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Adenosine receptor-mediated relaxation of guinea-pig precontracted, isolated trachea

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1 We have investigated the pharmacological profile of the adenosine receptor mediating relaxation of the carbachol pre-contracted guinea-pig trachea.

2 5'-N-Ethylcarboxamidoadenosine (NECA) and 2-chloroadenosine elicited concentration-dependent relaxations with pD_2 ($-\log_{10}$ half-maximal values) of 6.37 ± 0.04 and 5.25 ± 0.09, with maximal relaxations of 73 ± 7 and $208 \pm 38\%$, respectively. In the presence of 10 μM NECA, 2-chloroadenosine was able to relax the tissue further with a pD_2 value of 4.74 ± 0.11 and a maximal response of $252 \pm 68\%$.

3 CGS 21680, APEC and adenosine failed to elicit significant relaxations of precontracted tracheal rings at concentrations below 10 μM. At 10 μM, adenosine analogues elicited relaxations with the following order of magnitude (% relaxation): 2-chloroadenosine ($75 \pm 16\%$) = NECA ($69 \pm 16\%$) > APEC ($25 \pm 8\%$) > CGS 21680 ($11 \pm 2\%$) > adenosine ($6 \pm 4\%$).

4 NECA-induced relaxation of precontracted trachea was antagonized by adenosine receptor antagonists with the rank order of apparent affinity (K_i, nM): PD 115,199 (27 ± 8) = XAC (43 ± 11) > CP 66,713(285 ± 89) = DPCPX (316 ± 114).

5 We conclude that the adenosine analogue-induced relaxation of guinea-pig tracheal rings fails to fit into the current classification of A_2 adenosine receptors.

Keywords: guinea-pig tracheal smooth muscle; A_2 adenosine receptor

Introduction

Adenosine receptors may be divided into two major classes, based on the functional coupling to adenylyl cyclase activity. Thus, A_1 receptors are generally linked to inhibition of adenosine 3':5'-cyclic monophosphate (cyclic AMP) generation (Alexander *et al.*, 1994a; Fredholm *et al.*, 1994), while A_2 receptors stimulate the production of cyclic AMP (Alexander *et al.*, 1994b; Fredholm *et al.*, 1994). In recent years, however, the subclassification of adenosine receptors has become more complex, with the acceptance of A_3 receptors (Fredholm *et al.*, 1994; Linden, 1994) and subtypes of A_2 receptors (Fredholm *et al.*, 1994). Both A_{2a} and A_{2b} subtypes appear to be coupled to stimulation of adenylyl cyclase. Although selective agents for the A_{2b} adenosine receptor have not yet been described, these subtypes may be differentiated through the use of A_{2a}-selective agents (Fredholm *et al.*, 1994). Thus, the 2,5'-disubstituted adenosine analogues, CGS 21680 and APEC have been shown to stimulate A_{2a} adenosine receptors preferentially without stimulating A_{2b} receptors (Lupica *et al.*, 1990; Fredholm *et al.*, 1994). Similarly, the antagonists PD 115,199 and CP 66,713 appear to antagonise A_{2a} receptors without significant activity at A_{2b} receptors (Bruns *et al.*, 1987; Sarges *et al.*, 1990). Adenosine itself also appears to be more potent at the A_{2a} than at the A_{2b} adenosine receptor, leading to the former initially being termed the 'high-affinity' and the latter the 'low-affinity' A_2 adenosine receptor (Daly *et al.*, 1983).

In the periphery it has been suggested that A_2 receptors are present in the cardiovascular, immune and respiratory systems (Olsson & Pearson, 1990; Collis & Hourani, 1993). However, relatively few examinations have been made in these systems of the pharmacology with respect to the particular subtype of adenosine receptor present. Recently, we reported that the A_2 adenosine receptor mediating relaxation of the precontracted guinea-pig aorta was identical to the CNS A_{2b} adenosine receptor mediating stimulation of cyclic AMP generation if agonist and antagonist rank order of potencies were compared (Alexander *et al.*, 1994b). However, the intrinsic activities of 5'-N-ethylcarboxamidoadenosine (NECA) and adenosine were distinct in the two preparations, raising the possibility that CNS and peripheral variants of the A_{2b} subtype might exist. In the current study, we have examined the pharmacology of the adenosine receptor which mediates relaxation of the precontracted guinea-pig trachea, an A_2 adenosine receptor response (Brown & Collis, 1982; Caparotta *et al.*, 1984; Ghai *et al.*, 1987; Collis *et al.*, 1989; Collis & Hourani, 1993). We have compared profiles of this receptor with the A_{2b} adenosine receptors of the CNS and aorta (Alexander *et al.*, 1994b).

Methods

Relaxation of the precontracted guinea-pig isolated trachea

Contraction of tracheal smooth muscle was carried out with minor modifications of a method previously described for guinea-pig isolated aorta (Alexander *et al.*, 1994b). Guinea-pigs (Dunkin-Hartley, 200-600 g) of either sex were used. In some experiments, tracheal rings were denuded of their epithelium by gently rolling the rings on a cotton tissue, whilst a blunt metal pointer was inserted into the lumen of the ring. After dissection to remove connective tissue, tracheal rings (4/5 of approximately 3 mm width) with intact or denuded epithelium were attached to one another by cotton suture and attached to an isometric transducer under an initial tension of 1 g in a warmed organ bath (10 ml). After several changes of Krebs medium, repeated contractions in the presence of a submaximally-effective concentration of carbachol (0.3 μM) were carried out until a reproducible contraction was apparent. Relaxation of tracheal rings was carried out with cumulative additions of adenosine analogues after tone was induced with 0.3 μM carbachol. Cumulative concentration-relaxation curves to NECA were carried out in the absence and presence

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of adenosine receptor antagonists to calculate antagonist affinities (Alexander *et al.*, 1994b). Tissue contractility was monitored using Chart software (CED, Cambs, UK) running on a IBM-compatible PC.

Data analysis

Concentration-response profiles were fitted to sigmoidal curves using the computer programme Prism (GraphPad, California, U.S.A.), to generate pD_2 and E_{max} values. Antagonist affinities were calculated with fixed concentrations of antagonists and examination of shifts in the agonist concentration-response curves, as previously defined (Alexander *et al.*, 1994b).

Chemicals

APEC ((2-[(2-aminoethylamino)carbonylethylphenylethylamino-5′-*N*-ethylcarboxamidoadenosine), PD 115,199 (N-[2-dimethylamino) ethyl]N-methyl-4-(1,3-dipropylxanthine) benzenesulphonamide) and CP 66,713 (4-amino-8-chloro-1-phenyl[1,2,4]triazolo[4,3-a]quinoxaline were kind gifts from Dr Ken Jacobson, NIH, U.S.A.; Warner-Lambert, Michigan, U.S.A. and Pfizer Research, U.S.A., respectively. CGS 21680, (2-p-(2-carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine) NECA, 2-chloroadenosine, DPCPX (8-cyclopentyl-1,3-dipropylxanthine) and XAC (xanthine amine congener) were obtained from RBI Semat, Herts. All other chemicals were obtained from either Sigma Chemicals, Dorset, UK or Fisons Chemicals, Leics.

Adenosine receptor antagonists were dissolved initially to 10 mM in dimethylsulphoxide. NECA, CGS 21680 and APEC were dissolved in dimethylsulphoxide to 50 or 100 mM, while 2-chloroadenosine and adenosine were dissolved in dilute aqueous NaOH to a concentration of 100 mM.

Results

Agonist contraction of guinea-pig tracheal rings

Histamine evoked a concentration-dependent contraction of guinea-pig tracheal rings with a pD_2 of 5.2 ± 0.19 and maximal contractile response of 0.71 ± 0.05 g ($n = 3$, slope $= 1.52 \pm 0.06$). Carbachol evoked a more potent contraction with a greater maximal response (pD_2 6.52 ± 0.09, E_{max} 1.32 ± 0.40 g, slope 0.88 ± 0.03, $n = 3$). Carbachol-evoked contractions were also better maintained than those evoked by histamine, and so carbachol at a submaximally-active concentration (0.3 μM) was used to investigate adenosine receptor-evoked relaxation in subsequent experiments.

Adenosine analogue-evoked relaxations of carbachol-contracted tracheal rings

In tracheal rings precontracted with 0.3 μM carbachol, the adenosine analogue NECA evoked a concentration-dependent relaxation (e.g. Figure 1a). From a number of such observations, a mean pD_2 value of 6.37 ± 0.04 (IC_{50} value of 0.46 μM) was calculated with a maximal relaxation of $73 \pm 7\%$ of the carbachol-induced contraction ($n = 18$, slope -1.18 ± 0.05, Figure 2). 2-Chloroadenosine also evoked a concentration-dependent relaxation (e.g. Figure 1b) with a calculated pD_2 of 5.25 ± 0.09 (IC_{50} value of 6.1 μM). However, 2-chloroadenosine evoked a greater maximal relaxation than NECA, relaxing the tracheal rings past their basal tone (E_{max} $208 \pm 38\%$, slope -1.38 ± 0.11, $n = 5$, Figure 2).

Concentration-response curves were attempted for a number of other adenosine analogues. However, since adenosine and the 2,5′-disubstituted analogues CGS 21680 and APEC failed to elicit significant relaxations at concentrations below 10 μM, responses were compared at 10 μM (Table 1). The analogues exhibited the following rank order of magnitude: NECA = 2-chloroadenosine > APEC > CGS 21680 = adenosine. Epithelium-denuded rings showed no significant alteration in the response to adenosine, 2-chloroadenosine or CGS 21680 (Table 1).

In order to ascertain whether NECA and/or CGS 21680 were acting as partial agonists at the adenosine receptor mediating relaxation of the guinea-pig trachea, concentration-response curves to NECA and 2-chloroadenosine were carried out in the presence of 10 μM CGS 21680 and 10 μM NECA, respectively. In the presence of 10 μM CGS 21680, NECA evoked a concentration-dependent relaxation of the tissue with a pD_2 of 5.99 ± 0.22 (IC_{50} value of 1.3 μM) and a maximal relaxation of $74 \pm 12\%$ (slope $= -1.13 \pm 0.21$, $n = 3$, Figure 2). In the presence of 10 μM NECA, 2-chloroadenosine was able to relax the tissue further with a pD_2 of 4.74 ± 0.11 (IC_{50} of 19.4 μM) and a maximal response of $252 \pm 68\%$ relaxation (slope $= -1.62 \pm 0.05$, $n = 3$, Figure 2).

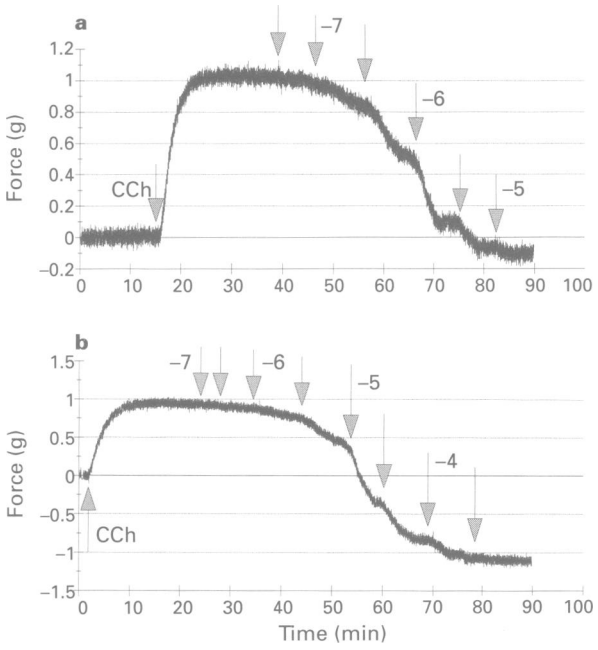

Figure 1 Contractility of guinea-pig tracheal rings: The addition of 0.3 μM carbachol (CCh) and subsequent relaxation by cumulative additions of either NECA (a) or 2-chloroadenosine (b) over the ranges $10^{-7.5}$ to 10^{-5} and 10^{-7} to $10^{-3.5}$ M are indicated by the arrows.

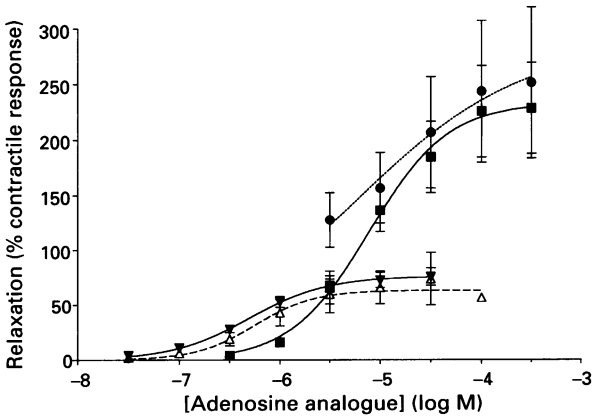

Figure 2 Concentration-dependent relaxation of precontracted guinea-pig tracheal rings by NECA (▼, solid line) and 2-chloroadenosine (■, solid line). Also shown is the response to 2-chloroadenosine in the presence of 10 μM NECA (●, dotted line), and NECA in the presence of 10 μM CGS 21680 (△, dashed line). Data are means ± s.e.mean of 18, 5, 3 and 3 experiments, respectively.

Antagonist affinities at the guinea-pig tracheal adenosine receptor

Antagonist affinities were estimated by examining the effect of fixed concentrations of antagonist on the NECA concentration-relaxation profile. Thus, the concentrations of antagonists used were 100–300 nM (XAC), 25–2500 nM (PD 115,199) and 1–2 μM (DPCPX and CP 66,713). Using a single tissue preparation, Schild analysis of the antagonist effect of PD 115,199 over the indicated concentration-range gave an apparent pA_2 value of 7.61 (an apparent K_i value of 24.5 nM), with a slope factor of 0.76 and $r^2 = 0.9998$. The rank order of apparent antagonist affinity observed was PD 115,199 = XAC > CP 66713 = DPCPX (Table 2).

Discussion

The investigation of adenosine receptors on smooth muscle has established the presence of A_1 and A_2 subtypes of receptor. However, in the main, little further classification of A_2 receptors has been attempted. We recently investigated A_2 receptors in guinea-pig cerebral cortex and cerebellum (Hernández *et al.*, 1993) and observed little pharmacological distinction between A_{2b} receptors mediating cyclic AMP generation in those two brain regions. An identical profile of agonist potency and antagonist affinity was also observed for the A_2 adenosine receptor mediating relaxation of the guinea-pig aorta (Alexander *et al.*, 1994b). However, in the CNS, adenosine exhibited partial agonism with respect to NECA, while in the aorta, the reverse was true. This raises the possibility that CNS and peripheral variants of the A_{2b} receptor exist.

In the current study, we have examined the A_2 adenosine receptor which mediates a relaxation of guinea-pig trachea (Brown & Collis, 1982; Caparotta *et al.*, 1984; Ghai *et al.*, 1987; Collis *et al.*, 1989; Collis & Hourani, 1993). It is apparent that this receptor is distinct from both CNS and aortic A_{2b} receptors (Hernández *et al.*, 1993; Alexander *et al.*, 1994b).

Table 1 Relaxant responses to adenosine and its analogues compared at 10 μM in control and epithelium-denuded tracheal rings

Agonist (10 μM)	*% relaxation (control)*	*% relaxation (epithelium-denuded)*
NECA	69 ± 16 (3)	–
2-Chloroadenosine	75 ± 16 (4)	67 ± 20 (3)
APEC	25 ± 8 (3)	–
CGS 21680	11 ± 2 (4)	3 ± 3 (3)
Adenosine	6 ± 4 (3)	8 ± 4 (3)

Data are means ± s.e.mean of the number of experiments shown in parentheses. – indicates that the effect of the agonist was not determined in the epithelium-denuded tissue.

Table 2 K_i values determined by examiniation of concentration-response curves to NECA in the absence and presence of fixed concentrations of the antagonist (see Methods)

Antagonist	K_i (nM)
PD 115,199	27 ± 8 (10)
XAC	43 ± 11 (6)
CP 66,713	285 ± 89 (4)
DPCPX	316 ± 114 (5)

Data are means ± s.e.mean of the number of experiments shown in parentheses.

Agonist pharmacology of the tracheal receptor

The agonist profile observed for relaxation of precontracted isolated trachea is consistent with our previous observations of the potency of adenosine analogues at A_{2b} receptors of guinea-pig aorta, that is: NECA (pD_2 6.17) > 2-chloroadenosine (pD_2 -5.33) > adenosine (pD_2 3.98) > CGS 21680 (Alexander *et al.*, 1994b). Preliminary studies in the guinea-pig trachea showed an approximate pD_2 value for adenosine in this tissue of 3.43 (EC_{50} value 370 μM, E_{max} 53 ± 31%) which was left shifted in the presence of 1 μM dipyridamole to a pD_2 value of 4.37 (EC_{50} value 42 μM, E_{max} 26 ± 2%, J. Moore & S.P.H. Alexander). In the trachea, as in the aorta (Alexander *et al.*, 1994b), NECA appears to elicit a smaller maximal response compared to 2-chloroadenosine. In the presence of a maximally active concentration of NECA (10 μM), 2-chloroadenosine was able to relax the tissue further to levels observed with 2-chloroadenosine in the absence of NECA. However, the shift in the pD_2 for 2-chloroadenosine (ca. 3 fold) is less than might be expected in the presence of a concentration of a partial agonist approximately 30 fold its EC_{50} value. A potential explanation for this may derive from the observation that responses to high concentrations of 2-chloroadenosine (but not NECA) are blunted in the presence of purine nucleoside uptake inhibitors in the guinea-pig trachea (Collis & Brown, 1982). Thus, it appears that some of the effects of 2-chloroadenosine may be mediated through an intracellular site distinct from the extracellular A_2 adenosine receptor.

On the basis of the low potencies of CGS 21680, APEC and adenosine, therefore, it appears unlikely that the tracheal A_2 receptor is of the A_{2a} subtype (formerly termed the 'high-affinity A_2 adenosine receptor', Daly *et al.*, 1983). We investigated whether the epithelium contributed to the low potency of CGS 21680 in this preparation, since it has previously been suggested that removal of this barrier enhances the potency of adenosine in the guinea-pig trachea (Holroyde, 1986). However, we were unable to observe any significant enhancement of the response to CGS 21680 in this tissue after removal of the epithelium (Table 1). Indeed, there appeared to be no change in the effect of adenosine or 2-chloroadenosine after this intervention either. We also investigated the possibility that CGS 21680 might be acting as a high affinity partial agonist in this system, by conducting concentration-relaxation curves to NECA in the absence and presence of 10 μM CGS 21680. The presence of 10 μM CGS 21680 led to a non-significant, rightward shift in the potency of NECA (from 6.37 to 5.99), indicating that it is unlikely that CGS 21680 is a partial agonist in this tissue.

Data obtained from the cloned guinea-pig A_{2a} adenosine receptor expressed in Chinese hamster ovary (CHO) cells (Meng *et al.*, 1993) indicates that the guinea-pig A_{2a} receptor exhibits submicromolar potency and affinity for CGS 21680 in functional and radioligand binding assays, respectively. Thus, comparison of agonist potencies suggests that the tracheal receptor is of the A_{2b} adenosine receptor subtype.

Antagonist pharmacology of the tracheal receptor

Competition for [^{3}H]-CGS 21680 in radioligand binding assays conducted on Chinese hamster ovary cells expressing the guinea-pig A_{2a} adenosine receptor (Meng *et al.*, 1994) used a limited range of agents but showed an antagonist profile of XAC > > DPCPX, which is not inconsistent with the profile of the tracheal receptor. Northern hybridization of mRNA from guinea-pig tissues showed the presence of abundant transcript in guinea-pig neostriatum, heart, spleen and kidney, but not in lung, liver, stomach or intestine (Meng *et al.*, 1994). The potential presence of A_{2a} receptor message in guinea-pig tracheal smooth muscle was not investigated. Comparison of antagonist affinities suggests that the tracheal receptor is distinct from A_{2b} receptors we have previously investigated in the cerebellum (Hernández *et al.*, 1993), cerebral cortex and aorta (Alexander *et al.*, 1994b). In these latter tissues, the antagonist rank order

of apparent affinities was XAC (K_i 15–35 nM) > DPCPX (81–171 nM) ≥ PD 115,199 (117–407 nM). Thus, PD 115,199 shows an affinity at the tracheal receptor which is greater than one order of magnitude more potent than its affinity in the aorta and the CNA. Also CP 66,713, a putatively A_{2a}-selective antagonist (Sarges *et al.*, 1990), which fails to reverse significantly NECA-stimulated cyclic AMP generation in the guinea-pig cerebral cortex at concentrations up to 10 μM (SPH Alexander, unpublished observation), exhibits relatively high affinity at the tracheal A_2 receptor (Table 2). Thus, comparison of antagonist potencies suggests that the tracheal receptor is of the A_{2a} adenosine receptor subtype.

Concluding remarks

The adenosine analogue-induced relaxation of guinea-pig precontracted, isolated tracheal rings shows a pharmacology inconsistent with either A_{2a} or A_{2b} adenosine receptors. That is, the agonist profile is compatible with the presence of an A_{2b} adenosine receptor while the antagonist profile is more similar to an A_{2a} adenosine receptor. It seems most likely, therefore, that the tracheal adenosine receptor is a further subtype of A_2 adenosine receptor, distinct from A_{2b} adenosine receptors which we have previously defined, and also distinct from an A_{2a} receptor cloned from guinea-pig neostriatum. The sustained relaxation evoked by adenosine analogues and the dissimilarity of this receptor compared with other guinea-pig adenosine receptors suggests that it may be a suitable target for future therapeutic exploitation.

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