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Functional characterization of three adenosine receptor types

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1 The purpose of the present study was to classify adenosine receptors into A_1 and A_2 subtypes in a wide range of isolated tissues and cell types (rat adipocytes and atria, guinea-pig ileum and atria (A_1); guinea-pig aorta, dog coronary artery and human platelets and neutrophils (A_2)) using the **R**- and **S**-diastereoisomers of N-phenylisopropyladenosine (PIA), N-cyclopentyladenosine (CPA), the novel compound, N-[(1S,*trans*)-2-hydroxycyclopentyl]adenosine (GR79236), N-[(2-methylphenyl)methyl]adenosine (metrifudil), 2-(phenylamino)adenosine (CV1808), and 2-[[2-[4-(2-carboxyethyl)phenyl]ethyl]amino]-*N*-ethylcarboxamidoadenosine (CGS21680); N-ethylcarboxamidoadenosine (NECA) was used as a standard.

2 Results obtained in all tissue preparations previously reported to contain A_1-receptors could be described by a single rank order of agonist potency: CPA $\geqslant$ GR79236, **R**-PIA $\geqslant$ NECA $>>$ **S**-PIA $\geqslant$ metrifudil $\geqslant$ CV1808, CGS21680.

3 In contrast, two distinct rank orders of agonist potency were observed in preparations previously reported to contain A_2-receptors. In dog coronary artery, human neutrophils and platelets the rank order of potency was: CV1808, CGS21680 $\geqslant$ NECA $>$ **R**-PIA $\geqslant$ metrifudil $\geqslant$ CPA $>$ GR79236, **S**-PIA. However, in guinea-pig aorta the rank order was: NECA $>$ metrifudil $>$ **R**-PIA, CPA $>$ CV1808, GR79236 $\geqslant$ **S**-PIA, CGS21680.

4 The results of this study are consistent with the existence of three types of adenosine receptor: A_1- and two subtypes of A_2-receptor. The receptor present in dog coronary artery, human platelets and neutrophils, probably corresponds to the A_{2a} subtype, whilst that present in the guinea-pig aorta may be of the A_{2b} subtype.

Keywords: Adenosine receptors; A_1, A_2; GR79236; metrifudil; P_1-purinoceptors

Introduction

It is now generally accepted that adenosine receptors can be divided into two types, termed A_1 and A_2. This classification was first proposed by Van Calker *et al.* (1979), based on the relative potencies of adenosine and some of its derivatives as stimulants or inhibitors of adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation in cultured brain cells. Although antagonists selective for adenosine A_1- or A_2-receptors have been described (Trevidi *et al.*, 1990), to date pharmacological classification of these receptors has usually depended on the use of selective agonists.

Studies investigating the structure-activity relationships for adenosine derivatives at A_1- and A_2-receptors have led to a number of key conclusions. Firstly, certain N^6-substituted compounds, such as cyclopentyladenosine (CPA) and **R**-phenylisopropyladenosine (**R**-PIA), show selectivity for A_1- versus A_2-receptors (Londos *et al.*, 1980; Bruns *et al.*, 1986). Furthermore, the work of Smellie *et al.* (1979) suggested that the relative potency of the **R**- and **S**-isomers of PIA is higher at A_1- than A_2-receptors. Secondly, certain 2-substitituted derivatives, such as CV1808 (2-(phenylamino)adenosine) and CGS21680 (2-[[2-[4-[-(2-carboxyethyl)phenyl]ethyl]amino]-N-ethylcarboxamidoadenosine) show selectivity for A_2- versus A_1-receptors (Bruns *et al.*, 1986; Jarvis *et al.*, 1989). Finally, although N-ethylcarboxamidoadenosine (NECA) is sometimes considered to be selective for A_2-receptors, it is, in fact, a potent, non-selective agonist (Bruns *et al.*, 1986). This profile makes NECA a useful standard with which other agonists may be compared in receptor classification studies.

Although these conclusions are generally accepted, anomalies have been reported. Thus, variations in the relative potencies of NECA and N^6-substituted derivatives such as PIA and CPA led Ribeiro & Sebastiao (1986) to suggest the existence of a sub-type of the adenosine A_1-receptor, which they termed A_3. However, our own work (Kennedy *et al.*, 1992a) failed to confirm any key differences, leading us to conclude that comparisons of the relative potencies of these agonists provides no evidence to support a sub-classification of adenosine A_1-receptors.

The classification of receptors as being of the A_2-type originally depended upon the low potency, relative to NECA, of agonists such as **R**-PIA and CPA. However, in some tissues generally considered to contain A_2-receptors, the differences in potency between NECA and the N^6-substituted derivatives were small. Thus, we have reported that the adenosine receptors mediating inhibition of U-46619-stimulated platelet aggregation (Foster *et al.*, 1987) and relaxation of dog coronary artery (Bhalla *et al.*, 1985) could not be classified as being either of the A_1- or A_2-type according to these criteria. Similar results obtained in the dog coronary circulation *in vivo* led Kusachi *et al.* (1983) to propose the existence of a 'hybrid' receptor, having characteristics of both adenosine A_1- and A_2-receptors.

Alternatively, these anomalies may be explained by a proposal advanced by Bruns and co-workers (1986). These authors compared the binding affinities of a range of compounds for the rat striatal A_2-receptor with their known potencies as stimulants of cyclic AMP formation in the human fibroblast and concluded that the preparations contained different receptors. Those present in the striatum were termed A_{2a}- and those in the fibroblast A_{2b}-receptors. The compounds showing the greatest apparent degree of selectivity (for the putative A_{2a}- versus A_{2b}-sub-type) were 2-substituted analogues such as CV1808. More recently, 2-substituted derivatives of NECA, such as CGS21680, have been reported

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to be potent ligands at the rat striatal A_2-receptor (Jarvis *et al.*, 1989).

The purpose of the present study was two fold. Firstly, to extend our previous observations on the relative potencies of agonists in preparations containing adenosine A_1 receptors (Kennedy *et al.*, 1992a) to include a wider range of tissues and compounds, and secondly to determine the relative potencies of agonists in a range of preparations usually considered to contain adenosine A_2-receptors (guinea-pig aorta, Collis & Brown, 1983; human neutrophil, Cronstein *et al.*, 1988; human platelet, Haslam & Cusack, 1981; dog coronary artery, Kusachi *et al.*, 1983), and so investigate the utility of the A_{2a}-/A_{2b}-receptor classification scheme. The compounds used here include a novel adenosine A_1-receptor agonist, N-[(1S, *trans*)-2-hydroxycyclopentyl]adenosine (GR 79236) and N-[(2-methylphenyl)methyl]adenosine (metrifudil) which was selected since its reported cardiovascular profile in man suggests that it may be a vasodilator (Schaumann & Kutscha, 1972). A preliminary account of part of this work has been published in abstract form (Gurden & Kennedy, 1992; Kennedy *et al.*, 1992b).

Methods

Tissues were obtained from male Dunkin Hartley guinea-pigs (250–300 g), male AH/A rats (Glaxo Group Research, 200–300 g; 100–120 g for adipocyte experiments), pure-bred (Glaxo Group Research) beagle dogs of either sex and blood from healthy human volunteers.

Isolated smooth and cardiac muscle preparations

Rings of isolated thoracic aorta (guinea-pig), left anterior descending coronary arteries (beagle dogs) and left and right atria (rat and guinea-pig) were bathed in a modified Krebs solution of the following composition (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.7, $MgSO_4$ 0.6, KH_2PO_4 1.2, D-glucose 11.2, $CaCl_2$ 1.3. Indomethacin (2.8×10^{-6} M) was included for experiments with guinea-pig aorta and dog coronary artery.

Rings of guinea-pig aorta were suspended (resting tension 0.5–0.75 g) on parallel steel wires (one fixed and one attached to the tension transducer) in organ baths. Following equilibration for 1 h, during which time preparations were washed regularly, phenylephrine (1×10^{-4} M) was added to the organ baths to induce a maximal level of tone. The tissues were then washed repeatedly to reverse the effect of the phenylephrine. The procedure was then repeated with a concentration of phenylephrine (usually 3×10^{-6} M) which produced approximately 50% of the maximum tone, and was repeated until constant responses were obtained. The effects of adenosine derivatives were then studied, and responses expressed as the percentage reversal of the phenylephrine-induced tone.

Rings of dog coronary artery were suspended on parallel steel wires (resting tension 0.4 g) in organ baths. Following equilibration for 2 h, during which preparations were washed regularly, the stable thromboxane-mimetic, U-46619, was added to the baths at a concentration (usually 1×10^{-7} M) sufficient to produce a maximal contraction of the tissues. The preparations were then washed repeatedly to reverse the effect of U-46619. A concentration of U-46619 (1×10^{-8}–3×10^{-8} M) producing approximately 50% of the maximum response was then added and repeated, following washing, until constant responses were obtained. Preliminary experiments indicated that U-46619-induced tone was not well maintained in some preparations. The fade seen, however, could be reduced by inclusion of adenosine deaminase (5 u ml^{-1}) in the bathing fluid. The effects of adenosine derivatives on U-46619-induced tone were therefore assessed in the presence of the enzyme.

Methods and experimental protocols for the other preparations used have been described in detail previously (Kennedy *et al.*, 1992a). In brief, strips of guinea-pig ileum longitudinal muscle were stimulated electrically at 0.2 Hz (1 ms pulse width; supra-maximal voltage), and the ability of adenosine derivatives to inhibit the electrically-evoked twitch response was measured. Left atrial preparations were paced electrically at a frequency of 1 (rat) or 3 (guinea-pig) Hz (1 ms pulse width; twice threshold voltage), and the ability of adenosine derivatives to inhibit orciprenaline-induced postive inotropic responses was measured. The negative chronotropic effects of adenosine derivatives were determined in spontaneously-beating right atria.

Isolated cell preparations

Rat adipocytes were obtained from epididymal fat pads using the methods described by Stratton *et al.* (1985), and the ability of adenosine derivatives to inhibit noradrenaline-induced lipolysis was assessed as described previously (Kennedy *et al.*, 1992a).

Neutrophils were prepared from venous blood drawn from the forearm of healthy human volunteers. The anticoagulated blood (10 units heparin ml^{-1} blood) was mixed with plasmagel (2/3 the volume of blood) and left to sediment at 37°C for 35–40 min. The supernatant buffy-coat was removed by aspiration and Ficoll-paque (1/5 volume of buffy coat) underlaid. A granulocyte pellet was prepared by centrifugation, and then washed twice in calcium-free, Tris buffer (mM: NaCl 122, KCl 2.7, Tris 25, glucose 5.6, $MgCl_2$ 1.0, bovine serum albumin 0.1% (w/v); pH 7.4), washed again in buffer containing calcium chloride (1×10^{-3} M) and then finally suspended in this buffer. Aliquots of the suspensions were taken to determine total white blood cell numbers. Granulocyte suspensions were normally found to comprise >95% neutrophils. Cells were resuspended to a concentration of $1-2 \times 10^6$ cells ml^{-1}, and indomethacin (3×10^{-6} M), adenosine deaminase (1 u ml^{-1}), cytochalasin B (5 $\mu g\ ml^{-1}$) and cytochrome C (1×10^{-4} M) were added. Concentration-inhibition curves for adenosine derivatives were then constructed (10 min pre-incubation, 37°C) versus N-formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated (2×10^{-7} M) release of O_2^- by monitoring the reduction of cytochrome C at 550 nm. The effects of each concentration of agonist were assessed in triplicate.

Platelet rich plasma (PRP) was prepared by centrifugation (2500 *g* for 2 min) of human venous blood, collected into 1/10 volume of 0.13 M trisodium citrate containing 1×10^{-4} M aspirin. Gel-filtered platelets (GFP) were prepared by the application of PRP to a column of Sephadex CL2B (Hornby *et al.*, 1989). The platelets were eluted with a modified HEPES buffered Tyrode solution. The GFP suspension was reconstituted with fibrinogen (3 mg ml^{-1}, $CaCl_2$ (2×10^{-3} M) and $MgCl_2$ (1×10^{-3} M)). Concentration-effect curves for the aggregatory response to U-46619, assessed with an optical aggregometer (Born, 1962), were carried out and the just-maximal concentration of U-46619 selected. Concentration-inhibition curves to adenosine derivatives were then constructed, following a 5 min pre-incubation at 37°C using the selected U-46619 concentration to stimulate aggregation.

Analysis of results

The responses obtained to NECA in each preparation were measured and a log concentration-effect curve constructed. From this, the agonist concentration producing 50% of its own maximum response (EC_{50}), was estimated. In the guinea-pig ileum and all four atrial preparations, the effects of NECA were rapidly reversed by washing. Consequently, the concentration-effect relationships for other compounds were determined from a second curve constructed 30–60 min after the NECA curve had been completed. However, NECA responses were not readily reversible in dog coronary artery and the guinea-pig aorta. In these preparations, therefore, and in

rat isolated adipocytes and human isolated neutrophils and platelets, concentration-effect curves to NECA and test agonists were constructed in parallel. Where the maximum response to a test agonist was equivalent to that of NECA, the equipotent molar concentration ratio (EMCR) was then calculated by dividing the EC_{50} value for the test compound (calculated as above) by the appropriate value for NECA. However, where a full concentration-effect curve could not be obtained, the EMCR was calculated using a lower equieffective concentration (greater than 20% and from the parallel portion of the concentration-effect curves).

Experiments using adenosine receptor antagonists

The sensitivity of agonist responses to either 8-phenyltheophylline (8-PT) or 1,3-diethyl-8-phenylxanthine (DPX) has been investigated in a range of tissues. In the guinea-pig ileum and all atrial preparations, 8-PT was added to the bathing fluid and kept in contact with the tissue 45 min before, and during, the construction of the second agonist concentration-effect curve. However, in the dog coronary artery and guinea-pig aorta preparations, only one agonist concentration-effect curve could be obtained in each tissue. Here, 8-PT was added to the bathing fluid 60 min before the agonist concentration-effect curve was constructed.

In rat adipocyte experiments, cells were incubated with 8-PT for 30 min prior to addition of the agonist, and throughout the subsequent incubation with noradrenaline. Thus, cells were in contact with the antagonist for 45 min before the lipolytic stimulant was added. In human neutrophil experiments, cells were incubated with the antagonist for 10 min before addition of the agonist, and throughout the subsequent stimulation with fMLP. Thus, cells were in contact with 8-PT for 20 min before the stimulant of O_2^- formation was added. However, our preliminary experiments with human platelets demonstrated that adenosine-receptor blocking concentrations of 8-PT potentiated the anti-aggregatory effects of NECA. Accordingly, DPX, but not 8-PT was used as an antagonist in these studies. The suspension of GFP was prepared as described previously. Concentration-effect curves for the aggregatory response to U-46619 were constructed in an optical aggregometer and the concentration causing 90–100% aggregation calculated and used as a standard stimulus throughout the experiment. GFP was incubated in the presence or absence of DPX (1×10^{-7}–3×10^{-6} M) for 5 min at 37°C, before the addition of NECA.

Antagonist potency was expressed as a concentration ratio (CR), determined by dividing the EC_{50} of the agonist in the absence of the antagonist by that obtained in its presence. If only one antagonist concentration, [B], was used, an estimate of the affinity of the antagonist for the receptor being studied was then calculated as an apparent pK_b (see Gaddum, 1957) using:

$$pK_b = \log (CR-1) - \log [B]$$

Where CRs had been determined from 3 (or more) antagonist concentrations, a pA_2 value was calculated by the method of Arunlakshana & Schild (1959).

Drugs

The following compounds were used: **R**-PIA and **S**-PIA (Boehringer Mannheim), CV1808 (2-(phenylamino)adenosine) and CGS21680 (2-[[2-[4-(2-carboxyethyl)phenyl]ethyl] amino]-N-ethylcarboxamidoadenosine) and 8-phenyltheophylline and 1,3-diethyl-8-phenylxanthine (batches from both Research Biochemicals Inc and Chemistry Division, Glaxo Group Research, Ware), indomethacin, L-phenylephrine, noradrenaline ((−)-arterenol) and orciprenaline (metaproterenol hemisulphate; Sigma Chemical Co), and U-46619 (11α,9α-epoxymethanoprostaglandin H_2; Cayman Chemicals). NECA, CPA, metrifudil (N-[2-(methylphenyl)methyl]adenosine) and GR79236 (N-[(1S,*trans*)-2-hydroxycyclopentyl]adenosine) were synthesized in the Chemistry Division (Glaxo Group Research, Ware). All other chemicals were standard laboratory reagents of analytical grade where possible.

GR79236, CPA, **R**- and **S**-PIA, NECA, metrifudil, CV 1808 and CGS21680 were prepared as stock solutions in HCl (0.01–0.1 M) or in 10% dimethylsulphoxide (DMSO) for experiments with GFP. A stock solution of 8-phenyltheophylline (1×10^{-2} M) was prepared in 0.1 M NaOH. Indomethacin and U-46619 were dissolved in 1% (w/v) $NaHCO_3$. L-Phenylephrine and orciprenaline were dissolved in distilled water. Further dilutions of all solutions were prepared in distilled water or Krebs buffer.

Results

Preparations reported to contain A_1 receptors

NECA produced a concentration-dependent inhibition of lipolysis by rat isolated adipocytes, an inhibition of the electrically-evoked twitch response in the guinea-pig ileum,

Table 1 Relative potencies of adenosine analogues in tissues containing A_1-receptors

Preparation	*NECA EC_{50}* (nM)	*EMCR* (NECA = 1) *R-PIA*	*CPA*	*GR79236*	*S-PIA*	*Metrifudil*	*CV1808*	*CGS21680*
Rat adipocyte	20 (17–24) $n=20$	0.54 (0.34–0.86) $n=6$	0.21 (0.10–0.43) $n=4$	0.34 (0.16–0.65) $n=6$	32 (18–58) $n=6$	56 (17–188) $n=4$	114 (32–408) $n=4$	253 (79–808) $n=6$
Guinea-pig ileum	52 (44–61) $n=20$	1.15 (0.81–1.64) $n=7$	0.37 (0.30–0.50) $n=8$	1.33 (0.80–2.21) $n=5$	56 (41–75) $n=15$	51 (37–71) $n=8$	101 (59–175) $n=6$	299 (161–553) $n=5$
Rat left atrium	46 (28–76) $n=16$	0.50 (0.31–0.82) $n=6$	0.24 (0.14–0.40) $n=6$	0.45 (0.22–0.95) $n=6$	18 (11–28) $n=6$	149 (52–431) $n=6$	1128 (738–1724) $n=6$	419 (301–583) $n=6$
Guinea-pig left atrium	30 (24–37) $n=22$	1.74 (1.07–2.81) $n=6$	0.57 (0.52–0.62) $n=6$	1.62 (0.71–3.69) $n=6$	32 (24–43) $n=7$	115 (81–162) $n=7$	257 (166–399) $n=6$	386 (209–713) $n=5$
Rat right atrium	93 (72–119) $n=20$	0.70 (0.31–1.57) $n=6$	0.21 (0.17–0.26) $n=6$	0.77 (0.45–1.32) $n=6$	25 (12–52) $n=7$	59 (24–149) $n=4$	1041 (736–1473) $n=4$	382 (326–447) $n=3$
Guinea-pig right atrium	148 (115–189) $n=23$	1.66 (0.99–2.78) $n=6$	0.45 (0.23–0.88) $n=6$	1.71 (0.3–8.7) $n=3$	49 (22–107) $n=5$	139 (109–176) $n=3$	1322 (494–3536) $n=3$	430 (141–1306) $n=3$

Equipotent molar concentration ratios (EMCR), together with the EC_{50} of NECA, are presented as geometric mean values, with 95% confidence limits shown in parentheses and where n = the number of observations.

and negative inotropic or chronotropic responses in both guinea-pig and rat left and right atria, respectively. Geometric mean EC_{50} values for NECA ranged from 2.0×10^{-8} M for inhibition of lipolysis by rat adipocytes, to 14.8×10^{-8} M for negative chronotropic activity in guinea-pig right atria. The other agonists evaluated produced responses which were qualitatively similar to those of NECA. In addition, where full concentration-effect curves could be constructed, the maximal responses which were elicited were not different from that of NECA. Comparison of the relative potencies of the compounds evaluated here in these six preparations (Table 1) shows a common rank order of agonist potency: CPA $\geqslant$ GR79236, **R-PIA** $\geqslant$ NECA $>>$ **S-PIA** $\geqslant$ metrifudil $\geqslant$ CV18-08, CGS21680.

It is noteworthy, however, that whilst CPA was consistently the most potent agonist in the 6 preparations, it was at the most, only 5 times more potent than NECA.

The agonist effects of NECA in all six tissues were antagonized by 8-PT (Figure 1, Table 2). In those tissues examined (rat adipocyte, guinea-pig ileum, rat left atrium and guinea-pig right atrium), the agonist effects of GR79236 were similarly antagonized by 8-PT. Furthermore, the pA_2/pK_b values obtained in this series of experiments appeared to be agonist-independent. However, there was a trend for affinity estimates for 8-PT to be higher in tissue preparations from the rat than those from the guinea-pig.

Table 2 Antagonist potency of 8-phenyltheophylline in tissues containing A_1-receptors

Preparation	pA_2 (Slope)/pK_b vs NECA	GR79236
Rat adipocyte	7.0 (0.73)* $n=4$	6.6 (0.85) $n=3$
Guinea-pig ileum	6.36 (0.95) $n=13$	6.14 $n=4$
Rat left atrium	7.32 (1.09) $n=7$	7.10 $n=3$
Guinea-pig left atrium	6.49 (0.93) $n=6$	NT
Rat right atrium	7.81 (0.83) $n=5$	NT
Guinea-pig right atrium	6.25 (1.11) $n=3$	NT

Results are presented as the mean of either pA_2 values (slopes shown in brackets) or pK_b values, where n = the number of observations. NT = not tested.
*Slope significantly ($P<0.05$, t test) less than unity.

Preparations reported to contain A_2-receptors

NECA produced concentration-dependent relaxations of the guinea-pig aorta and coronary artery preparations, inhibition of U-46619-induced human platelet aggregation and inhibition of fMLP-induced O_2^- production by human neutrophils. The other agonists evaluated produced qualitatively similar responses. Furthermore, where full concentration-effect curves could be constructed, the maximal responses obtained were not different from those to NECA. Comparison of the relative potencies of the eight compounds used in this study, however, show that the rank order of agonist potency characteristic of preparations containing adenosine A_1-receptors did not apply to these tissues and secondly, rank order of agonist potency did not apply to all of these preparations (Table 3). In the case of dog coronary artery, human neutrophils and platelets, the rank order of agonist potency was: CV1808, CGS21680 $\geqslant$ NECA $>$ **R-PIA** $\geqslant$ metrifudil $\geqslant$ CPA $>$ GR79236, S-PIA. However, for guinea-pig aorta, the rank order of agonist potency was: NECA $>$ metrifudil $>$ **R-PIA**, CPA $>$ CV1808, GR79236 $\geqslant$ S-PIA, CGS21680.

The agonist effects of NECA in dog coronary artery, guinea-pig aorta and human neutrophils were antagonized by 8-PT (Figure 2, Table 4). The agonist effects of metrifudil in guinea-pig aorta, and of CGS21680 in dog coronary artery, were found to be antagonized similarly. Here, the affinity estimates for 8-PT were apparently both agonist- and tissue-independent (Table 4). In the human platelet the inhibitory effects of NECA on U-46619-induced aggregation were antagonized by DPX. Schild analysis of these data resulted in a pA_2 value ($\pm$ s.e.mean) for DPX of 7.31 ($\pm$ 0.14) with a slope of 0.85 ($\pm$ 0.1), a value similar to the compound's reported affinity for adenosine receptors (Bruns *et al.*, 1986).

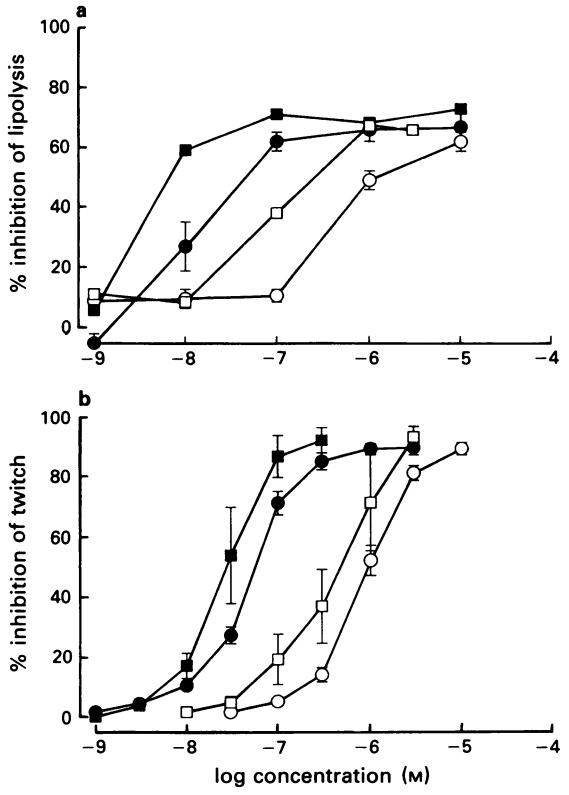

Figure 1 Antagonism of the effects of NECA and GR79236 on rat adipocytes and guinea-pig ileum by 8-phenyltheophylline (8-PT). Mean concentration-effect curves to NECA (● and ○) and GR 79236 (■ and □) in the absence (filled symbols) and presence (open symbols) of 8-PT (1×10^{-5} M) on rat adipocytes (a) and guinea-pig ileum (b). Results are shown as mean ($\pm$ s.e.mean, where $n>3$) values from 4 (NECA) or 3 (GR79236) observations for rat adipocytes and 13 (NECA) and 4 (GR79236) observations for guinea-pig ileum. For abbreviations, see text.

Discussion

In the present study, the effects of the adenosine analogues tested appear to be mediated via activation of adenosine receptors. Although responses have not been shown to be blocked by adenosine receptor antagonists (8-PT or DPX) in every tissue, we have shown that the effects of GR79236, metrifudil and CGS21680 are antagonized in preparations containing receptors for which the agonists show selectivity. In addition, the effects of the non-selective agonist, NECA, are sensitive to antagonists in all preparations.

The rank order of agonist potency obtained in guinea-pig ileum, rat adipocytes and the atrial preparations is consistent with the reported presence of adenosine A_1-receptors in these

Table 3 Relative potencies of adenosine analogues in tissues containing A_2-receptors

Preparation	*NECA* EC_{50} (nM)	*EMCR* (NECA = 1) *Metrifudil*	*CV1808*	*CGS21680*	*R-PIA*	*CPA*	*GR79236*	*S-PIA*
Dog coronary artery	44 (29–67) $n = 30$	16.5 (8–33) $n = 6$	0.75 (0.37–1.52) $n = 5$	1.23 (0.4–3.5) $n = 6$	10.5 (5.7–19.3) $n = 10$	20.8 (8–51) $n = 6$	131 (90–190) $n = 6$	80 (52–123) $n = 7$
Guinea-pig aorta	250 (222–281) $n = 42$	4.6 (2.7–7.5) $n = 8$	102 (52–199) $n = 7$	626 (430–912) $n = 6$	31 (16–63) $n = 8$	29 (21–41) $n = 8$	145 (93–227) $n = 6$	304 (136–684) $n = 6$
Human platelet	54 (34–84) $n = 19$	6.0 (2.41–14.9) $n = 4$	1.14 (0.61–2.13) $n = 6$	0.88 (0.18–4.24) $n = 4$	2.74 (1.73–4.3) $n = 19$	14.3 (5.3–38.3) $n = 7$	156 (50–494) $n = 4$	50.9 (34–75.5) $n = 19$
Human neutrophil	5.0 (4.1–6.1) $n = 46$	21.1 (13.3–33.4) $n = 4$	0.57 (0.21–1.60) $n = 6$	1.10 (0.22–5.6) $n = 4$	9.0 (3.5–23) $n = 4$	72 (38–137) $n = 4$	494 (266–917) $n = 5$	204 (134–310) $n = 4$

Equipotent molar concentration ratios (EMCR), together with the EC_{50} of NECA, are presented as geometric mean values, with 95% confidence limits shown in parentheses and where n = the number of observations.

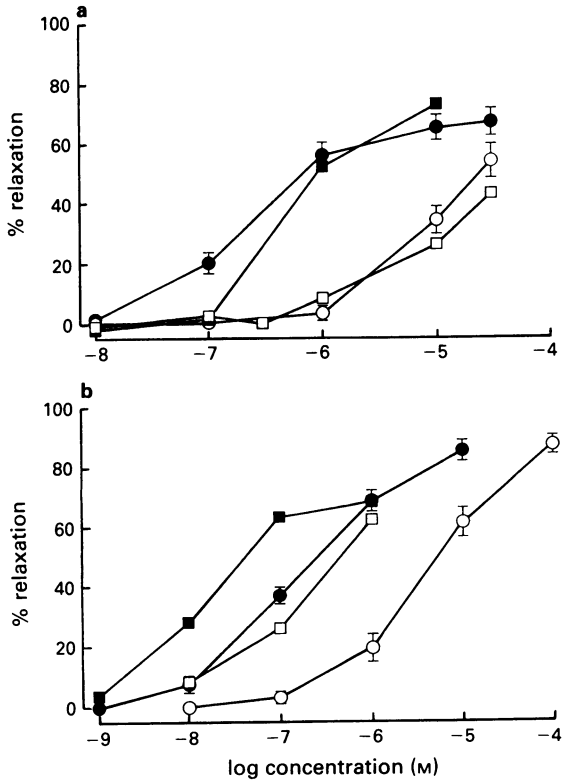

Figure 2 Antagonism of the effects of NECA and metrifudil on guinea-pig aorta and of NECA and CGS21680 on dog coronary artery by 8-phenyltheophylline (8-PT). Mean concentration-effect curves to NECA (● and ○; $n = 13$) and metrifudil (■ and □; $n = 2$) on the guinea-pig aorta (a) and NECA (● and ○; $n = 6$) and CGS21680 (■ and □; $n = 6$) on the dog coronary artery (b) in the absence and presence of 8-PT (1×10^{-5} M). Results are shown as mean values (± s.e.mean where $n > 3$).

Table 4 Antagonist potency of 8-phenyltheophylline in tissues containing A_2-receptors

Preparation	pA_2 (Slope)/pK_b vs *NECA*	*Metrifudil*	*CGS21680*
Guinea-pig aorta	6.78 (0.90) $n = 13$	6.5 $n = 2$	NT
Dog coronary artery	6.5 $n = 6$	NT	6.28 $n = 3$
Human neutrophil	7.1 (0.89) $n = 6$	NT	NT

Results are presented as the mean of either pA_2 values (slopes shown in brackets) or pK_b values, where n = the number of observations. NT = not tested.

tissues (see Kennedy *et al.*, 1992a,b for relevant literature). In particular the potency of N^6-substituted compounds, such as GR79236, **R**-PIA and CPA, was similar to or greater than that of NECA. In addition, in this study, we have found that CV1808 and CGS21680 were substantially weaker than NECA in these tissues, consistent with data from ligand binding studies (Bruns *et al.*, 1986; Jarvis *et al.*, 1989). However, the results obtained in this study and those from our previous work (Kennedy *et al.*, 1992a), do not provide any evidence for the existence of sub-types of the A_1-receptor. It is possible that there are receptor sub-types, but the compounds and preparations used in the present study do not allow them to be detected. In marked contrast, however, two distinct rank orders of agonist potency have been obtained from tissues previously characterized as containing adenosine A_2-receptors (Collis & Brown, 1983; Cronstein *et al.*, 1988; Haslam & Cusack, 1981; Kusachi *et al.*, 1983). Thus, in dog coronary artery, human neutrophils and human platelets, CV1808 and CGS21680 were similar in potency to NECA, whilst in guinea-pig aorta, both compounds were substantially less active than NECA.

Ligand binding studies have shown that CGS21680 and CV1808 have high affinity for the adenosine A_{2a}-receptor in rat striatum, and in the case of CV1808, selectivity for this receptor versus the adenosine A_{2b}-receptor in human fibroblasts (Bruns *et al.*, 1986; Jarvis *et al.*, 1989). It is, therefore, likely that the receptors which we have studied in dog coronary artery, human platelets and human neutrophils are similar to, if not identical with, the striatal adenosine A_{2a}-receptor. Furthermore, it is possible that the guinea-pig aorta contains an adenosine receptor similar to that in human fibroblasts (see Hargreaves *et al.*, 1991, Martin, 1992). However, since no ligands which are selective for this receptor have yet been described, no firm conclusions may be drawn at present. Although the guinea-pig aorta appears to be the only example of this receptor type studied here, it should be noted that receptors which show similar characteristics with respect to CGS21680 and CV1808 mediate relaxation of dog saphenous vein, guinea-pig fundus and rat bladder and duodenum (Hargreaves *et al.*, 1991; Gurden & Kennedy, 1992; Nicholls *et al.*, 1992).

Although **R**-PIA, CPA and GR79236 showed selectivity for adenosine A_1- over A_2-receptors, the degree of selectivity was variable. The three compounds have similar potencies in tissues containing A_1 receptors, so their selectivity is dependent upon their abilities to stimulate A_2 receptors. **R**-PIA was the least selective of the three, whilst GR79236 appeared to

be the most selective, showing up to 1453 fold (rat adipocytes versus human neutrophils) selectivity for A_1 versus A_2 receptors.

Smellie *et al.* (1979) have suggested that the ratio of the potencies of the **R**- and **S**-isomers of PIA could be used to differentiate between adenosine A_1- and A_2-receptors. We have found that although this ratio does tend to be higher in the tissues containing A_1-receptors, the difference can be modest. Thus, the highest value was 59 for rat adipocyte (A_1), and the lowest value was 7.6 for dog coronary artery (A_{2a}). However, the lowest value obtained for the guinea-pig left atrium in which the receptor is characterized as being of the A_1-sub-type, was 18.4. This overlaps with the highest value obtained for a tissue containing adenosine A_2-receptors, namely 22.7 for human neutrophils (A_{2a}). Our findings therefore support the proposal of Bruns *et al.* (1986) that the potency ratio is of strictly limited value in the classification of adenosine receptors.

The 2-substituted compounds CV1808 and CGS21680 showed a high degree of selectivity for adenosine A_{2a}- over either A_1- or the putative A_{2b}-receptor (guinea-pig aorta). Thus, comparing EMCR values, CGS21680 showed a minimum of 206 fold selectivity for adenosine A_{2a}- versus A_1-receptors (dog coronary artery versus rat adipocyte) and 509 fold selectivity for A_{2a}- versus the putative A_{2b}-receptor (dog coronary artery versus guinea-pig aorta). Our inability to distinguish between the selectivity profiles of CV1808 and CGS 21680 was suprising, since previous binding and functional studies have reported that CGS21680 is the more selective compound (Jarvis *et al.*, 1989; Hutchison *et al.*, 1989). We have no explanation for this difference at present. In contrast, metrifudil did not differentiate between adenosine A_{2a}- and the putative A_{2b}-receptor, but did display modest selectivity for A_2- versus A_1-receptors.

In conclusion, the results of the present study provide further evidence for the existence of three types of adenosine receptor, corresponding to the A_1-, A_{2a}- and, possibly, A_{2b}-subtypes. Key compounds for distinguishing these sub-types are the N^6-substituted compounds, CPA and GR79236 and the 2-substituted compounds, CV1808 and CGS21680. Using these compounds, together with the reference agonist NECA, adenosine A_1-receptors can be defined by the rank order of agonist potency: CPA $\geqslant$ GR79236 $\geqslant$ NECA $>>$ CV1808, CGS21680, whilst the adenosine A_{2a}-receptor can be defined as: CV1808, CGS21680 $\geqslant$ NECA $>>$ CPA $>$ GR79236. The putative adenosine A_{2b}-receptor may be defined by a rank order of agonist potency of: NECA $>$ CPA $>$ CV1808, GR 79236 $\geqslant$ CGS21680. However, although a more satisfactory definition in these terms must await the discovery of selective agonists or antagonists for this receptor site, receptors reported to correspond to the A_1, A_{2a} and A_{2b} subtypes have apparently been cloned and expressed (see Pierce *et al.*, 1992 for references).

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