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A_1 adenosine receptor inhibition of cyclic AMP formation and radioligand binding in the guinea-pig cerebral cortex

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1 A_1 adenosine receptors were investigated by radioligand binding and functional studies in slices and particulate preparations from guinea-pig cerebral cortex.

2 Binding of the adenosine receptor antagonist radioligand, 8-cyclopentyl-[^{3}H]-1,3-dipropylxanthine (DPCPX) to guinea-pig cerebral cortical membranes exhibited high density (1410 ± 241 fmol mg^{-1} protein) and high affinity (K_d 3.8 ± 0.3 nM).

3 [^{3}H]-DPCPX binding to guinea-pig cerebral cortical membranes was displaced in a monophasic manner by adenosine receptor antagonists with the rank order of affinity (K_i values, nM): DPCPX (6) < xanthine amine congener (XAC, 153) < PD 115,199 (308).

4 Agonist displacement of [^{3}H]-DPCPX binding was biphasic and exhibited the following rank order at the low affinity site (K_i values): 2-chloro-N^6-cyclopentyl-adenosine (CCPA, 513 nM) = N^6-**R**-phenyl-isopropyladenosine (**R**-PIA, 526 nM) = N^6-cyclopentyladenosine (CPA, 532 nM) < 2-chloroadenosine (2CA, 3.2 μM) = 5'-N-ethylcarboxamidoadenosine (NECA, 4.6 μM) < N^6-S-phenylisopropyladenosine (S-PIA, 19.9 μM).

5 In cerebral cortical slices, [^{3}H]-DPCPX binding was displaced by antagonists and agonists in an apparently monophasic manner with the rank order of affinity (K_i values, nM): DPCPX (14) < XAC (45) < **R**-PIA (266) < PD 115,199 (666) < S-PIA (21000).

6 Cyclic AMP accumulation stimulated by 30 μM forskolin in guinea-pig cerebral cortical slices was inhibited by **R**-PIA, CCPA and CPA up to 1 μM in a concentration-dependent fashion with IC_{50} values of 14, 18, and 22 nM, respectively. All three analogues inhibited the forskolin response to a similar extent (82–93% inhibition). NECA, S-PIA and 2CA failed to inhibit the forskolin response, but rather enhanced the accumulation of cyclic AMP at concentrations of 100 nM or greater, presumably through activation of A_{2b} adenosine receptors coupled to stimulation of cyclic AMP accumulation in guinea-pig cerebral cortical slices.

7 The inhibition of forskolin-stimulated cyclic AMP accumulation by CPA was antagonized with the rank order of affinity (K_i values, nM): DPCPX (6) < XAC (52) < PD 115,199 (505).

8 Xanthine-based antagonists inhibited the adenosine receptor augmentation of histamine-induced phosphoinositide turnover in guinea-pig cerebral cortical slices with the rank order of affinity (K_i, nM): DPCPX (12) = XAC (17) < PD 155,199 (640).

9 In summary, we observe a good correlation between antagonist affinity at A_1 receptors defined by radioligand binding, inhibition of cyclic AMP generation or augmentation of histamine-evoked phosphoinositide turnover in guinea-pig cerebral cortex.

Keywords: A_1 adenosine receptor; xanthines; cyclic AMP; phosphoinositide turnover; guinea-pig cerebral cortex

Introduction

The role of extracellular adenosine as a neuromodulator in the central nervous system is now well established. Two classes of extracellular adenosine receptor were originally delineated from studies of the effects of adenosine in mouse astroglial primary culture, subserving an inhibition (A_1) and a stimulation (A_2 of adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels (van Calker *et al.*, 1979; Londos *et al.*, 1980; Daly *et al.*, 1983). However, to date in the literature there exist relatively few examples (such as the work of Fredholm in the rat hippocampus: Fredholm *et al.*, 1982; 1986; Dunwiddie & Fredholm, 1985) of investigations of the pharmacological profile of A_1 adenosine receptor-evoked alterations in second messenger levels in tissue slice preparations from the CNS. Quantitative investigations of the A_1 adenosine receptor have in the main been limited to applying radioligand binding assays using either agonists (e.g. [^{3}H]-**R**-PIA: Schwabe & Trost, 1980) or antagonists (e.g. [^{3}H]-DPCPX: Bruns *et al.*, 1987; Lohse *et al.*, 1987). Studies of adenylyl cyclase activity in cell-free preparations from the CNS have also been carried out to investigate A_1 adenosine receptors (e.g. Schubert *et al.*, 1979). We are particularly interested in adenosine receptors of guinea-pig cerebral cortical slices since we have previously observed a selective enhancement of histamine H_1 receptor-stimulated phosphoinositide turnover in this tissue through activation of an adenosine receptor (Hill & Kendall, 1987). The agonist rank order of potency, whereby the N^6-derivative CPA was more potent than the 5'-derivative NECA could indicate mediation of this response through an A_1 adenosine receptor. However, the agonist potencies observed were relatively high (ca. micromolar), which is not entirely compatible with a typical A_1 receptor. We have additionally reported that the receptor mediating this potentiation is distinct from the A_{2b} adenosine receptor of guinea-pig cerebral cortical slices which mediates a stimulation of cyclic AMP levels (Alexander *et al.*, 1989). We have recently further characterized the latter A_{2b} adenosine receptor in guinea-pig cerebellum and cerebral cortex and identified a rank order of antagonist potency at this receptor of xanthine amino congener (XAC) > DPCPX > PD 115, 199 (Hernández *et al.*, 1993; Alexander *et al.*, 1994).

Comparisons between affinity constants estimated from functional and radioligand binding studies are relatively few in the adenosine receptor field, and are often conducted

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across species (e.g. rat brain radioligand binding data compared with guinea-pig atrial preparations. This situation is obviously far from ideal. In the current paper, we present an investigation of the A_1 adenosine receptor-mediated inhibition of forskolin-stimulated cyclic AMP accumulation in guinea-pig cerebral cortical slices and compare data obtained from this approach with data from radioligand binding techniques.

Methods

Inhibition of forskolin-stimulated cyclic AMP accumulation

After equilibration of cerebral cortex slices (350 × 350 μm) for 60 min with several changes of Krebs-Henseleit medium, slices were exposed to [^{3}H]-adenine (74 kBq ml^{-1}) for 40 min, then washed to remove excess radioactivity. Aliquots of gravity-packed slices (25 μl) were placed in flat-bottomed vials to which Krebs-Henseleit solution was added to give a final volume of 300 μl. Adenosine deaminase (1 u ml^{-1}) was also included at this time in the assay, together with antagonist, where indicated. After an interval of at least 10 min, adenosine analogues were added in 10 μl, followed rapidly by the addition of forskolin to a final concentration of 30 μM. The slices were allowed to incubate for a further 10 min, before termination of the reaction with 200 μl of 1 M hydrochloric acid containing 30–35 Bq of [^{14}C]-cyclic AMP, followed by 750 μl of ice-cold water. Cyclic AMP was isolated by the Dowex-alumina method (Salomon *et al.*, 1974).

Augmentation of histamine-stimulated phosphoinositide turnover

Histamine-stimulated phosphoinositide turnover was assessed by measuring the accumulation of [^{3}H]-inositol phosphates over a 45 min incubation period in [^{3}H]-inositol pre-labelled cerebral cortex slices in the presence of 5 mM LiCl, as previously described (Alexander *et al.*, 1989).

Radioligand binding studies

[^{3}H]-DPCPX binding to a particulate fraction A particulate preparation was obtained from guinea-pig cerebral cortex by homogenization of tissue in 10 volumes of 1 mM EDTA in 50 mM Tris, pH 7.4 and centrifugation at 20 000 *g* for 15 min. The membranes were re-suspended by homogenization in 10 volumes of the same buffer and the centrifugation process repeated. After a second homogenization/centrifugation step the membranes were re-suspended in the same buffer to a protein concentration of approximately 1 mg ml^{-1}.

[^{3}H]-DPCPX binding was carried out in a final volume of 200 μl in 1 mM EDTA, 50 mM Tris pH 7.4 containing 1.25 u ml^{-1} adenosine deaminase and 0.01% Triton X-100. Non-displaceable binding was defined by the presence of 5 mM theophylline, and was typically 5–10%. The incubation was allowed to proceed for 90 min at room temperature before termination by rapid filtration over Whatman GF/B filters using a cell harvester (Brandel, Semat, Herts, UK). Filters were extracted overnight in scintillation cocktail prior to estimation of radioactivity by liquid scintillation spectrometry.

[^{3}H]-DPCPX binding in cerebral cortical slices [^{3}H]-DPCPX binding was carried out using 10 μl of gravity-packed cerebral cortical slices in a final volume of 2 ml, in Krebs Henseleit medium, pH 7.4 containing 1.25 u ml^{-1} adenosine deaminase. Non-displaceable binding was defined by the presence of 5 mM theophylline, and was typically 25–35% of total binding for 0.5–1.2 nM [^{3}H]-DPCPX. The incubation was allowed to proceed for 60–90 min at 37°C in a shaking water bath before termination by rapid filtration over Whatman GF/B filters using a 10 place manifold (Shearline, Cambridge, UK). Filters were extracted overnight in scintillation cocktail prior to estimation of radioactivity by liquid scintillation spectrometry.

Data analysis and statistics

Data are expressed as means ± s.e.mean. Saturation and competition data were analysed with the computer programme InPlot (GraphPad, California, U.S.A.) using non-linear equations for rectangular hyperbolae and sigmoidal curves, respectively. Apparent inhibition constants were calculated from radioligand binding data using the Cheng-Prusoff transformation (Cheng & Prusoff, 1973). Functional affinity constants were calculated as previously described (Alexander *et al.*, 1989).

Materials

8-cyclopentyl-[^{3}H]-1,3-dipropylranthine [^{3}H]-DPCPX, 4040 GBq mmol^{-1}) and [^{3}H]-adenine (880 GBq mmol^{-1}) were obtained from DuPont NEN, Herts. [^{14}C]-cyclic AMP (10.3 GBq mmol^{-1}) was from Amersham International, Bucks. Adenosine analogues (CPA, N^6-cyclopentyladenosine; CCPA, 2-chloro-N^6-cyclopentyladenosine; 2CA, 2-chloroadenosine; NECA, 5'-N-ethylcarboxamidoadenosine; **R**-PIA, **R**-N^6-phenylisopropyladenosine; and **S**-PIA, **S**-N^6-phenylisopropyladenosine) were obtained from Research Biochemicals Incorporated, Herts and were dissolved to 50 mM in dimethylsulphoxide. Adenosine receptor antagonists (DPCPX, 8-cyclopentyl-1,3-dipropylxanthine and XAC, xanthine amino congener) were also obtained from RBI (with the exception of PD 115,199, a gift from Warner-Lambert, U.S.A.), and were also initially dissolved in dimethylsulphoxide to 10 mM. Adenosine deaminase was obtained from Boehringer Mannheim (Sussex). All other chemicals were from Fisons (Leics) or Sigma Chemicals (Dorset).

Guinea-pigs of the Hartley strain, 200–600 g, of either sex were used throughout.

Results

[^{3}H]-DPCPX radioligand binding

Analysis of [^{3}H]-DPCPX binding to guinea-pig cerebral cortical membranes gave estimates of the maximal capacity for this tissue of 1410 ± 241 fmol mg^{-1} protein, with a K_d of 3.8 ± 0.3 nM. Antagonist competition curves for [^{3}H]-DPCPX binding showed a rank order of potency of DPCPX > XAC > PD 115,199, each of which exhibited Hill slopes near to unity (Figure 1a, Table 1). Displacement of [^{3}H]-DPCPX by agonists, in contrast, was biphasic exhibiting high and low affinity sites for all the agonists examined (e.g. **R**- and **S**-PIA, Figure 1a). At the low affinity site, constituting *c.* 66% of displaceable binding, displacement of [^{3}H]-DPCPX by adenosine receptor agonists showed the rank order of potency **R**-PIA = CCPA = CPA > 2CA > NECA > S-PIA (Table 1).

[^{3}H]-DPCPX bound to cerebral cortical slices in a time-dependent manner, reaching steady-state levels at 30–45 min, remaining constant for at least a further 45 min. Subsequent binding experiments were allowed to proceed over 60–90 min. The antagonists DPCPX, XAC and PD 115,199 and the agonists **R**-PIA and S-PIA competed for [^{3}H]-DPCPX binding (Figure 1b, Table 2). Radioligand binding in slices was observed to be somewhat more variable than the comparable particulate binding assays. B_{max} values estimated from DPCPX competition curves were 4078 ± 1285 fmol mg^{-1} protein. This value derives from an average protein content of 40 mg ml^{-1} slices (data not shown) which includes both soluble and particulate proteins and so must be taken to be

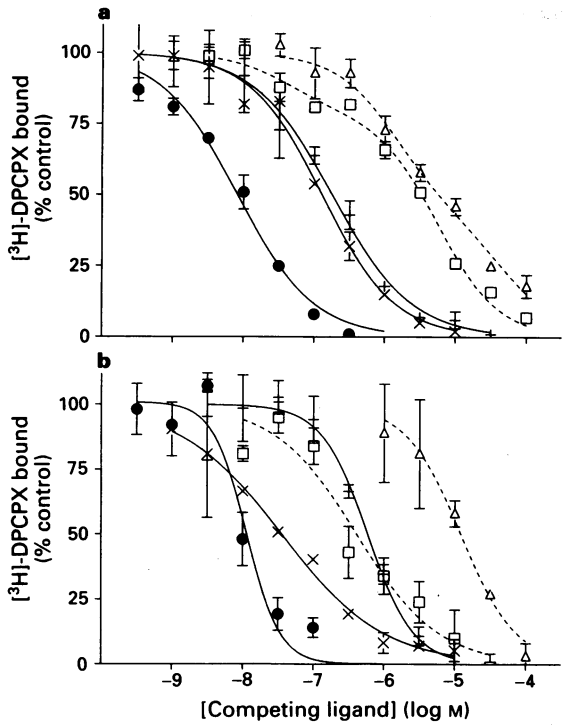

Figure 1 Displacement of [^{3}H]-DPCPX (0.5–1.2 nM binding to (a) a particulate preparation or (b) intact slices from guinea-pig cerebral cortex. Data are means and s.e.mean from single experiments conducted in triplicate (a) or quadruplicate (b) in the presence of increasing concentrations of DPCPX (●), XAC (×), PD 115,199 (+), R-PIA (□) or S-PIA (△). For abbreviations, see text.

Table 1 Inhibition constants, K_i, for displacement of [^{3}H]-DPCPX binding to a cerebral cortical particulate preparation by adenosine receptor antagonists and agonists

Antagonist	K_i (nM)	n_H
DPCPX	5.5 ± 1.6	− 1.14 ± 0.07
XAC	153 ± 30	− 1.07 ± 0.10
PD 115,199	264 ± 54	− 1.15 ± 0.08

Agonist	*Low affinity binding site* K_i (nM)	*% low affinity binding site*	*High affinity binding site* K_i (nM)
CPA	529 ± 123	70 ± 7	4 ± 1
CCPA	905 ± 430	59 ± 8	19 ± 4
R-PIA	1618 ± 101	75 ± 6	18 ± 12
2CA	4913 ± 1904	65 ± 7	37 ± 16
NECA	10580 ± 1320	52 ± 10	107 ± 15
S-PIA	22150 ± 538	62 ± 10	751 ± 344

Data are means ± s.e.mean of experiments conducted on at least four (up to eight) separate preparations of guinea-pig cerebral cortex membranes. Agonist competition data were fitted to a two-site model for competition of [^{3}H]-DPCPX binding (using the computer programme InPlot, in all cases $r^2 > 0.98$) to generate high and low affinity binding sites for the agonists. For abbreviations, see text.

an underestimate of binding capacity for comparison with data derived using the particulate preparation.

Forskolin-stimulated [^{3}H]-cyclic AMP accumulation

Since A_1 adenosine receptors are commonly associated with an inhibition of adenylyl cyclase activity (Jacobson *et al.*, 1992), we investigated the possible functional linkage of A_1 receptors in guinea-pig cerebral cortical slices using adenosine analogues and forskolin as a stimulus of cyclic AMP formation. When [^{3}H]-cyclic AMP accumulation was stimulated in the presence of 30 μM forskolin and 1 u ml^{-1} adenosine deaminase, a variety of responses was observed with adenosine analogues (Figure 2). The putative A_{2a}-selective agonist, CGS 21680 was without significant effect on basal (Alexander *et al.*, 1994) or forskolin-stimulated cyclic AMP generation at concentrations up to 10 μM (Figure 2). In contrast, the non-selective adenosine receptor agonists NECA and 2CA elicited concentration-dependent enhancements of the forskolin response (Figure 2) with EC_{50} values of 89 ± 47 nM and 1.5 ± 0.2 μM, and a maximal effect of 374 ± 59% and 352 ± 66% of the forskolin response, respectively ($n=3$). S-PIA also increased forskolin-induced cyclic AMP generation without reaching saturation. Assuming a similar maximal response to S-PIA compared with NECA and 2CA, an EC_{50} value could be estimated at 13 and 14 μM ($n=2$). In comparison, stimulation of cyclic AMP generation in the absence of forskolin is evoked by NECA and 2CA with EC_{50} values of 3.1 and 10 μM, respectively (Alexander *et al.*, 1994).

Certain N^6-derivatives of adenosine elicited biphasic effects on the forskolin response. Thus, at concentrations below micromolar, concentration-dependent inhibitions of [^{3}H]-cyclic AMP generation were observed, while at higher concentrations, increased cyclic AMP responses were apparent (e.g. R-PIA, Figure 2). Thus, R-PIA, CCPA and CPA in the concentration range 1–1000 nM all inhibited the forskolin response to a maximal level of 82 ± 3, 92 ± 4, and 91 ± 2% inhibition, respectively (Table 3). The stimulation of [^{3}H]cyclic AMP accumulation by NECA, 2CA and S-PIA

Table 2 Inhibition constants, K_i, for displacement of [^{3}H]-DPCPX binding to cerebral cortical slices adenosine receptor antagonists and agonists

	K_i (nM)	n_H
DPCPX	14 ± 4	− 1.37 ± 0.26
XAC	45 ± 10	− 0.80 ± 0.20
PD 115,199	666 ± 117	− 1.22 ± 0.26
R-PIA	266 ± 55	− 0.82 ± 0.16
S-PIA	21 000 ± 6000	− 1.15 ± 0.47

Data are means ± s.e.mean of experiments conducted on at least four separate preparations of guinea-pig cerebral cortical slices. Competition data were fitted to a sigmoidal using the computer programme InPlot (in all cases $r^2 > 0.98$). For abbreviations, see text.

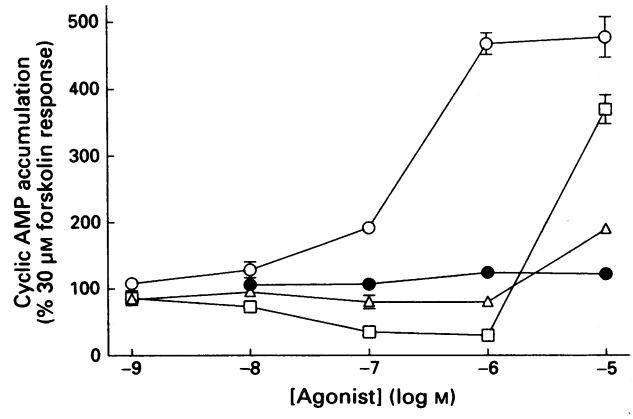

Figure 2 Modulation of forskolin-stimulated cyclic AMP accumulation in guinea-pig cerebral cortical slices by adenosine analogues. [^{3}H]-adenine-prelabelled slices were incubated with increasing concentrations of R-PIA (□), S-PIA (△), NECA (○) or CGS 21680 (●) immediately prior to addition of 30 μM forskolin. Results are means ± s.e.mean of single experiments expressed as a percentage of the forskolin response. The response to each agonist was investigated on at least two further occasions. For abbreviations, see text.

and higher concentrations of R-PIA, CCPA and CPA is presumably mediated through activation of A_{2b} adenosine receptors (Jacobson *et al.*, 1992; Alexander *et al.*, 1994). Since no selective antagonist for this receptor exists, however, it is not possible to assert unequivocally that these effects are A_{2b} receptor-mediated.

Estimation of antagonist apparent affinity constants

The CPA-elicited inhibition of forskolin-stimulated [^{3}H]cyclic AMP generation was investigated to determine the apparent affinity constants of the three antagonists (e.g. DPCPX, Figure 3). Using concentrations of antagonists at approximately one order of magnitude greater than the K_i calculated from the radioligand binding studies presented above, rightward shifts in the agonist concentration-inhibition curves were analysed in order to calculate antagonist apparent inhibition constants. The antagonist rank order of potency observed was DPCPX > XAC > PD 115,199 (Table 3).

Table 3 Agonist potency, IC_{50} or EC_{50} values (nM), and antagonist affinity, K_i (nM), at adenosine receptors of guinea-pig cerebral cortical slices

Agonist	*Cyclic AMP inhibition*	*PI turnover potentiation*
R-PIA	14 ± 2 (4)	815 ± 110[1]
CCPA	18 ± 7 (14)	573 ± 123 (4)
CPA	22 ± 3 (14)	410 ± 100[1]
NECA	–	920 ± 95[1]
2CA	–	1900 ± 150[1]
S-PIA	–	> 75 000 (3)[2]
CGS 21680	–	–

Antagonist	*Cyclic AMP inhibition*	*PI turnover potentiation*
DPCPX	6.0 ± 2.3 (3)	12 ± 3 (8)
XAC	52 ± 10 (3)	17 ± 8 (4)
PD 115,199	521 ± 109 (4)	640 ± 133 (3)

[1]Data taken from Hill & Kendall (1987). [2]The augmentatory response to S-PIA failed to reach an asymptote at concentrations up to 1 mM in two out of three experiments, while in the third an EC_{50} value of 75 μM could be calculated. Data are means ± s.e.mean of the number of experiments shown in parentheses. For abbreviations, see text.

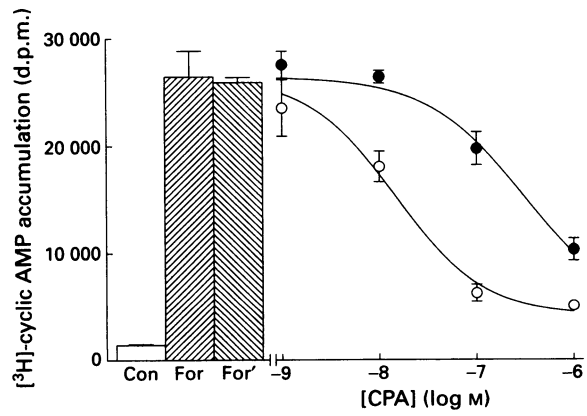

Figure 3 Antagonism by DPCPX of the CPA-elicited inhibition of forskolin-stimulated cyclic AMP accumulation. [^{3}H]-adenine-prelabelled slices were pre-incubated in the absence (○) or presence (●) of 50 nM DPCPX before the addition of increasing concentrations of CPA immediately prior to addition of 30 μM forskolin. Also shown are the basal response (Con) and the response to 30 μM forskolin in the absence (For) and presence (For') of 50 nM DPCPX. Results are means ± s.e.mean of a single experiment. Essentially identical results were obtained on two further occasions.

Augmentation of histamine-stimulated phosphoinositide turnover

We have previously reported potencies of a number of adenosine analogues for augmentation of H_1 histamine receptor-evoked phosphoinositide turnover in guinea-pig cerebral cortical slices (Hill & Kendall, 1987). In the present investigation, we further assayed the potency of a number of these agents together with two further analogues, CCPA and S-PIA. Using data from these two series of experiments, agonist potency at this adenosine receptor was observed to be CPA ≥ CCPA ≥ R-PIA = NECA > 2CA > S-PIA (Table 3). Antagonist apparent affinities at this receptor were in the rank order DPCPX ≥ XAC > PD 115,199 (Table 3).

Discussion

In this study, we compare the ability of adenosine receptor agonists and antagonists to displace binding of [^{3}H]-DPCPX to guinea-pig cerebral cortical membranes and slices, with their activity at functional adenosine receptor responses in intact tissue slices. The three antagonists show good correlation of receptor affinity estimates in these paradigms, while the agonists may be divided into A_1-selective and non-selective groups based on the functional responses to these agents.

Radioligand binding to membranes

Results presented here show that DPCPX exhibits high affinity for the A_1 receptor of guinea-pig cerebral cortex with a K_i of 4 nM and a maximal capacity of 1410 fmol mg^{-1} protein (Table 1). These values compare well with other investigations of [^{3}H]-DPCPX binding in guinea-pig cerebral cortex (Stroeher *et al.*, 1989; Klotz *et al.*, 1991). The xanthine-derived antagonists exhibited a monophasic displacement of [^{3}H]-DPCPX binding with a rank order of potency of DPCPX > XAC > PD 115,199, consistent with the binding site labelled being the A_1 adenosine receptor. Agonist displacement of [^{3}H]-DPCPX was biphasic, a phenomenon expected for a G-protein-coupled receptor, with a rank order of potency at the low affinity site of CCPA = CPA = R-PIA > 2CA = NECA > S-PIA. The higher potency of N^6-adenosine analogues CCPA, CPA and R-PIA compared to the 5'- derivative NECA, together with the high degree of stereoselectivity exhibited for the isomers of PIA, are consistent with the binding site being the A_1 adenosine receptor (Jacobson *et al.*, 1992).

Radioligand binding to cerebral cortical slices

In cerebral cortical slices, [^{3}H]-DPCPX was observed to bind with relatively high affinity (ca. 14 nM) and high capacity (ca. 4100 fmol mg^{-1} protein). This latter value is subject to some error, since protein content was not assayed in each tube, and was likely to be variable, no doubt increasing the variability of the parameters described for radioligand binding in slices. The binding capacity in slices is expressed relative to total protein content and not simply particulate protein as is the case for the binding carried out in cell-free preparations (see above, 1410 fmol mg^{-1} protein), and is therefore likely to be an underestimate of binding capacity expressed per unit membrane protein. Increases in affinity and capacity are commonly found when comparing binding data in slices and in cell-free preparations (Shaw & Wilkinson, 1994). Nevertheless, the competition data for radioligand binding in slices is in good agreement with data obtained with the particulate preparation. Significantly, binding parameters for the agonists, R- and S-PIA showed closer

agreement with K_is identified for the low affinity site in the particulate preparation, a phenomenon also observed for A_1 receptors in intact cells and particulate preparations of the DDT1 MF-2 cell line (Gerwins *et al.*, 1990). It is likely that the presence of intracellular GTP allows only the low affinity state of the A_1 receptor to be visualised using steady-state radioligand binding.

It should also be noted that the non-displaceable binding observed with this technique was rather higher than that obtained with membranes (typical values of 25–35% and 5–10% of total binding, respectively).

Inhibition of forskolin-stimulated cyclic AMP accumulation

Forskolin-stimulated cyclic AMP generation was inhibited by three adenosine analogues (CPA, CCPA and **R**-PIA) but not NECA, 2CA or S-PIA, although all six analogues competed for [^{3}H]-DPCPX binding. However, the three 'active' analogues were more potent at competing for [^{3}H]-DPCPX binding than the three 'inactive' compounds (Table 2). It appears likely that, since all six compounds stimulate cyclic AMP formation at higher concentrations, all six are agonists at the A_{2b} adenosine receptor linked to cyclic AMP generation in guinea-pig cerebral cortex (Alexander *et al.*, 1994). Thus, the 'active' analogues probably exhibit greater differences in potency for A_1 versus A_{2b} receptors than the 'inactive' analogues. The affinity of the three 'active' analogues for [^{3}H]-DPCPX binding to the high affinity site of the A_1 receptor is similar to the IC_{50}s for agonist inhibition of cyclic AMP generation. A similar observation was made in DDT_1 MF-2 cells (Gerwins *et al.*, 1990), where **R**-PIA inhibited β-adrenoceptor-stimulated cyclic AMP generation with an IC_{50} value of 0.4 nM, and competed for [^{3}H]-DPCPX binding in cell membranes with K_i values of 1.7 and 93 nM. Interestingly, however, inhibition of [^{3}H]-DPCPX binding to intact cells with **R**-PIA revealed only a single site with a K_i value of 77 nM. The low affinity of **R**-PIA in intact cells presumably reflects high intracellular levels of GTP leading to uncoupling of the G-protein from the receptor and formation of the low agonist affinity state.

The cyclic AMP response to 30 μM forskolin was inhibited by more than 80% by the three N^6-derivatives of adenosine, CPA, CCPA and **R**-PIA. A similar maximal inhibition (>90%) is observed when forskolin-stimulated cyclic AMP formation is inhibited by metabotropic glutamate receptor agonists (Cartmell *et al.*, 1992). The question therefore arises as to whether 90% of cells in the guinea-pig cerebral cortex express A_1 adenosine receptors or metabotropic glutamate receptors. Intuitively, one would suggest that this is unlikely, and that it is more likely that the stimulation of cyclic AMP by forskolin (which is not maximal at 30 μM, data not shown) may be selective, in that a subpopulation of cells and/or adenylyl cyclase isoforms may be activated by forskolin. These possibilities require further investigation.

It has been suggested that adenosine antagonists may have 'negative efficacy' effects on A_1 adenosine receptor function (Parsons *et al.*, 1988; Ramkumar & Stiles, 1988a,b). Also, GTP analogues have been observed to enhance antagonist radioligand binding to A_1 adenosine receptors (Stiles, 1988; Klotz *et al.*, 1990). If this were the case, then we would observe an enhancement of forskolin-stimulated cyclic AMP formation in guinea-pig cerebral cortical slices in the presence of DPCPX, XAC and PD 115,199. However, this was not the case (data not shown). Therefore, we would support the hypothesis of Prater *et al.* (1992), who suggested that the effects of guanine nucleotide on antagonist radioligand binding were due to release of endogenous adenosine from 'cryptic' binding sites in vesicles hidden from adenosine deaminase action. In the radioligand binding assays in the present study, we have included a low concentration of the detergent Triton X-100 to improve access of adenosine deaminase (Schiemann *et al.*, 1990).

Comparison of data from functional and radioligand binding assays

It is common for the selectivity of compounds to be inferred from radioligand binding assays, to the extent that agents may be given *in vivo* at relatively large doses, and functional responses ascribed to particular receptor subtypes. In the case of adenosine receptors, it is clear from the present results that CCPA, CPA and **R**-PIA are A_1 selective (eliciting an inhibition of forskolin-stimulated cyclic AMP generation) in their action at concentrations in the nanomolar range. However, at higher concentrations, these agents were able to enhance cyclic AMP generation, presumably through activating A_{2b} receptors. Thus, in different tissues exhibiting a higher relative density of A_{2b} receptors (at the present time it is not possible to define A_{2b} receptor density due to the lack of a radioligand for this receptor), it is possible that the functional inhibition of cyclic AMP generation by the three A_1-selective agents might be lost. This may possibly explain our recent findings that CPA fails to inhibit forskolin-stimulated cyclic AMP generation in slices from the guinea-pig cerebellum (Hernández *et al.*, 1993).

In the case of the adenosine receptor antagonists as opposed to the agonists, there should be greater parity between affinity defined by radioligand binding techniques compared to functional assays. Comparing antagonist affinities in the various assays, DPCPX affinity varies between 4–14 nM, while PD 115,199 and XAC exhibited greater variation (521–666 and 17–52 nM respectively). However, these variations appear to be within the experimental error of the assays conducted, and it appears justifiable to assume that the antagonist affinities are not different in the different assays. One potential explanation for the greater variation of PD 115,199 and XAC compared to DPCPX, concerns the charges of the sidechains of the compounds which are all based on a 1,3-dipropylxanthine core. The 8-substituent of DPCPX (a cyclopentyl group) is uncharged, while XAC and PD 115,199 are charged at neutral pH. Thus, although binding in both membranes and slices was carried out at pH 7.4, factors such as ionic strength and sequestration of antagonist in the tissues may lead to alterations in the distribution of XAC and PD 115,199 in the intact slice preparations, and hence their apparent affinity. Since the receptor is an integral membrane protein subject to influence by agents from both cytoplasmic and extracellular faces of the plasma membrane, it may not be possible to replicate the same conditions in the broken cell particulate preparation which the receptor experiences in the slice preparation.

A further difference between radioligand binding and functional approaches to establishing antagonist affinities is the concentrations of agents used relative to their affinities. Thus, in the radioligand binding assays, accurate determination of K_is relies heavily on the linear portion of the graph, which is most likely to be spread less than one log unit either side of the K_i (or IC_{50}). In the functional assays, accurate determinations of K_is can only be effected using concentrations of antagonists at least 10 fold the K_i value. Therefore, should sequestration of compounds occur it is more likely to underestimate the K_i determined using lower concentrations of antagonist, that is, the radioligand binding assay.

A further potential source of error arises from the assumption that these agents are competitive antagonists. Proper Schild analysis of the functional A_1 inhibition of cyclic AMP over a 2–3 log unit range of antagonist concentrations is not possible in this tissue, since a greater than 10 fold rightward shift of the agonist concentration-response curves will result in concentrations of agonist being required which are active in the A_{2b} activation range leading to stimulation of cyclic AMP generation, thereby confounding the experiment.

Modulation of histamine-stimulated phosphoinositide turnover

All the adenosine analogues tested exhibited the ability to enhance the accumulation of [^{3}H]-inositol phosphates stimulated by histamine H_1-receptor activation (Table 3, see also Hill & Kendall, 1987). When potencies for the N^6-analogues are compared for inhibition of forskolin-stimulated cyclic AMP generation and phosphoinositide turnover potentiation, it is apparent that these agents are approximately one order of magnitude more potent in the cyclic AMP assay. This was not a factor of the incubation period of the assay since preliminary results indicated a similar inhibitory response to 100 nM CPA after prolonged incubation in the presence of forskolin (data not shown). The rank order of agonist potency appears to suggest an A_1-type adenosine receptor since the N^6-derivatives of adenosine CPA and CCPA were more potent than NECA and 2CA. Similarly, the S-isomer of N^6-phenylisopropyladenosine was much less potent than the R-isomer, again indicating that the adenosine receptor responsible for the potentiation of the H_1 receptor response was of the A_1 class. Antagonist rank order of potency at these two adenosine receptors is identical for the three antagonists investigated.

The mechanism of potentiation of histamine-stimulated phosphoinositide turnover remains to be defined, but it is attractive to hypothesize that the G-protein through which the A_1 receptor couples to elicit inhibition of adenylyl cyclase also mediates the enhancement of the histamine response. The latter response may require the recruitment of more intermediary G-proteins than the latter, and this may therefore underlie the difference in agonist potency (and presumably occupancy) between the two systems. It is relevant to note, therefore, that A_1 receptors transfected into Chinese hamster ovary cells and in DDT1 MF-2 hamster vas deferens smooth muscle cells couple to phosphoinositide turnover and calcium mobilisation through a pertussis toxin-sensitive mechanism (Dickenson & Hill, 1993; Iredale *et al.*, 1994).

Concluding remarks

In the present study, we show that functional A_1 adenosine receptors may be demonstrated in intact brain slices from the guinea-pig and that using this method there is good coincidence of apparent antagonist affinity compared with radioligand binding studies. There is also good correlation in apparent antagonist affinity between the A_1 receptor-induced inhibition of cyclic AMP generation compared to the A_1-type receptor which is responsible for potentiation of histamine-evoked phosphoinositide turnover.

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