible for mediating the increase of production/secretion of this peptide. These authors also showed that the A_1-adenosine receptors also lead to an increase of intracellular calcium and the production/secretion of ET-1. The relationship between the generation of cyclic AMP and the inhibition of irET production/secretion were also reported in some other cell population such as rat mesengial cells and airway epithelial cells (Sakamoto *et al*., 1992; Yang *et al*., 1997). Interestingly, studies also suggested that G_s-coupled receptors mediated the decrease in ET-1 production/secretion in endothelial cells but through the activation of cyclic GMP and independently of the cyclic AMP formation (Prins *et al*., 1994; Razandi *et al*., 1996). In contrast, other reports suggested that the activation of G_s-coupled receptors, phosphodiesterase inhibitors and cyclic AMP analogues stimulated the production/secretion of ET-1 (Durieu-Trautman *et al*., 1993; Patel *et al*., 1997). In our model, PGE_2, acting through the EP_4 receptor subtype (a G_s coupled receptor), also inhibit basal production/secretion of irET without affecting cyclic GMP generation (unpublished results). Furthermore, both adenosine and PGE_2 mediated inhibition of basal irET production/secretion are reversed by the presence of 100 μM of Rp-cAMP-S, a competitive and selective protein kinase A (PKA) inhibitor (Schaap *et al*., 1993) which strongly suggests the involvement of PKA in adenosine or PGE_2 induced-inhibition of irET production/secretion.

In conclusion, we reported that A_{2B}-adenosine receptors are expressed on guinea-pig tracheal epithelial cells. The activation of this receptor leads to the increase in cyclic AMP generation and concomitantly inhibits the basal and LPS-stimulated release of ET-1 by these cells. This suggests that adenosine, through the activation of A_{2B} receptors, can play the role of an anti-inflammatory mediator since it reduces LPS-stimulated ET-1 release from intact epithelium.

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