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Short communication

The affinity of adenosine for the high- and low-affinity states of the human adenosine A_1 receptor

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Abstract

The affinity of adenosine for the human adenosine A_1 receptor expressed on Chinese hamster ovary cell membranes has been measured in the presence and absence of GTP. The competitive effect of endogenous adenosine on the binding properties of adenosine A_1 receptors was estimated from differences in the binding of N^6-cyclohexyladenosine measured in the absence and presence of adenosine deaminase. From these data, the affinity of adenosine for the high- and low-affinity states of the human adenosine A_1 receptor (7×10^7 and 1.3×10^5 M^{-1}, respectively) was calculated.

Keywords: Adenosine A_1 receptor; Binding study; Adenosine

1. Introduction

Adenosine is present in binding assays on membrane preparations from a variety of tissues and cells. It is thought to be released into the incubation medium from cryptic sites or small vesicles (Schiemann et al., 1990; Prater et al., 1992). Attempts have been made to reduce or eliminate this contamination by the use of detergents or permeabilising agents, but these have not been successful. Therefore, in binding assays of adenosine A_1 receptors in membrane preparations, adenosine deaminase is routinely added to metabolise endogenous adenosine which would otherwise inhibit the binding of the radiolabelled ligands (for a review, see Linden, 1989). As a consequence, there has been, to our knowledge, no *detailed* characterisation of the adenosine binding properties of adenosine A_1 receptors. In this paper we determine the affinity of adenosine for the human adenosine A_1 receptor, when coupled to and uncoupled from its G-protein. A preliminary report of part of these data has been published (Cohen et al., 1994).

2. Materials and methods

2.1. Materials

All cell culture reagents were obtained from Gibco Laboratories. Chinese hamster ovary (CHO) cells stably transfected with the human adenosine A_1 receptor were obtained from Glaxo Group Research (Greenford, UK). 8-Cyclopentyl-1,3-dipropyl-2,3-^{3}H(N)-xanthine ([^{3}H]-DPCPX) was from DuPont NEN. $R(-)$-N^6-(2-phenylisopropyl)adenosine and N^6-cyclohexyl adenosine were from Research Biochemicals (Natick, MA, USA). Adenosine deaminase, adenosine and saponin were from Sigma.

2.2. Cell culture

Cells were grown in 1 : 1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium containing 10% newborn calf serum, 2 mM L-glutamine and geneticin (0.25 mg/ml) at 37°C in 5% CO_2. Cells were subcultured twice weekly at a ratio of 1 : 10 and once weekly the cells were transferred to large 24 × 24 cm plates.

2.3. Membrane preparation

Cells were washed with phosphate-buffered saline and scraped from the plates in 5 ml of ice-cold homogenization buffer (20 mM Tris, 10 mM EDTA, pH 7.4, saponin 0.1 mg/ml). Cells were disrupted in a Polytron homogenizer

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(setting 6) at 4°C, twice for 5 s at a 30-s interval. Plasma membranes and the cytosolic fraction were separated by centrifugation at 100 000 × *g* for 15 min. The membrane pellet was resuspended in buffer (20 mM Tris, 0.1 mM EDTA, pH 7.4), again disrupted in a Polytron (5 s) and the homogenate centrifuged at the same speed for 15 min. The pellet was resuspended in binding assay buffer at approximately 3 mg protein/ml and stored in 0.5-ml aliquots at −70°C. Protein determinations were performed with the Bio-Rad protein assay, using bovine serum albumin as a standard. The yield of protein was approximately 3 mg protein/plate.

2.4. Radioligand binding

All binding assays were performed in triplicate in a final volume of 1 ml. Varying concentrations (0.1 nM–300 μM) of agonist were included in the assay with the antagonist [^{3}H]DPCPX (0.2 nM) as radioligand. Studies were performed with cell membranes that were untreated or previously treated with 3 U/ml adenosine deaminase for 30 min at 25°C and in the absence and presence of GTP (1 mM) where appropriate. Binding assays were initiated by the addition of membranes at a final protein concentration of 40–80 μg/ml in assay buffer (NaCl 100 mM, Hepes 20 mM, $MgCl_2$ 10 mM pH 7.4). Reactions were continued for 1 h at 25°C. Non-specific binding was determined by the addition of 3 mM theophylline.

Bound and free ligand were separated by filtration through Whatman GF/B filter strips which were presoaked in 0.3% polyethyleneimine for 1 h. Filtration was performed on a cell harvester (Brandel); filters were washed 3 times with 5-ml aliquots of ice-cold H_2O, transferred into vials and 5 ml of scintillation fluid was added. Samples were counted after they had been stored at room temperature for at least 6 h to permit the glass-fibre filters to become uniformly translucent.

2.5. Data analysis

Binding data were analysed by non-linear least squares analysis using the appropriate 1- or 2-site models and the programs Enzfitter and Sigmaplot. All results are expressed as means ± S.E.M. Parameters obtained by this form of analysis are numerically within 10% of the equivalent parameters obtained by fitting the data to a ternary complex model (Jones et al., 1995).

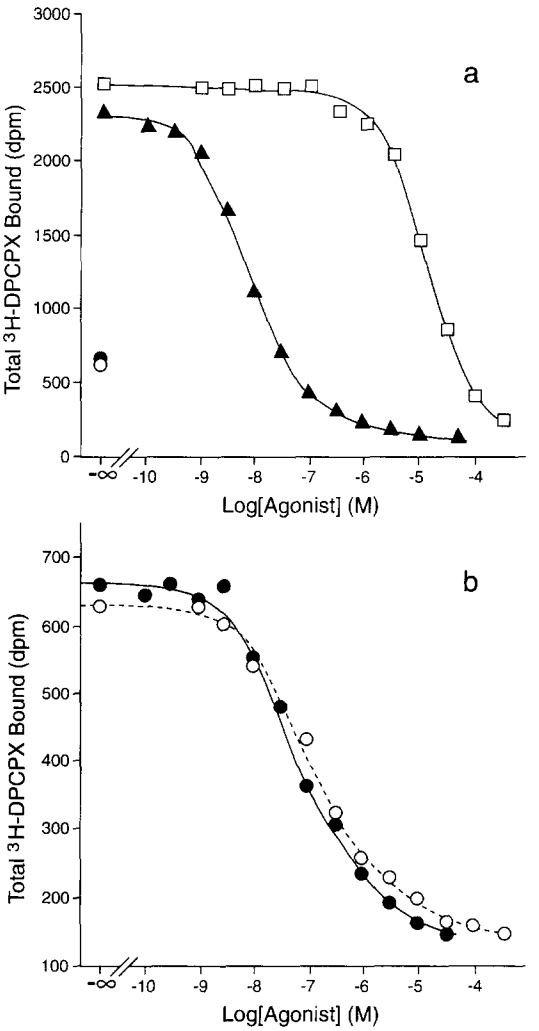

Fig. 1. Competition of [^{3}H]DPCPX binding to adenosine A_1 receptors on saponin pretreated CHO membranes by cyclohexyladenosine and adenosine in the presence and absence of GTP and adenosine deaminase. The presence of adenosine deaminase or GTP minimises the inhibitory effect of endogenous adenosine and results in high levels of [^{3}H]DPCPX binding (Fig. 1a). The absence of both adenosine deaminase and GTP gives low levels of [^{3}H]DPCPX binding (Fig. 1b and 1a, circles). Binding assays were carried out by competition against a fixed concentration of [^{3}H]DPCPX (0.2 nM) using membranes that were untreated (○, □ and ●) or previously treated (▲) with 3 U/ml adenosine deaminase for 30 min at 25°C. The competing agonist was adenosine (○ and □) or cyclohexyladenosine (● and ▲). The binding of [^{3}H]DPCPX was measured in the absence (○, ● and ▲) or presence (□) of 1 mM GTP. Data points are means of triplicate determinations in 1 of 3 experiments. The error bars generally fall within the data points. Curves are computer-generated best fits to a 1- or 2-site model, where appropriate.

3. Results

The CHO cell membranes used in this assay had been pretreated with the permeabilising agent saponin (0.1 mg/ml). This procedure facilitates access of guanine nucleotides to G-proteins and increases coupling and function without affecting the binding constants of ligands (Cohen et al., 1994, 1996).

In the presence of GTP, the adenosine/[^{3}H]DPCPX curve is well described by a mass action inhibition curve with a mean log K_{APP} of 5.02 ± 0.15 ($n = 3$) where K_{APP} is the reciprocal of the IC_{50} (Fig. 1a). Previous experiments have demonstrated that, under these conditions, the concentration of endogenous adenosine is too low to affect substantially the binding constant of agonists to the low-affinity state of the receptor which is generated in the

presence of GTP (Cohen et al., 1996). The log affinity constant of adenosine for the low-affinity form of the receptor of 5.10 ± 0.15 is simply obtained by applying the small correction factor for occupancy (20%) of the adenosine A_1 receptors by the radioligand (the Cheng-Prusoff correction). The K_d of [^{3}H]DPCPX was determined to be 1.0 ± 0.1 nM ($n = 6$). This value and the B_{max} value (1.3 pmol/mg protein) were unaffected by adenosine deaminase (Cohen et al., 1996).

In the case of [^{3}H]DPCPX competition curves, measured in the absence of GTP and adenosine deaminase, the endogenous adenosine had a profound inhibitory effect on [^{3}H]DPCPX binding (Fig. 1a, circles). The specific binding of [^{3}H]DPCPX decreased to approximately 20% of the value observed in the presence of GTP. The [^{3}H]DPCPX/adenosine inhibition curve in the absence of GTP (Fig. 1b, open circles) can be fitted well to a 2-site model. The high-affinity site (70 ± 5%) has a log K_{APP} of 7.21 ± 0.06 ($n = 3$) and the remaining site has a log K_{APP} of 5.39 ± 0.04 ($n = 3$), a value which is not significantly different from the value found in the presence of GTP ($P > 0.05$, unpaired t-test).

In order to estimate the true affinity constant of adenosine for the high-affinity state of the adenosine A_1 receptor it is necessary to correct the K_{APP} value of 7.2 for the competitive effects of endogenous adenosine and the radioligand [^{3}H]DPCPX. To do this we took advantage of a previous approach in which the competitive effect of endogenous adenosine on the binding of a non-metabolised agonist was analysed (Cohen et al., 1996).

The effects of adenosine deaminase on the affinity constant of the high-affinity binding sites for cyclohexyladenosine in [^{3}H]DPCPX-cyclohexyladenosine competition experiments were measured (Fig. 1a,b, closed symbols). These experiments were carried out in parallel to the [^{3}H]DPCPX-adenosine competition experiments on the same membrane preparations and on the same day.

In the presence of adenosine deaminase, the [^{3}H]DPCPX/cyclohexyladenosine competition curve (Fig. 1a, closed triangles) could be analysed by a 2-site model with 93% of the sites having high affinity (log $K_{APP} = 8.08$) and the remainder having low affinity (log $K_{APP} = 5.65$). The mean log K_A value of cyclohexyladenosine for the high-affinity state of the receptor in the presence of adenosine deaminase was 8.30 ± 0.08, $n = 3$ (after application of the Cheng-Prusoff correction) with 91 ± 2% of the receptors existing in that state. These parameters agree well with those determined previously (Cohen et al., 1996).

In the absence of adenosine deaminase the [^{3}H]DPCPX/cyclohexyladenosine competition curve was well described by a 2-site model (Fig. 1b, closed circles). Under these conditions, 70 ± 1% ($n = 3$) of the binding of [^{3}H]DPCPX was associated with a high-affinity site of log K_{APP} of 7.55 ± 0.07 with the remaining sites having a log K_{APP} of 5.59 ± 0.06. Thus there is a clear difference in the potency of cyclohexyladenosine at the high-affinity state of the adenosine A_1 receptor when measured in the presence and absence of adenosine deaminase.

Table 1
Affinity constants (log values) of adenosine and two adenosine A_1 agonists for the high- and low-affinity states of the adenosine A_1 receptor

	High-affinity state	Low-affinity state
Adenosine	7.9 ±0.1	5.10±0.15
Cyclohexyladenosine	8.30±0.03 [a]	5.76±0.02 [a]
Phenylisopropyladenosine	8.48±0.06 [a]	6.03±0.09

Values are the means ± S.E.M. of 3–5 experiments. [a] Data from Cohen et al. (1996).

The fold change in the cyclohexyladenosine potency due to endogenous adenosine is a factor which is independent of the nature of the ligand which is binding to or monitoring the binding to the high-affinity state of the receptor. Therefore, the factor can be used to correct the log K_{APP} of adenosine to give the true affinity constant. This competitive factor (5.0 ± 0.6 fold or 0.7 log units, $n = 3$) gives a calculated log affinity of adenosine for the high-affinity binding component of adenosine A_1 receptors of 7.9 ± 0.1.

Since the fold shift on the cyclohexyladenosine affinity constant is $1 + K_{ADO}$ [adenosine]/$(1 + K_{DP}$[DPCPX]), where K_{DP} and K_{ADO} are the affinity constants of DPCPX and adenosine for the receptor, it is possible to estimate the concentration of adenosine in the assay as approximately 60 nM.

The affinity constants for adenosine are compared to those of cyclohexyladenosine and phenylisopropyladenosine in Table 1. The data for phenylisopropyladenosine at the low-affinity state of the receptor was obtained from a 1-site fit of a [^{3}H]DPCPX/phenylisopropyladenosine competition curve measured in the presence of GTP and adenosine deaminase. At both states of the receptor adenosine is 3–7-fold weaker than the other two agonists.

4. Discussion

There have been to our knowledge only two reports on the estimation of the potency (IC_{50} value) of adenosine (in the absence of GTP) when it binds to rat (Lohse et al., 1988) and human (Libert et al., 1992) adenosine A_1 receptors. In neither report was the inhibition curve analysed by a 1- or 2-site model nor did these studies account for the presence of endogenous adenosine or, in the former case, residual adenosine deaminase activity. There has also been a report on the measurement of the affinity of adenosine for adenosine A_1 receptors on DDT_1 MF-2 cells (Gerwins et al., 1990). This study was carried out on intact cells at 4°C and the measured affinity constant was assumed to reflect the affinity of adenosine for the uncoupled receptor. The value (log $K_A = 5.78$) and the values for cyclohexyladenosine and phenylisopropyladenosine are 5–10-fold

higher than those reported in this paper for the uncoupled receptor (Table 1). These differences could be explained by the different temperatures of incubation in the two studies or by species variations in the binding properties of the hamster and human receptors.

It is somewhat surprising that there have not been reports on the detailed determination of the binding of adenosine, the endogenous ligand, to adenosine A_1 receptors. A major reason has been that adenosine seems to be present in all membrane binding assays at concentrations at which it interferes with the binding of radioligands, most notably the binding of radiolabelled agonists. As a consequence, from the earliest studies, adenosine deaminase has been included routinely in adenosine A_1 receptor binding assays. However, we have demonstrated in this paper and elsewhere (Cohen et al., 1996) that [^{3}H] DPCPX-agonist competition experiments can be performed readily in the absence of adenosine deaminase.

Thus it has been possible to measure the affinity constant of adenosine for the uncoupled receptor (log $K_A = 5.10 \pm 0.15$) directly by performing [^{3}H]DPCPX-adenosine competition experiments in the presence of GTP (Fig. 1a). The affinity constant of adenosine for the high-affinity state of the receptor was somewhat more difficult to calculate because of the enhanced inhibition by the endogenous adenosine. However, a value (log $K_A = 7.9 \pm 0.1$) could be obtained by calculating the competitive effect of endogenous adenosine on the high-affinity state of cyclohexyladenosine (an agonist not catabolised by adenosine deaminase) and applying the appropriate correction factor to the estimated apparent affinity constant of adenosine obtained from a [^{3}H]DPCPX-adenosine competition experiment in the absence of GTP and adenosine deaminase. Interestingly, adenosine only binds 3–7 times weaker than cyclohexyladenosine and phenylisopropyladenosine to both states of the human adenosine A_1 receptor. The ratio of affinities of the three agonists for the coupled and uncoupled forms of the receptor is 300–600 (Table 1). This ratio is somewhat higher than some corresponding values in rat brain (90–150; Lohse et al., 1984) and bovine brain (15–30; Lohse et al., 1987). The differences are not caused by the presence of saponin (Cohen et al., 1996) but could be due to species variations or different incubation conditions. This ratio is often considered to be a monitor of agonist efficacy and thus it would therefore be predicted that cyclohexyladenosine, phenylisopropyladenosine and adenosine would have comparable efficacies. It is now possible to relate the binding and actions of adenosine A_1 agonists to those of the natural transmitter adenosine.

It has also been possible to estimate the concentration of endogenous adenosine in the binding assay at approximately 60 nM. This is in general agreement with levels measured directly in assays using membranes from brain and smooth muscle (Prater et al., 1992; Schiemann et al., 1990) and in studies of adenosine stimulation of GTPγ-^{35}S binding to G-proteins coupled to adenosine A_1 receptors on CHO cell membranes (Cohen et al., manuscript in preparation). At these concentrations, adenosine occupies approximately 80% of the adenosine A_1 receptor G-protein complexes and less than 1% of the uncoupled receptors, illustrating the profound and selective effects of endogenous adenosine on binding.

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References

Cohen, F.R., S. Lazareno and N.J.M. Birdsall, 1994, Binding and functional properties of the cloned human adenosine A_1 receptor, Br. J. Pharmacol. 112, 135P.

Cohen, F.R., S. Lazareno and N.J.M. Birdsall, 1996, The effect of saponin on the binding and functional properties of the human A_1 adenosine receptor, Br. J. Pharmacol. 117, 1521.

Gerwins, P., C. Norstedt and B.B. Fredholm, 1990, Characterisation of adenosine A_1 receptors in intact DDT, MF-2 smooth muscle cells, Mol. Pharmacol. 38, 660.

Jones, P.G., C.A.M. Curtis and E.C. Hulme, 1995, The function of a highly conserved arginine residue in activation of the muscarinic receptor, Eur. J. Pharmacol. 288, 251.

Libert, F., J. Van Sander, A. Lefort, A. Czernilofsky, J.E. Dumont, G. Vassart, H.A. Ensinger and K.D. Mendla, 1992, Cloning and functional characterization of the human A_1 adenosine receptor, Biochem. Biophys. Res. Commun. 187, 919.

Linden, J., 1989, Adenosine deaminase for removing adenosine: how much is enough?, Trends Pharmacol. Sci. 10, 260.

Lohse, M.J., V. Lenschow and U. Schwabe, 1984, Two affinity states of the R_1 adenosine receptors in brain membranes, Mol. Pharmacol. 26, 1.

Lohse, M.J., K.-N. Klotz, J. Lindenborn-Fotinos, M. Reddington, U. Schwabe and R.A. Olsson, 1987, 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) – a selective high affinity antagonist radioligand for A_1 adenosine receptors, Naunyn-Schmiedeberg's Arch. Pharmacol. 336, 204.

Lohse, M.J., K.-N. Klotz, E. Diekmann, K. Friedrich and U. Schwabe, 1988, 2′,3′-Dideoxy-N^6-cyclohexyladenosine: an adenosine derivative with antagonist properties at adenosine receptors, Eur. J. Pharmacol. 156, 157.

Prater, M.R., H. Taylor, R. Munshi and J. Linden, 1992, Indirect effect of guanine nucleotides on antagonist binding to A_1 adenosine receptors: occupation of cryptic binding sites by endogenous vesicular adenosine, Mol. Pharmacol. 42, 765.

Schiemann, W.P., J.M. Walther and I.L.O. Buxton, 1990, On the ability of endogenous adenosine to regulate purine nucleoside receptor binding of antagonists in smooth muscle membranes, J. Pharm. Exp. Ther. 256, 886.