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# An endogenous $A_{2B}$ adenosine receptor coupled to cyclic AMP generation in human embryonic kidney (HEK 293) cells

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**1** Cyclic AMP generation by adenosine analogues was examined in human embryonic kidney (HEK 293) cells by use of a [$^3$H]-adenine pre-labelling methodology.

**2** Adenosine analogues showed the following rank order of potency ($pD_2$ value): 5′-*N*-ethylcarboxamidoadenosine (NECA, 5.24) > 2-chloroadenosine (4.41) ⩾ adenosine (4.19) = $N^6$-(2-(4-aminophenyl)-ethylamino)adenosine (APNEA, 4.11). The $A_{2A}$-selective agonist CGS21680 failed to elicit a significant stimulation of cyclic AMP generation at concentrations below 30 $\mu$M.

**3** Of these agents, NECA was observed to exhibit the greatest intrinsic activity, while in comparison maximal responses to adenosine (76±8% NECA response), 2-chloroadenosine (70±6%) and APNEA (40±3%) were significantly reduced.

**4** Antagonists of the NECA-evoked cyclic AMP generation showed the rank order of apparent affinity (apparent $pA_2$ value): CGS 15943 (7.79) = XAC (7.74) > DPCPX (7.01) = PD115199 (6.93) = 8FB-PTP (6.80) > KF 17837 (5.98) > 3-propylxanthine (5.13).

**5** Agarose gel electrophoresis of the products of the polymerase chain reaction, with cDNA generated from HEK 293 cell total RNA showed virtually identical patterns and nucleotide sizes in comparison with the vector for the full length human brain $A_{2B}$ adenosine receptor.

**6** We concluded that HEK 293 cells express an endogenous adenosine receptor coupled to cyclic AMP generation which is of the $A_{2B}$ subtype.

**Keywords:** $A_{2B}$ adenosine receptors; human embryonic kidney (HEK 293) cells; cyclic AMP

## Introduction

Adenosine receptors may be divided into classes which are coupled to inhibition ($A_1$ and $A_3$) or stimulation ($A_{2A}$ and $A_{2B}$) of adenylyl cyclase activity (Fredholm *et al*., 1994). The latter classes are distinguishable on the basis of the greater potency for activation or antagonism of $A_{2A}$ adenosine receptors by the agonists CGS21680 (Lupica *et al*., 1990) and adenosine (Daly *et al*., 1983) and the antagonist PD115199 (Bruns *et al*., 1987b). For example, in human intact platelets, *N*-ethylcarboxamidoadenosine (NECA) and CGS21680 show a similar high potency ($pD_2 > 6$) for stimulation of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) generation (Feoktistov & Biaggioni, 1993; Cooper *et al*., 1995b) and inhibition of 5-hydroxytryptamine (5-HT) release (Cooper *et al*., 1995b). PD115199 displays high affinity as an antagonist of NECA-evoked responses in human platelets with an apparent $pA_2$ value of approximately 7.5. This pharmacology therefore suggests the presence of an $A_{2A}$ adenosine receptor subtype on human platelets (Feoktistov & Biaggioni, 1993; Cooper *et al*., 1995b). In general, $A_{2B}$ adenosine receptors have been classified on the basis of the reduced potency of agents selective for other adenosine receptor subtypes. For example, $A_{2B}$ adenosine receptors, which we have recently been characterized in rat astrocytes and guinea-pig brain, mediate stimulation of cyclic AMP accumulation and were unresponsive to concentrations of CGS21680 below 10 $\mu$M (Hernández *et al*., 1993; Alexander *et al*., 1994; Peakman & Hill, 1994). Similarly, PD115199 ($A_1 - A_{2A}$-selective) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; $A_1$-selective) were poor antagonists at these receptors. At adenosine receptors in the guinea-pig isolated aorta and trachea, also, CGS21680 appeared to be a poor agonist (Alexander *et al*., 1994b; Losinski & Alexander, 1995). In the former tissue, PD115199 and DPCPX were less potent than xanthine amine congener (XAC) indicating that this receptor was also of the $A_{2B}$ subtype. However, in the trachea PD115199 showed relatively high affinity, indicating that the adenosine receptor present in this tissue may fall outside the simple $A_{2A}/A_{2B}$ adenosine receptor classification. We have also recently characterized cyclic AMP responses in a Chinese hamster ovary cell line expressing cDNA for a putative $A_{2B}$ adenosine receptor derived from human brain (Alexander *et al*., 1996). Cyclic AMP responses in this line were evoked by adenosine analogues with the rank order of $pD_2$ values: NECA > adenosine > 2-chloroadenosine > $N^6$-(2-(4-aminophenyl)-ethylamino)adenosine. Antagonist inhibition of NECA-stimulated cyclic AMP responses showed the rank order of apparent $pA_2$ values: XAC ⩾ CGS 15943 > DPCPX > PD 115199 > 8FB-PTP > 3-propylxanthine. The antagonist rank order corresponded to the rank order of antagonist potency at the $A_{2B}$ adenosine receptor of guinea-pig cerebral cortex, similarly coupled to cyclic AMP generation, indicating that it was indeed the human homologue of this receptor.

The HEK 293 cell is a common recipient of exogenous DNA coding for receptors, including adenosine receptors (Furlong *et al*., 1992). It is useful to have some knowledge of which receptors are endogenously expressed in such cells, to provide both a yardstick for those receptors which exhibit similar signal transduction profiles, or to provide stimuli which may be modulated by exogenous receptors. In this study, we demonstrated the presence in HEK 293 cells of a robust (ca. 10 fold) stimulation of cyclic AMP levels by a receptor distinct from the $A_{2A}$ adenosine receptors, that we have previously identified in human, intact platelets, but more similar to our previously defined $A_{2B}$ adenosine receptors. Furthermore, analysis of the RNA content of these cells provides strong supporting evidence for the presence of the $A_{2B}$ receptor. A preliminary account of some of these findings has been presented to the British Pharmacological Society (Cooper *et al*., 1995a).

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## Methods

### Cell culture

HEK 293 cells were grown in Eagle's minimum essential medium (MEM) with Earle's balanced salt solution containing 1% MEM non-essential amino acids 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.

### Measurement of [$^3$H]-cyclic AMP accumulation

[$^3$H]-cyclic AMP was monitored in [$^3$H]-adenine-prelabelled HEK 293 cells as previously described (Alexander *et al*., 1996). 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 $\mu$M), and where indicated, adenosine receptor antagonists. After 15 min, agonist was added and the incubation continued for a further 15 min, before being stopped with HCl. [$^3$H]-cyclic AMP was quantified by the dual column methodology of Salomon *et al*. (1974) with [$^{14}$C]-cyclic AMP as a recovery marker. Data were derived from experiments conducted on at least three separate cell preparations and were initially expressed as [$^3$H]-cyclic AMP production as a percentage conversion from total [$^3$H]-adenine nucleotides.

### PCR amplification of HEK 293 $A_{2B}$ adenosine receptor cDNA

Total RNA was isolated from HEK 293 cells by use of the Nucleon total RNA kit (Scotlab) as described by the manufacturers. First strand synthesis of cDNA was performed from total RNA with Superscript II reverse transcriptase (Life Technologies) and oligo $(dT)_{12-18}$ primer. Target cDNA was amplified by use of the primers described in Table 1 by the polymerase chain reaction (PCR). Briefly, the amplification primer pairs described in Table 1b (PE Applied Biosystems) were added to PCR buffer containing 1.5 mM $MgCl_2$, 1 mM dNTP mix, 2 $\mu$l of cDNA and Taq DNA polymerase in a total volume of 50 $\mu$l. PCR amplification was performed on a Biometra Trio thermal cycler. An initial denaturation period (95°C for 5 min) was followed by 25 cycles of denaturation (95°C for 1 min), annealing (50–60°C for 1 min) and extension (72°C for 1 min), followed by a final extension at 72°C for 5 min. Samples were normally subjected to a second round of PCR amplification (25 cycles) in which 2 $\mu$l of the initial PCR reaction was used in place of the cDNA in a second 50 $\mu$l PCR reaction. A single round of PCR amplification was also applied to each primer pair, as a control, with a pRC-CMV vector containing the full length human $A_{2B}$ adenosine receptor (Pierce *et al*., 1992). Reaction products were separated by use of electrophoresis in 1% agarose (Appligene).

### Data analysis

Sigmoidal curves were fitted to concentration-response data, after responses in the absence of agent had been subtracted, by use of the computer programmes Prism and InPlot (GraphPad, California, U.S.A.) to generate estimates of $EC_{50}$ and $E_{max}$ values:

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

where X is the drug concentration, $E_{max}$ is the maximal response, and $n_H$ is the Hill coefficient. Antagonist $IC_{50}$ values were calculated from the same equation (substituting $IC_{50}$ for $EC_{50}$) to fit a curve for data points of increasing antagonist concentrations in the presence of a fixed concentration of NECA (10 $\mu$M). Antagonist $pA_2$ values ($-\log K_i$ values) were calculated from the $IC_{50}$ values by use of the null method described by Lazareno & Roberts (1987):

$$pA_2 = -\log[IC_{50}/(C/C' - 1)]$$

where C is the NECA concentration (i.e. 10 $\mu$M) and C′ is the NECA concentration evoking 50% of the response achieved at C.

### Chemicals

PD115199 (N-[2-(dimethylamino)ethyl]N-methyl-4-(1,3-dipropylxanthine)benzenesulphonamide) was a gift from Warner-Lambert (Michigan, U.S.A.); while KF 17837 ((E)7-methyl-8-(3,4-dimethoxystyryl)-1,3-dipropyl-xanthine) and 8FB-PTP (5-amino-8-(4-fluorobenzyl)-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*] pyrimidine) were kindly 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. CGS21680 (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 Fisher Scientific (Leics, U.K.).

Adenosine receptor antagonists were dissolved initially to 10 mM in dimethylsulphoxide (DMSO). NECA and CGS-21680 were dissolved in DMSO to 50 or 100 mM, while 2CA,

**Table 1** PCR primers used for amplification of fragments of the human $A_{2B}$ adenosine receptor (full length coding region 1-996 base pairs)

a Primer details

| *PCR Primer* | *Nucleotide position* | *Sequence* | *Strand* |
|---|---|---|---|
| A2B5 | 1–20 | ATGCTGCTGGAGACACAGGA | Sense |
| A2B6 | 287–306 | CACAGATGGCCAGGTATCTG | Antisense |
| A2B3 | 482–501 | CCACGAATGAAAGCTGCTGC | Sense |
| A2B | 775–794 | TGCCCACTTGGGCTTATTTT | Antisense |
| A2B2 | 1003–1022 | CCTGGAAGAGGCGAGAGCCT | Antisense |

b Predicted product size

| *Lane number* | *Primer pair* | *Predicted product length (base pairs)* |
|---|---|---|
| 1 | A2B5 : A2B6 | 325 |
| 2 | A2B3 : A2B | 332 |
| 3 | A2B3 : A2B2 | 541 |
| 4 | A2B5 : A2B | 813 |
| 5 | A2B5 : A2B2 | 1022 |

APNEA and adenosine were dissolved in dilute aqueous NaOH to a concentration of 100 mM.

## Results

### *Cyclic AMP generation*

Basal accumulation of [$^3$H]-cyclic AMP in HEK 293 cells was $0.36 \pm 0.06\%$ 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 > 2-CA $\geqslant$ adenosine $\geqslant$ APNEA (Figure 1, Table 2). The maximal response to NECA was approximately 10 fold over basal ($4.08 \pm 0.30\%$ conversion, $n = 16$). The $A_{2A}$-selective agonist CGS21680 (Lupica *et al*., 1990) failed to stimulate cyclic AMP generation significantly at concentrations below 30 $\mu$M (data not shown).

Responses to a submaximally effective concentration of NECA (10 $\mu$M) were inhibited in the presence of adenosine receptor antagonists (Figure 2), with the rank order of apparent $pA_2$ values: CGS 15943 = XAC > DPCPX $\geqslant$ PD115199 $\geqslant$ 8FB-PTP > KF 17837 > 3-propylxanthine (Table 3). Linear regression analysis of the antagonist affinities obtained in the present study, compared with affinities obtained in a clone of the human brain $A_{2B}$ adenosine receptor expressed in Chinese hamster ovary cells (Alexander *et al*., 1996), showed a high degree of correlation (Figure 3, slope = 0.81, $r^2 = 0.99$). A similar comparison with the $A_{2B}$ adenosine receptor of guinea-pig cerebral cortex (Alexander *et al*., 1994b; 1996) also showed good correlation (data not shown, slope = 0.91, $r^2 = 0.93$).

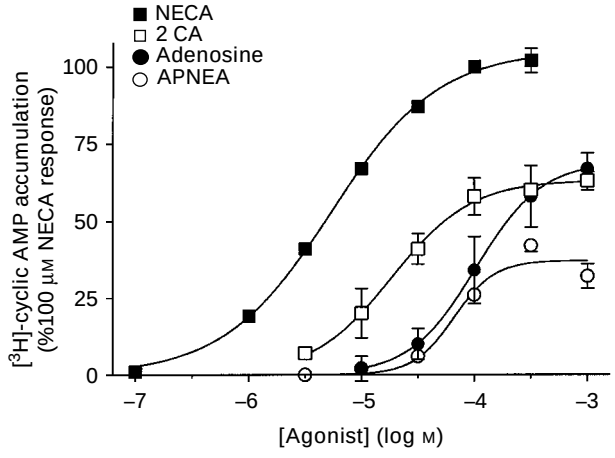


**Figure 1** Cyclic AMP accumulation in HEK 293 cells. Concentration-dependent generation of cyclic AMP in the presence of adenosine and its analogues. Data points are means, and vertical lines indicate s.e.mean, of 3–16 experiments conducted in triplicate.

**Table 2** Potency and intrinsic activity of adenosine and its analogues as stimulants of cyclic AMP generation in HEK 293 cells

| *Agonist* | n | $pD_2$ | *Maximal response* (% 100 $\mu$M NECA response) |
|---|---|---|---|
| NECA | 16 | $5.24 \pm 0.05$ | |
| 2CA | 4 | $4.41 \pm 0.15$ | $75 \pm 5$ |
| Adenosine | 4 | $4.19 \pm 0.09$ | $79 \pm 7$ |
| APNEA | 3 | $4.11 \pm 0.03$ | $43 \pm 3$ |

Data are means ± s.e.mean of the indicated number of experiments conducted in triplicate.

### *PCR amplification of $A_{2B}$ adenosine receptor mRNA*

Agarose gel electrophoresis of the products of PCR amplification of HEK 293 cell cDNA (reverse transcribed from total RNA) and a control pRC-CMV vector containing the full length human $A_{2B}$ adenosine receptor cDNA, showed the presence of major amplified products with identical patterns of size (Figure 4). Of particular note is the fact that the same oligonucleotide primer (e.g. A2B5) produced products of different size (in combination with different secondary primers) which matched the predicted product lengths (Table 1b; Figure 4).

## Discussion

In this investigation, we have studied cyclic AMP responses in the human embryonic kidney (HEK 293) cell line and observed an endogenous adenosine receptor linked to cyclic AMP generation, which exhibits characteristics of the $A_{2B}$ receptor subtype. Furthermore, analysis of a total RNA fraction from these cells showed the presence of a sequence with the profile expected of a human $A_{2B}$ adenosine receptor.

### *An $A_{2B}$ adenosine receptor in HEK 293 cells*

$A_{2B}$ adenosine receptors are most frequently defined by signal transduction properties and the inactivity of agents used to

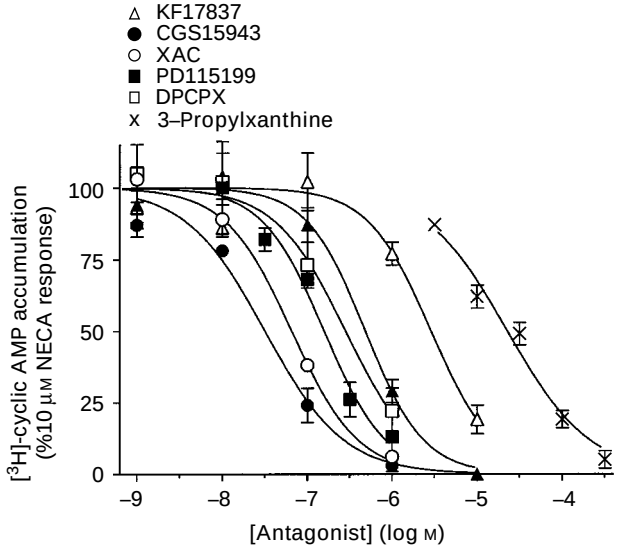


**Figure 2** Cyclic AMP accumulation in HEK 293 cells. Concentration-dependent inhibition of the cyclic AMP response to 10 $\mu$M NECA in the presence of adenosine receptor antagonists. Data points are means, and vertical lines indicate s.e.mean, of 3–4 experiments conducted in triplicate.

**Table 3** Apparent $pA_2$ values for antagonists at endogenously expressed (HEK 293 and guinea-pig cerebral cortex) and transfected (CHO.$A_{2B4}$ cells) $A_{2B}$ adenosine receptors

| *Antagonist* | *HEK 293 cells* | *Guinea-pig cerebral cortex*[1] | *CHO. $A_{2B4}$ cells*[1] |
|---|---|---|---|
| CGS 15943 | $7.79 \pm 0.10$ | 7.33 | 7.75 |
| XAC | $7.74 \pm 0.12$ | 7.46 | 7.89 |
| DPCPX | $7.01 \pm 0.14$ | 6.91 | 7.16 |
| PD115199 | $6.93 \pm 0.06$ | 6.39 | 6.95 |
| 8FB-PTP | $6.80 \pm 0.08$ | 6.55 | 6.52 |
| KF 17837 | $5.98 \pm 0.05$ | – | – |
| 3-Propylxanthine | $5.13 \pm 0.17$ | 4.59 | 4.63 |

[1]Alexander *et al*. (1996). Data are means ± s.e.mean of 3–4 determinations conducted in triplicate.

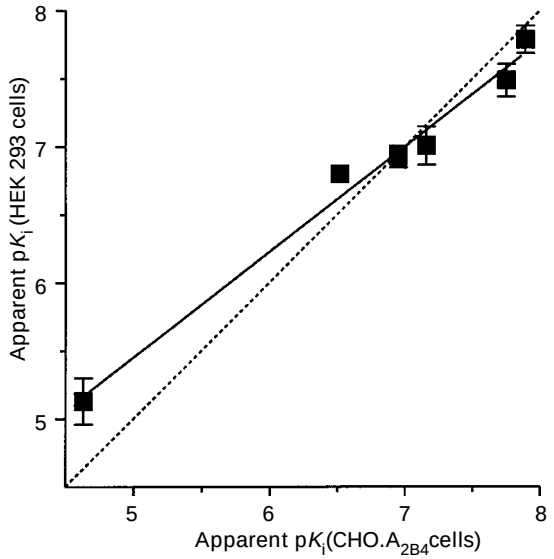


**Figure 3** Comparison of HEK 293 adenosine receptor antagonist affinities with those obtained for the human brain $A_{2B}$ adenosine receptor expressed in Chinese hamster ovary cells (Alexander *et al.*, 1996). The dashed line shows the line of identity. Linear regression analysis gives the solid line (CHO.$A_{2B4}$ vs HEK 293 cells: slope = 0.807, coefficient of determination ($r^2$) = 0.985). Data points are mean $pK_i$ values, while vertical lines indicate s.e.mean, from 3–4 experiments conducted in triplicate.

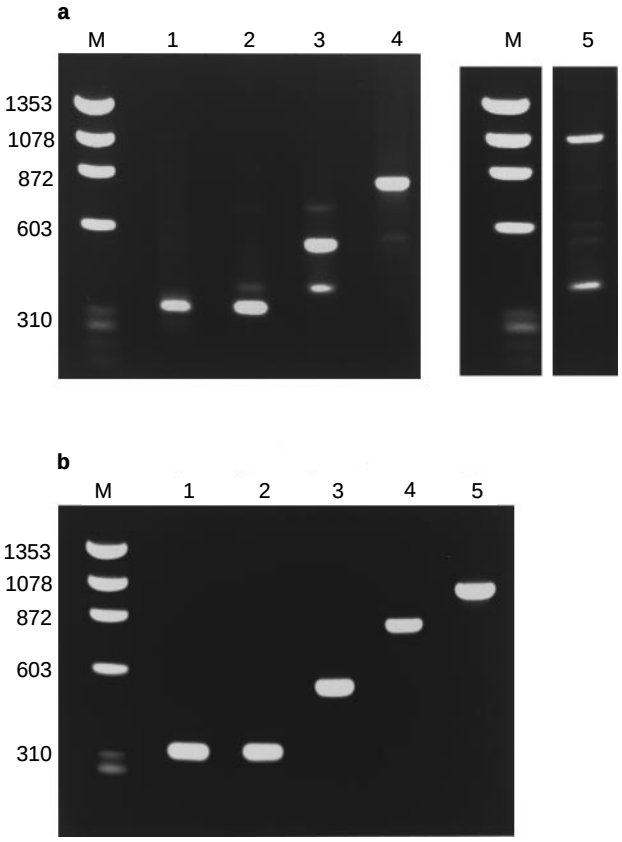


**Figure 4** PCR amplification of the human $A_{2B}$ adenosine receptor in HEK 293 cells. Agarose gel micrographs showing the PCR products (stained with ethidium bromide) following PCR amplification of (a) HEK 293 cell cDNA (reverse transcribed from total RNA) and (b) a control pRC-CMV vector containing the full length human $A_{2B}$ adenosine receptor cDNA. Lanes 1–5 refer to the primer pairs described in Table 1b. M indicates the lane containing molecular weight standards. In (a) all samples were subjected to two rounds of PCR amplification as described under Methods. The annealing temperature for both rounds was 60°C for all primer pairs with the exception of lane 5 (primers A2B5 and A2B2) which was annealed at 50°C during the first PCR reaction and 60°C during the second round. In (b) all samples were subjected to a single round of PCR amplification with an annealing temperature of 55°C.

define other subtypes of 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). Of the $A_2$ receptor ligands, the $A_{2A}$-selective agonist CGS21680 (Lupica *et al.*, 1990; Feoktistov & Biaggioni, 1993) and adenosine, which also is able to differentiate $A_{2A}$ and $A_{2B}$ subtypes (Daly *et al.*, 1983), both exhibited low potency as stimuli for cyclic AMP generation in HEK 293 cells. Thus, analysis of the agonist profile suggests the receptor to be an $A_{2B}$ adenosine receptor.

Perusal of the antagonist data suggests a similar conclusion. Thus, the relatively high affinities of the xanthine-based compounds which are effective at antagonizing the NECA-evoked cyclic AMP generation in the current study indicate that the receptor is not of the $A_3$ subtype, since the latter appears to exhibit low affinity for xanthine derivatives (Fredholm *et al.*, 1994). The low affinity of the $A_1$-selective antagonist DPCPX (Bruns *et al.*, 1987a; Lohse *et al.*, 1987) suggests that the receptor is not an $A_1$ subtype. Similarly, the low affinities of the antagonists with high affinity at $A_{2A}$ receptors (PD115199 (Bruns *et al.*, 1987b; 8FB-PTP (Dionisotti *et al.*, 1994); KF 17837 (Shimada *et al.*, 1992)) further indicates that the receptor is not an $A_{2A}$ receptor. Taken together, the antagonist data also indicate that this receptor is of the $A_{2B}$ subtype.

The virtually identical pattern of PCR products obtained with cDNA manufactured from HEK 293 cell total RNA and the full-length human $A_{2B}$ receptor (Pierce *et al.*, 1992), is substantial supporting evidence for the presence of the $A_{2B}$ receptor in these cells.

### *Comparisons with other $A_{2B}$ adenosine receptors*

The non-selective adenosine analogue NECA showed the highest potency of the agonists examined ($pD_2$ value 5.24). This is similar to estimates we have previously made for the $A_{2B}$ adenosine receptor in the guinea-pig brain (ca 5.5, Alexander *et al.*, 1994b; 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), and a putative $A_{2B}$ receptor cloned from human brain (5.91, Alexander *et al.*, 1996). However, comparison of agonist potencies between tissues and indeed between distinct responses in the same tissue is fraught with problems, such as differences in receptor reserve. Indeed, it is notable that 2-chloroadenosine, adenosine and APNEA do not produce full agonism in this cell line. It is usually more reliable to compare receptors through the analysis of antagonist apparent affinities. In this respect, the HEK 293 receptor compares well with data from our previous examinations of human $A_{2B}$ adenosine receptors (Table 3). A comparison of antagonist apparent $pA_2$ values with the human brain $A_{2B}$ adenosine receptor by use of linear regression analysis provided an estimated slope of 0.81 with an $r^2$ of 0.99, indicating the high degree of similarity of the two adenosine receptors (Figure 3). A good correlation was also observed with antagonist affinities at the guinea-pig cerebral cortex $A_{2B}$ receptor.

In conclusion, the discovery of an endogenous receptor coupled to stimulation of cyclic AMP generation in HEK 293 cells, which are widely used as recipients of exogenous DNA coding for receptors, ion channels and effector enzymes, allows a comparison for other adenylyl cyclase-coupled receptors to be made. Furthermore, the identification of

a readily available cell line expressing a human, native $A_{2B}$ adenosine receptor should allow further advances in the medicinal chemistry and pharmacology of this receptor to be made.

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