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Characterization of the human brain putative A_{2B} adenosine receptor expressed in Chinese hamster ovary ($CHO.A_{2B4}$) cells

[1]Stephen P.H. Alexander, Jacqui Cooper, *John Shine & Stephen J. Hill

Department of Physiology & Pharmacology, University of Nottingham Medical School, Queen's Medical Centre, Nottingham NG7 2UH, U.K. and *Garvan Institute of Medical Research, St Vincent's Hospital, Sydney, New South Wales, Australia

1 An [^{3}H]-adenine pre-labelling methodology was employed to assay cyclic AMP generation by adenosine analogues in Chinese hamster ovary ($CHO.A_{2B4}$) cells, transfected with cDNA which has been proposed to code for the human brain A_{2B} adenosine receptor, and in guinea-pig cerebral cortical slices.

2 Adenosine analogues showing the following rank order of potency in the $CHO.A_{2B4}$ cells (pD_2 value): 5'-N-ethylcarboxamidoadenosine (NECA, 5.91) > adenosine (5.69) > 2-chloroadenosine (5.27) > N^6-(2-(4-aminophenyl)-ethylamino)adenosine (APNEA, 4.06). The purportedly A_{2A}-selective agonist, CGS 21680, failed to elicit a significant stimulation of cyclic AMP generation at concentrations up to 10 μM in $CHO.A_{2B4}$ cells. In the guinea-pig cerebral cortex, NECA was more potent than APNEA with pD_2 values of 5.91 and 4.60, respectively.

3 Of these agents, NECA was observed to exhibit the greatest intrinsic activity in $CHO.A_{2B4}$ cells (ca. 10 fold stimulation of cyclic AMP), while, in comparison, maximal responses to adenosine (32% NECA response), 2-chloroadenosine (61%), and APNEA (73%) were reduced.

4 Antagonists of NECA-evoked cyclic AMP generation showed the rank order of apparent affinity (apparent pA_2 value in $CHO.A_{2B4}$ cells: guinea-pig cerebral cortex): XAC (7.89: 7.46) > CGS 15943 (7.75: 7.33) > DPCPX (7.16: 6.91) > PD 115,199 (6.95: 6.39) > 8FB-PTP (6.52: 6.55) > 3-propylxanthine (4.63: 4.59).

5 We conclude that, using the agents tested, the A_{2B} adenosine receptor cloned from human brain expressed in Chinese hamster ovary cells exhibits an identical pharmacological profile to native A_{2B} receptors in guinea-pig brain.

Keywords: A_{2B} adenosine receptors; cyclic AMP; human brain; $CHO.A_{2B4}$ cells

Introduction

A_2 adenosine receptors are regarded as coupling almost exclusively to stimulation of adenylyl cyclase activity and the production of adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Fredholm *et al.*, 1994). Subtypes of brain A_2 receptor were originally identified on the basis of regional selectivity, persistence of the stimulatory response in cell-free preparations compared to tissue slices and the potency of adenosine (Daly *et al.*, 1983). Indeed this latter property led to the original classification of these subtypes as high and low affinity A_2 adenosine receptors, since re-named A_{2A} and A_{2B} subtypes, respectively (Fredholm *et al.*, 1994). A number of agents have been developed which exhibit some degree of selectivity for the A_{2A} compared to the A_{2B} adenosine receptor, including the agonists CGS 21680 (Lupica *et al.*, 1990) and APEC (Fredholm *et al.*, 1994), and the antagonist PD 115,199 (Bruns *et al.*, 1987b). For example, in human platelets, NECA and CGS 21680 show a similar high potency ($pD_2 > 6$) for stimulation of cyclic AMP generation and inhibition of 5-hydroxytryptamine (5-HT) release (Cooper *et al.*, 1995b). PD 115,199 also showed high affinity for antagonism of 5'-N-ethylcarboxamidoadenosine (NECA)-evoked responses in human platelets with an apparent pA_2 value of approximately 7.5. This pharmacology therefore is positive evidence for the presence of an A_{2A} adenosine receptor subtype on human platelets (Cooper *et al.*, 1995b).

As yet there are no agents which exhibit selectivity for the A_{2B} subtype, and consequently A_{2B} adenosine receptors are typically identified using 'negative evidence', that is, by the reduced activity of A_{2A}-selective agents at cyclic AMP-stimulating adenosine receptors. For example, A_{2B} adenosine receptors which we have recently characterized in rat astrocytes and guinea-pig brain mediating a stimulation of cyclic AMP accumulation were unresponsive to concentrations of CGS 21680 below 10 μM (Hernández *et al.*, 1993; Alexander *et al.*, 1994b; Peakman & Hill, 1994). Similarly, PD 155,199 (A_1 : A_{2A}-selective) and DPCPX (A_1-selective) were poor antagonists at these receptors.

In tissue slices from the human brain taken at surgery, non-selective adenosine receptor agonists evoked cyclic AMP responses greater than those to forskolin or agonists of β-adrenoceptors or histamine receptors (Kendall *et al.*, 1992). However, analysis of adenosine receptors in 'vital' human brain samples is necessarily hindered by problems of tissue availability and the heterogeneity in the life history of the donors. These problems may be overcome through the use of molecular biological techniques which allow the cloning and expression of the protein of interest in naive host cells. We have therefore examined the pharmacological profile of a clone of the putative human brain A_{2B} adenosine receptor (Pierce *et al.*, 1992) expressed in the Chinese hamster ovary cell line. The preliminary pharmacology previously reported for this entity indicated that it was not an A_1 or A_{2A} adenosine receptor since an A_1-selective agonist (2-chloro-N^6-cyclopentyladenosine) and an A_{2A}-selective agonist (CGS 21680) were ineffective, whilst the non-selective adenosine receptor agonist, NECA, showed a robust cyclic AMP response (Pierce *et al.*, 1992). This response was reversed by the archetypal adenosine receptor antagonist, theophylline, which, however shows no selectivity between adenosine receptor subtypes (Daly *et al.*, 1983). In this paper, we have investigated the activity of a range of adenosine receptor agonists and antagonists to determine whether the human receptor expressed in CHO-K1 cells exhibits a pharmacological prolife comparable to the A_{2B} adenosine receptor of the guinea-pig brain (for which no sequence data are cur-

[1] Author for correspondence.

rently available). A preliminary account of some of these findings has previously been presented to the British Pharmacological Society (Cooper *et al.*, 1995a).

Methods

Cell culture

Chinese hamster ovary (CHO.K1) cells transfected with cDNA coding for the putative human A_{2B} adenosine receptor were generated as previously described (Pierce *et al.*, 1992) and grown in Dulbecco's Modified Eagles medium/Nutrient Mix F12 (1:1) supplemented with 2 mM L-glutamine and 10% foetal calf serum at 37°C in humidified air: CO_2 (95:5). Experiments were performed on confluent monolayers in 24 well cluster dishes.

[^{3}H]-cyclic AMP accumulation in CHO.A_{2B4} cells

[^{3}H]-cyclic AMP was monitored in [^{3}H]-adenine-prelabelled CHO.A_{2B4} cells as previously described (Peakman & Hill, 1994). Briefly, cells were incubated with [^{3}H]-adenine (74 kBq ml^{-1}) for 2 h at 37°C. Medium was then removed and replaced with fresh medium containing the phosphodiesterase inhibitor, rolipram (100 μM), and where indicated, adenosine receptor antagonists. After 15 min, agonist was added and the incubation continued for a further 15 min, before stopping with HCl. [^{3}H]-cyclic AMP was quantified by the dual column methodology of Salomon *et al.* (1974) using [^{14}C]-cyclic AMP as a recovery marker, and initially expressed as [^{3}H]-cyclic AMP production as a percentage conversion from total [^{3}H]-adenine nucleotides.

[^{3}H]-cyclic AMP accumulation in slices from the guinea-pig cerebral cortex

[^{3}H]-cyclic AMP was monitored in [^{3}H]-adenine-prelabelled slices (350 × 350 μm) from guinea-pig (Dunkin-Hartley, 250–270 g, either sex) cerebral cortex as previously described (Alexander *et al.*, 1994b). Briefly, slices were incubated with [^{3}H]-adenine (74 kBq ml^{-1}) for 40 min at 37°C. After careful washing, slices were incubated in the absence of phosphodiesterase inhibitors, and where indicated, adenosine receptor antagonists. After 10–15 min, agonist was added and the incubation continued for a further 15 min, before stopping with 1 M HCl. [^{3}H]-cyclic AMP was quantified as above, and expressed initially as a percentage conversion from total [^{3}H]-adenine nucleotides.

Data analysis

Experiments were conducted on at least three separate cell or tissue preparations. Sigmoidal curves were fitted to concentration-response data (after subtraction of basal cyclic AMP accumulation) using the computer programs Prism and InPlot (GraphPad, California, U.S.A.) to generate estimates of EC_{50} and E_{max} values:

$$\text{Response} = \frac{E_{max} \cdot X^{n_H}}{EC_{50}^{n_H} + X^{n_H}}$$

where X is the drug concentration, E_{max} is the maximal response, and n_H is the Hill coefficient. pD_2 values are defined as the negative log of the EC_{50} values. Antagonist IC_{50} values (CHO.A_{2B4} cells) were calculated by use of the same equation (substituting IC_{50} for EC_{50}) to fit a curve for increasing antagonist concentrations in the presence of a fixed concentration of NECA (10 μM, Peakman & Hill, 1994). Antagonist pA_2 values ($-\log K_i$ values) were then calculated from the IC_{50} values using the null method described by Peakman & Hill (1994), assuming competitive antagonism. Using tissue slices from the guinea-pig cerebral cortex, antagonist affinities were assessed at fixed antagonist concentrations (assuming competitive antagonism), observing the shift in the agonist concentration-response curve, calculating the pA_2 values using the equation:

$$pA_2 = \log (DR - 1) - \log (A)$$

where DR is the dose ratio of the EC_{50} values of NECA in the absence and presence of a fixed concentration, A, of antagonist.

Chemicals

PD 115,199 (N-[2-(dimethylamino)ethyl]N-methyl-4-(1,3-dipropylxanthine)benzenesulphonamide) was a gift from Warner-Lambert, Michigan, U.S.A.; 8FB-PTP (5-amino-8-(4-fluorobenzyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine) was donated by Dr Franco Gatta, Istituto Superiore de Sanità, Rome, Italy. APNEA (N^6-(2-(4-Aminophenyl)ethyl)-adenosine) was synthesized in the Department of Pharmaceutical Science, Nottingham University, by Dr E.A. Boyd. CGS 21680 (2-[*p*-(-carboxyethyl)-phenethylamino]-5'-N-ethylcarboxamidoadenosine), NECA (5'-N-ethylcarboxamidoadenosine), 2CA (2-chloroadenosine), DPCPX (8-cyclopentyl-1,3-dipropylxanthine), 3-propylxanthine, CGS 15943 (5-amino-9-chloro-2-(2-furyl)1,2,4-triazolo[1,5-c]quinazoline) and XAC (xanthine amine congener) were obtained from RBI Semat, Herts, U.K. All other chemicals were obtained from either Sigma Chemicals, Dorset, U.K. or Fisons Chemicals, Leics, U.K.

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

Results

Cyclic AMP generation in CHO.A_{2B4} cells

Basal accumulation of [^{3}H]-cyclic AMP in CHO.A_{2B4} cells was 0.25±0.03% conversion from total [^{3}H]-adenine nucleotides. In the presence of adenosine and its analogues, a concentration-dependent elevation of [^{3}H]-cyclic AMP accumulation was observed, with a rank order of pD_2 values of NECA > adenosine > 2CA > APNEA (Figure 1a, Table 1). The maximal response to NECA was approximately 10 fold over basal (2.46±0.21% conversion), while responses to adenosine (0.89±0.17% conversion), 2CA (1.92±0.05% conversion) and APNEA (2.25±0.43% conversion) appeared reduced compared to NECA (Table 1). The A_{2A}-selective agonist, CGS 21680 (Lupica *et al.*, 1990) failed to stimulate cyclic AMP generation significantly at concentrations below 10 μM (Figure 1a). Analysis of concentration-response profiles to adenosine in the presence of the purine nucleoside uptake inhibitor, dipyrimadole (1 μM), showed no change in basal cyclic AMP levels (control 0.26±0.02; dipyrimadole 0.26±0.02% conversion; $n=3$), pD_2 (control 5.38±0.56; dipyrimadole 5.76±0.18; $n=3$) or maximal response to adenosine (control 39±6; dipyrimadole 49±5% 100 μM NECA response; $n=3$).

Responses to a sub-maximally effective concentration of NECA (3–10 μM) were inhibited in the presence of adenosine receptor antagonists. Illustrated in Figure 1b are responses to the antagonists XAC, PD 115,199 and DPCPX, which we have previously used in tissues from the guinea-pig to define A_1, A_{2A} and A_{2B} adenosine receptor subtypes (Hernández *et al.*, 1993; Alexander *et al.*, 1994a,b; Alexander, 1995). A more thorough study of adenosine receptor antagonists of varying selectivities showed the rank order of apparent pA_2 values: XAC > CGS 15943 > DPCPX > PD 115,199 > 8FB-PTP > 3-propylxanthine (Table 2).

Cyclic AMP generation in the guinea-pig cerebral cortex

We have previously reported on the effects of a number of adenosine analogues on cyclic AMP generation in the guinea-pig cerebral cortex (Alexander *et al.*, 1994a,b). In this tissue, NECA is the most potent agonist followed by 2CA and adenosine (Alexander *et al.*, 1994b). In the present investigations, APNEA evoked a concentration-dependent increase in the accumulation of [^{3}H]-cyclic AMP with a calculated pD_2 value of 4.60 (Figure 2a, Table 1). The maximal response to APNEA was slightly, but not significantly reduced compared to 100 μM NECA (Table 1).

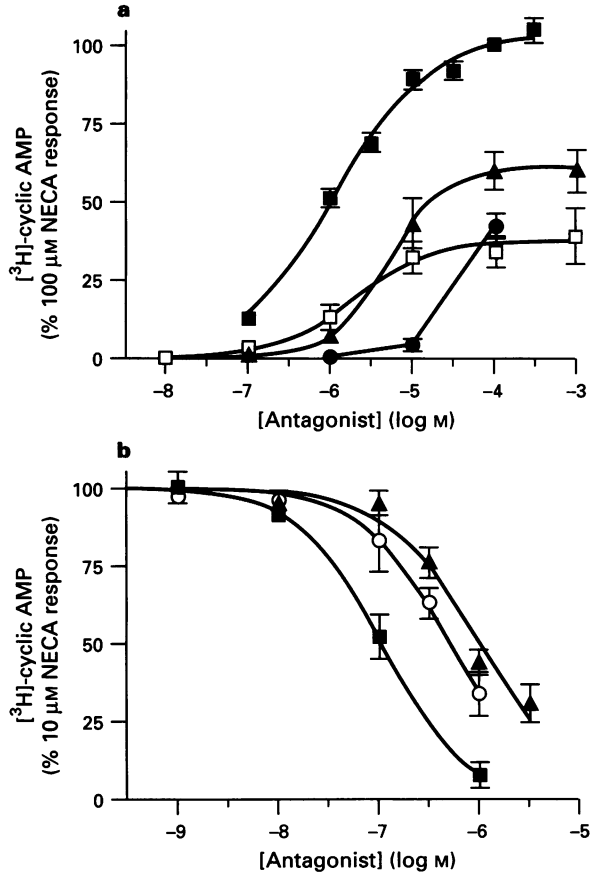

Figure 1 Generation of cyclic AMP accumulation in CHO.A_{2B4} cells in the presence of adenosine analogues. (a) The effect of increasing concentrations of NECA (■), 2CA (▲), adenosine (□) and CGS 21680 (●) on cyclic AMP generation. Data are means±s.e.mean of 3–16 experiments conducted in triplicate, expressed as a percentage of the response to 100 μM NECA. (b) Inhibition of 10 μM NECA-induced cyclic AMP generation in CHO.A_{2B4} cells in the presence of XAC (■), PD 115,199 (○) and DPCPX (▲). Data are means±s.e.mean of 3–4 experiments conducted in triplicate.

We had previously estimated antagonist apparent affinity constants for several xanthine-derived agents in the guinea-pig cerebral cortex with a rank order of affinity of XAC > DPCPX > PD 115,199 (Alexander *et al.*, 1994a,b). In the present investigation, we continued this characterization by assessing shifts in the concentration-response curve to NECA using fixed concentrations of CGS 15943 (0.3–1 μM), 8FB-PTP (3–10 μM), and 3-propylxanthine (100–500 μM) (see Figure 2b). Together with the previously published investigations of adenosine receptor antagonist affinities a rank order of apparent pA_2 values was established: XAC > CGS 15943 > DPCPX > PD 115,199 > 8FB-PTP > 3-propylxanthine (Table 2).

Discussion

In this investigation, we have undertaken a detailed examination of the pharmacological profile of the putative human brain A_{2B} adenosine receptor transfected into Chinese hamster ovary (CHO.A_{2B4}) cells. Previously, this clone had been shown to display characteristics incompatible with A_1, A_{2A} or A_3 adenosine receptors, that is the lack of activity of A_1 and A_{2A} subtype-selective agonists, and inhibition of the response by a methylxanthine (Pierce *et al.*, 1992). In the present investigation, comparison of the activities of a range of adenosine receptor agonists and antagonists of this receptor (in CHO.A_{2B4} cells) with the native A_{2B} adenosine receptor of guinea-pig cerebral cortex confirms the identity of the two receptor responses.

A human brain A_{2B} adenosine receptor

A_2 receptors are commonly associated with the stimulation of cyclic AMP generation, while A_1 and A_3 subtypes are proposed to couple principally to inhibition of adenylyl cyclase activity (Fredholm *et al.*, 1994). A_{2B} adenosine receptors are most frequently distinguished from A_{2A} adenosine receptors by the inactivity of agents used to define other subtypes of adenosine receptor. Indeed, this was the main criterion used to suggest

Table 2 Apparent pA_2 values for antagonists at A_{2B} adenosine receptors

Antagonist	*CHO.A_{2B4} cells*	*Guinea-pig cerebral cortex*
XAC	7.89±0.02	7.46[1]
CGS 15943	7.75±0.09	7.33±0.24
DPCPX	7.16±0.12	6.91[1]
PD 115,199	6.95±0.10	6.39[1]
8FB-PTP	6.52±0.04	6.55±0.12
3-Propylxanthine	4.63±0.12	4.59±0.05

[1] Alexander *et al.*, 1994b. Data are means±s.e.mean of 3–5 determinations conducted in triplicate (CHO.A_{2B4} cells) or quadruplicate (guinea-pig cerebral cortex).

Table 1 Potency and intrinsic activity of adenosine and its analogues at A_{2B} adenosine receptors

	CHO.A_{2B4} cells		*Guinea-pig cerebral cortex*	
Agonist	*Agonist potency* (pD_2 value)	*Maximal response* (% 100 μM NECA response)	*Agonist potency* (pD_2 value)	*Maximal response* (% 100 μM NECA response)
NECA	5.91±0.06		5.91±0.18	
Adenosine	5.69±0.08	32±5%	3.99±0.06[1]	71±6%[1]
2CA	5.27±0.08	61±6%	4.35±0.07[1]	97±6%[1]
APNEA	4.06±0.09	73±7%	4.60±0.14	83±7%

[1] Alexander *et al.*, 1994b. Data are means±s.e.mean of 3–16 experiments conducted in triplicate (CHO.A_{2B4} cells) or quadruplicate (guinea-pig cerebral cortex).

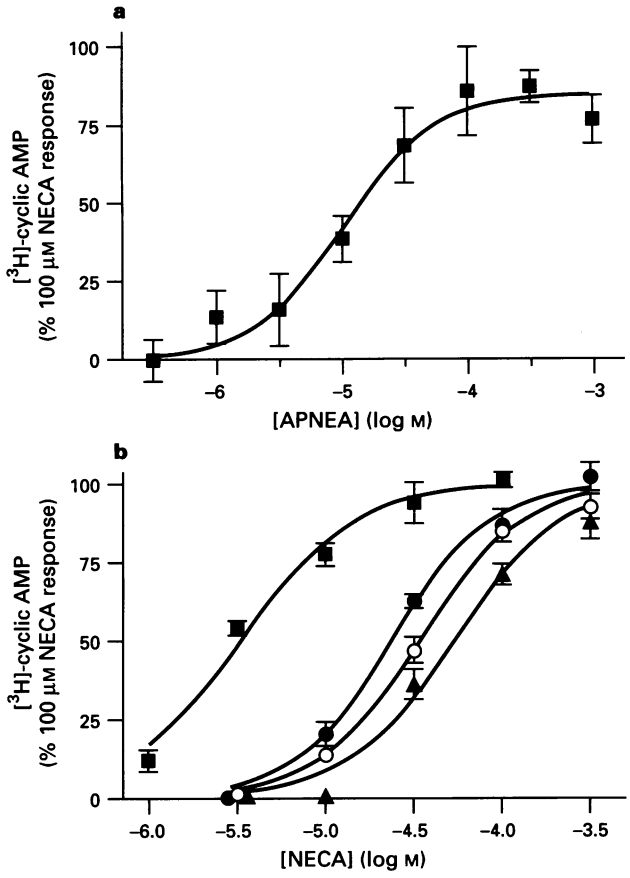

Figure 2 Generation of cyclic AMP accumulation in the guinea-pig cerebral cortex in the presence of adenosine analogues. (a) The effect of increasing concentrations of APNEA on cyclic AMP generation. Data are means ± s.e.mean from a single experiment representative of 4 experiments conducted in quadruplicate, expressed as a percentage of the response to 100 μM NECA. (b) The effect of increasing concentrations of NECA on cyclic AMP generation. Concentration-response curves to NECA were constructed in the absence (■) and in the presence of 3-propylxanthine (500 μM, ●), CGS 15943 (500 nM, ○) or 8FB-PTP (3 μM, ▲). Data are means ± s.e.mean from a single experiment representative of 4 experiments conducted in quadruplicate, expressed as a percentage of the response to 100 μM NECA.

that the clone examined in the present study was an A_{2B} adenosine receptor (Pierce *et al.*, 1992). Of the A_2 receptor ligands, the A_{2A}-selective agonist, CGS 21680 (Lupica *et al.*, 1990) exhibited low potency as a stimulus for cyclic AMP generation in CHO.A_{2B4} cells (present study and Pierce *et al.*, 1992). The potency of 2-chloroadenosine at the transfected human brain A_{2B} adenosine receptor (pD_2 value 5.27) is comparable with its potency in human cerebral cortex (ca. 5.3, Kendall *et al.*, 1992), guinea-pig cerebral cortical (5.00, Alexander *et al.*, 1994b) and cerebellar (5.22, Hernández *et al.*, 1993) slices. The potency of adenosine is increased at the transfected human brain A_{2B} adenosine receptor (5.69) compared to the values previously obtained in the guinea-pig brain (3.96–4.89, Hernández *et al.*, 1993; Alexander *et al.*, 1994b). It is likely that the figures from brain slice experiments are underestimates of the potency of adenosine, however, since they were conducted in the absence of uptake inhibitors. Preliminary experiments provided evidence for the lack of significant effects of adenosine uptake in CHO.A_{2B4} cells, since the inclusion of 1 μM dipyridamole failed to alter significantly the concentration-response profile to adenosine (see above).

Thus, analysis of the agonist profile suggests the receptor expressed in CHO.A_{2B4} cells has the pharmacological profile expected of a typical A_{2B} adenosine receptor. Perusal of the antagonist data suggests a similar conclusion. The relatively high affinities of the xanthine-based compounds which are effective in antagonizing the NECA-evoked cyclic AMP generation in the current study indicate that its pharmacology differs significantly from that of the A_3 subtype, since the latter appears to exhibit low affinity for many xanthine derivatives (Fredholm *et al.*, 1994). The low affinity of the A_1-selective antagonist, DPCPX (Bruns *et al.*, 1987a; Lohse *et al.*, 1987) and that of antagonists which have high affinity at A_{2A} receptors (PD 115,199 (Bruns *et al.*, 1987b); 8FB-PTP (Dionisotti *et al.*, 1994)) provide strong support for the original identification of the receptor expressed in CHO-K1 cells as an A_{2B} adenosine receptor.

Comparisons with other A_{2B} adenosine receptors

The non-selective adenosine analogue, NECA, showed the highest potency of the agonists examined with a pD_2 value of 5.91 in both CHO.A_{2B4} cells and guinea-pig cerebral cortex. This is similar to estimates we have previously made for the A_{2B} adenosine receptor in the guinea-pig cerebral cortex (ca. 5.5, Alexander *et al.*, 1994b), cerebellum (ca. 6.2, Hernández *et al.*, 1993) and aorta (ca. 6.2, Alexander *et al.*, 1994b), and rat astrocytes *in vitro* (ca. 5.9, Peakman & Hill, 1994). The pD_2 values for 2CA and APNEA are also similar in the CHO.A_{2B4} cells and in the guinea-pig cerebral cortex. However, comparison of agonist potencies between tissues and indeed between distinct responses (e.g. second messenger generation and smooth muscle contractility) in the same tissue is fraught with problems, such as differences in receptor reserve. Indeed, it is notable that the other adenosine analogues do not produce full agonism at adenosine receptors in either CHO.A_{2B4} cells (2CA, adenosine and APNEA; Table 1) or in the guinea-pig brain (2CA and adenosine; Hernández *et al.*, 1993; Alexander *et al.*, 1994b). It is usually more reliable to compare receptors through the analysis of antagonist apparent affinities. In this respect, the human brain A_{2B} adenosine receptor appears to be almost identical to A_{2B} adenosine receptors of guinea-pig cerebral cortex (Table 2). Comparison of the antagonist affinities in CHO.A_{2B4} and guinea-pig cerebral cortex using linear regression analysis provides an estimated slope of 1.12 with an r^2 value of 0.97, indicating the high degree of similarity between the two adenosine receptors.

Concluding remarks

The cloning of the human brain A_{2B} adenosine receptor, a receptor which appears to elicit the greatest receptor-evoked cyclic AMP generation in human brain slices, has allowed its pharmacological profile to be evaluated. The similarities between the transfected receptor and native A_{2B} adenosine receptors from guinea-pig brain confirm that the clone represents the human homologue of the A_{2B} adenosine receptor previously identified pharmacologically in the guinea-pig brain, guinea-pig aorta and rat astrocytes (Hernández *et al.*, 1993; Alexander *et al.*, 1994b; Peakman & Hill, 1994).

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