On the high affinity binding site for [³H]-1,3-dipropyl-8-cyclopentylxanthine in frog brain membranes

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1 Radioligand binding properties of the adenosine receptor ligands, [³H]-1,3-dipropyl-8-cyclopentylxanthine ([³H]-DPCPX), and [³H]-R-phenylisopropyladenosine ([³H]-R-PIA) were investigated in frog brain membranes.

2 The specific binding of the adenosine antagonist, [³H]-DPCPX to frog brain membranes showed one binding site with K_d and B_{max} values of 43.8 nM and 0.238 ± 0.016 pmol mg⁻¹ protein, respectively. Guanosine 5'-triphosphate (GTP, 100 μM) decreased to $72 \pm 7\%$ and Mg²⁺ (8 mM) increased to $121 \pm 3\%$ [³H]-DPCPX (40 nM) binding to frog brain membranes.

3 [³H]-DPCPX saturation binding experiments performed in the presence of Mg²⁺ (8 mM), or in the presence of GTP showed that Mg²⁺ ions decreased the K_d value of [³H]-DPCPX to 14 nM, and GTP increased this value to 65.6 nM. B_{max} values were not significantly ($P > 0.05$) modified (0.261 ± 0.018 pmol mg⁻¹ protein, with Mg²⁺, and 0.266 ± 0.026 pmol mg⁻¹ protein, in presence of GTP) by the presence of Mg²⁺ or GTP.

4 The specific binding of [³H]-R-PIA (15 nM) was decreased to $37 \pm 6\%$ by GTP (100 μM) and increased to $123 \pm 4\%$ by Mg²⁺ (8 mM). [³H]-R-PIA saturation binding experiments performed in the presence of Mg²⁺ (8 mM) showed one binding site with K_d and B_{max} values of 0.9 nM and 0.229 ± 0.008 pmol mg⁻¹ of protein, respectively.

5 The concentration-inhibition curves of adenosine agonists and antagonists versus [³H]-DPCPX binding showed the following order of potencies: CPA > R-PIA ≈ NECA > S-PIA >> CGS 21680, for the agonists, and XAC ≈ DPCPX >> XCC > PACPX, for the antagonists.

6 The present results suggest that the adenosine binding site in the frog brain membranes is G-protein coupled, but that the antagonist affinities and the pharmacological profile is different from the A₁ or A₂ adenosine receptors.

Keywords: Frog brain membranes; adenosine; adenosine receptors; [³H]-1,3-dipropyl-8-cyclopentylxanthine ([³H]-DPCPX) binding; [³H]-R-phenylisopropyladenosine ([³H]-R-PIA) binding

Introduction

Different subtypes of adenosine receptors have been proposed based on pharmacological and biochemical criteria (see e.g. Ribeiro & Sebastião, 1986). The adenosine receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), is a good tool to distinguish between adenosine receptor subtypes. The K_i value for this adenosine antagonist, at the rat neuromuscular junction ($K_i = 0.54$ nM; Sebastião *et al.*, 1990) is about the same as that obtained in the rat hippocampus ($K_i = 0.45$; Sebastião *et al.*, 1990), and very close to the A₁ high affinity binding site detected in the rat brain membranes ($K_d \leq 1$ nM; Bruns *et al.*, 1987). Besides this binding site, another high affinity binding site for [³H]-DPCPX ($K_d = 10.7$ – 13.5 nM) was detected in solubilized rat brain preparations; this binding site appears to be neither an A₁ nor an A₂ adenosine receptor (Oliveira *et al.*, 1991). At the frog neuromuscular junction the K_i value (35 nM) determined for DPCPX is about seventy times higher than that detected at the rat neuromuscular junction and within the same order of magnitude (2–3 times higher) as that found for the second high affinity binding site for [³H]-DPCPX in the solubilized rat brain membranes. It, therefore, is of interest to investigate the binding characteristics of [³H]-DPCPX to the frog brain membranes, as well as the potency of several adenosine agonists and antagonists in inhibiting [³H]-DPCPX binding. The involvement of G-proteins in the binding of the agonist, [³H]-phenylisopropyladenosine ([³H]-R-PIA), as well as in the binding of the antagonist [³H]-DPCPX was also investigated.

Brief accounts of some of this work already appeared (Oliveira *et al.*, 1992a,b).

Methods

Brain membrane preparation

Membranes were prepared according to Ruegg *et al.* (1981) with some modifications. Briefly, the frogs (*Rana ridibunda*) were killed by decapitation and brain membranes were prepared by homogenization with 20 ml of Buffer A per gram of tissue (Buffer A: Tris/HCl 50 mM (pH 7.4), NaCl 100 mM, MgCl₂ 10 mM, ethylenediaminetetraacetic acid (EDTA) 1 mM, diethylenetriaminepentaacetic acid (DTPA) 0.1 mM and a cocktail of proteinase inhibitors containing: dithiothreitol 1 mM, phenylmethylsulphonyl fluoride 0.1 mM and pepstatin A, leupeptin, chymostatin, antipain and aprotinin all at 1 μg ml⁻¹) and centrifuged at 2,000 r.p.m., (600 g) for 10 min (4°C). The supernatant was centrifuged at 15,000 r.p.m. (27,000 g), for 20 min (4°C), washed 3 times with the same buffer, and stored in fractions of 1 ml (~5 mg of protein) at -85°C. Protein content was measured according to the method of Lowry as modified by Peterson (1977).

Binding assay

In all the experiments, membranes were thawed, centrifuged at 15,000 r.p.m. to remove Buffer A and resuspended in Buffer B (50 mM Tris/HCl, pH 7.4). The effects of GTP on [³H]-DPCPX or [³H]-R-PIA binding were studied with membranes preincubated with 10 mM EDTA in Buffer B, for 30 min, at 25°C, washed twice and finally resuspended in Buffer B. In all experiments preparations were incubated with the ligands for 2 h, at 25°C, in presence of $2 \mu\text{M}$ of adenosine deaminase. In each experiment the assays were always performed in triplicate, in a final volume of 100 μl, using 12 × 75 mm polypropylene tubes.

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[³H]-DPCPX saturation binding studies (0.5–150 nM) were performed in control conditions (i.e. in the absence of added Mg²⁺ or GTP), in the presence of 8 mM MgCl₂, or in the presence of 100 μM GTP; nonspecific binding was determined in the presence of 10 mM theophylline; some experiments were performed with R-PIA (30 μM) to determine nonspecific binding. [³H]-R-PIA saturation binding studies in the frog brain membranes were performed, in the presence of 8 mM Mg²⁺, or in the presence of 100 μM GTP; nonspecific binding was determined in the presence of 10 μM xanthine amine congener (XAC). Concentration-inhibition curves for adenosine agonists (i.e. N⁶-cyclopentyladenosine (CPA), 5'-N-ethylcarboxamido adenosine (NECA), R- and S-phenylisopropyladenosine (R- and S-PIA), and 2-[4-(2-carboxyethyl) phenethylamino]-5'-N-ethylcarboxamidoadenosine, (CGS 21680)), and the antagonists, xanthine amine congener (XAC), DPCPX, 1,3-dipropyl-8-(carboxymethyl-oxyphenyl)xanthine (XCC), and 1,3-dipropyl-8-(2-amino-4-chlorophenyl)xanthine, (PACPX) versus [³H]-DPCPX (15 nM) binding were performed in the presence of 8 mM Mg²⁺; nonspecific binding was determined in the presence of 30 μM R-PIA.

Incubations were initiated by the addition of the tissue at a final protein concentration of about 80–100 μg/tube (0.8–1.0 μg ml⁻¹). Protein concentrations were determined by the method of Lowry as modified by Peterson (1977). After incubation, the binding was stopped by rapid filtration under reduced pressure over Whatman GF/C glass fibre filters; the preparation was then washed 3 times with 5 ml Buffer B containing 0.01% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulphonate (CHAPS) and dried. After gentle shaking overnight in a liquid-scintillation cocktail, the radioactivity was measured in a liquid-scintillation counter Beckman LS 6000 LL, for 1 min, with an efficiency of about 50%.

Data analysis

Binding parameters (K_d and B_{max} values) were determined from analysis of the binding data of all the experiments treated simultaneously with the LIGAND computer-assisted nonlinear curve fitting programme (Munson & Rodbard, 1980).

Statistics

Unless otherwise specified, data are expressed as means ± s.e.mean, and n represents the number of experiments. Because the K_d values are log normally distributed their confidence limits were calculated from the log of the K_d values. The significance of the differences between means was calculated by Student's t test, and was considered significant at $P < 0.05$.

Materials

Dithiothreitol, diethylenetriaminepentaacetic acid, ethylenediaminetetraacetic acid, phenylmethylsulphonyl fluoride, aprotinin, leupeptin, pepstatin A, adenosine deaminase (type VI), guanosine triphosphate, and theophylline were obtained from Sigma. [³H]-DPCPX (108.3 Ci mmol⁻¹) was obtained from NEN Dupont and [³H]-R-PIA (45.9 Ci mmol⁻¹) from Amersham. CPA, R- and S-PIA, NECA, CGS 21680, DPCPX, XAC and PACPX were from RBI (Research Biochemicals Inc., U.S.A.). All other chemicals were analytical grade.

Results

Figure 1 shows a typical binding experiment for [³H]-DPCPX, used in a range of concentrations between 5 and 100 nM. Concentrations of [³H]-DPCPX below 5 nM were used in two other experiments but specific binding was not detected. Concentrations up to 125 nM were used in three

Figure 1 Specific [³H]-1,3-dipropyl-8-cyclopentylxanthine ([³H]-DPCPX) binding to frog brain membranes in control conditions (i.e. in the absence of added Mg²⁺ or GTP). Specific [³H]-DPCPX binding was determined by subtracting nonspecific binding from the total binding. Nonspecific binding was determined in the presence of 10 mM theophylline, and for a [³H]-DPCPX concentration equal to its K_d value was $65 \pm 3\%$ of the total binding. The points correspond to one representative experiment performed in triplicate, out of four experiments with similar results. Inset: Scatchard plot of the specific [³H]-DPCPX binding. Binding parameters (K_d and B_{max} values) for the experiment showed in the figure were obtained by computer-assisted nonlinear curve fitting and were 40.8 nM and 0.245 ± 0.015 pmol mg⁻¹ of protein. B, bound (pmol mg⁻¹ protein) (i.e. specific binding); F, free [³H]-DPCPX in the incubation medium (nM) after occupation of the binding sites.

other experiments and the specific binding was not appreciably greater than that obtained with 100 nM [³H]-DPCPX. In the [³H]-DPCPX binding experiments only one binding site was detected and the averaged K_d and B_{max} values for [³H]-DPCPX were 43.8 nM and 0.238 ± 0.016 pmol mg⁻¹ protein ($n = 4$), respectively (see Table 1). Nonspecific binding determined in the presence of 10 mM theophylline was $65 \pm 3\%$ of the total binding at the K_d value for [³H]-DPCPX binding. This high nonspecific binding was not reduced when R-PIA (30 μM) was used as cold ligand instead of theophylline (see below).

Figure 2 illustrates a typical experiment on [³H]-R-PIA binding to frog brain membranes performed in the presence

Figure 2 Specific [³H]-R-phenylisopropyladenosine ([³H]-R-PIA) binding to frog brain membranes in the presence of 8 mM MgCl₂. Specific [³H]-R-PIA binding was determined by subtracting nonspecific binding from the total binding. Nonspecific binding was determined in the presence of 10 μM xanthine amine congener (XAC), and at the K_d values for [³H]-R-PIA was $12 \pm 0.5\%$ of the total binding. The points correspond to one representative experiment performed in triplicate, out of three experiments with similar results. Inset: Scatchard plot of the specific [³H]-R-PIA binding. Binding parameters (K_d and B_{max} values) for the experiment showed in the figure were obtained by computer-assisted nonlinear curve fitting and were 0.94 nM and 0.245 ± 0.243 pmol mg⁻¹ of protein. B, bound (pmol mg⁻¹ protein) (i.e. specific binding); F, free [³H]-R-PIA in the incubation medium (nM) after occupation of the binding sites.

of Mg^{2+} (8 mM). Only one [^3H]-R-PIA binding site was detected with a K_d value of 0.94 nM and a B_{max} value of 0.245 ± 0.024 pmol mg^{-1} of protein. Similar results were obtained in two other experiments, and K_d and B_{max} values calculated for the three experiments (treated simultaneously by LIGAND; Munson & Rodbard, 1980) were 0.9 nM and 0.229 ± 0.08 pmol mg^{-1} of protein, respectively. Nonspecific binding determined in the presence of 10 μM XAC represented $12 \pm 0.5\%$ of the total binding at the K_d value concentration of [^3H]-R-PIA.

It is well known that GTP modifies receptor G-protein interactions in an opposite manner to the changes induced by Mg^{2+} ; Mg^{2+} elicits the formation of R-G-complexes with high affinity for agonists, whereas GTP dissociates the R-G-complexes revealing the low affinity conformation for the agonists (Gilman, 1987). To investigate if the adenosine receptor present in frog brain membranes behaves as a receptor-G-protein complex, the effects of GTP (100 μM) or Mg^{2+} (8 mM) on the binding of the agonist, [^3H]-R-PIA (15 nM), were studied. The effects of these agents on the binding of the antagonist, [^3H]-DPCPX (40 nM), to frog brain membranes were also investigated. Figure 3 shows that

Figure 3 Effect of GTP (100 μM) and Mg^{2+} (8 mM) on the specific [^3H]-R-phenylisopropyladenosine ([^3H]-R-PIA, 15 nM) (open columns) and [^3H]-1,3-dipropyl-8-cyclopentylxanthine ([^3H]-DPCPX, 40 nM) (hatched columns) binding to frog brain membranes. Membranes were pretreated with EDTA (10 mM) for 30 min, at 25°C, centrifuged, washed twice with 50 mM Tris/HCl (pH 7.4), and resuspended in the same buffer. In each experiment the incubations were performed in triplicate, in control conditions (i.e. no added Mg^{2+} or GTP), in the presence of 8 mM MgCl_2 , and in the presence of 100 μM GTP. Nonspecific binding for the antagonist was determined in the presence of 10 mM theophylline, and for the agonist was determined with 10 μM xanthine amine congener (XAC). In each experiment the binding obtained in the presence of Mg^{2+} or GTP was calculated as a percentage of the binding obtained in control conditions, which was taken as 100%. Note that GTP decreased and Mg^{2+} increased the specific [^3H]-R-PIA and [^3H]-DPCPX binding to the frog brain membranes, when compared with the controls. Values are means $\pm$ s.e.mean obtained in $n=3$ experiments. * $P<0.05$ (Student's t test), when compared with the control (100%). ** $P<0.01$ (Student's t test), when compared with the control (100%).

GTP, and Mg^{2+} ions modified, in an opposite manner, the specific binding for [^3H]-R-PIA, as well as that of [^3H]-DPCPX. GTP decreased to $36.7 \pm 6\%$ (mean $\pm$ s.e.mean; $n=3$) and Mg^{2+} increased to $123 \pm 4.2\%$ ($n=3$) the specific binding of [^3H]-R-PIA; the binding of [^3H]-DPCPX was reduced by GTP to $71.5 \pm 7.4\%$ ($n=3$), and increased by Mg^{2+} to $120.7 \pm 3.4\%$ ($n=3$). The binding of ligands in the presence of Mg^{2+} was significantly different ($P<0.05$) from their binding in the presence of GTP. In one experiment several concentrations of Mg^{2+} (1–15 mM) were tested, and it was found that the specific binding of [^3H]-DPCPX (40 nM) increased as the concentrations of Mg^{2+} increased up to 4 mM; with concentrations of Mg^{2+} higher than 4 mM there was no further increase in the [^3H]-DPCPX binding (results not shown).

To ascertain whether K_d or B_{max} values were modified by Mg^{2+} or GTP, [^3H]-DPCPX and [^3H]-R-PIA saturation binding experiments were carried out in which we tested the influence of these agents. Typical [^3H]-DPCPX saturation binding experiments are shown in Figure 4; the K_d value found for [^3H]-DPCPX in the presence of Mg^{2+} was 12 nM, and in the presence of GTP was 77 nM. Similar values were obtained in two other experiments, and K_d values obtained for the three experiments treated simultaneously were 14 and 65.6 nM, for the [^3H]-DPCPX binding in the presence of Mg^{2+} and GTP, respectively. With the individual K_d values determined by LIGAND for each experiment, confidence limits of the K_d values for each group of experiments were determined, using the logarithms of the individual K_d values. The K_d values obtained in the presence of Mg^{2+} were significantly different ($P<0.05$) from the K_d values obtained in the presence of GTP (Table 1). B_{max} values determined in both conditions are presented in Table 1, and were not significantly different ($P>0.05$).

In the assays performed with GTP, nonspecific [^3H]-DPCPX binding determined in the presence of 10 mM theophylline was 72.2% of the total binding, and in the presence of Mg^{2+} ions (8 mM) it was $41 \pm 3\%$ of the total binding. [^3H]-DPCPX binding parameters were not markedly modified (i.e. $K_d = 15.8$ nM; $B_{\text{max}} = 0.260 \pm 0.024$ pmol mg^{-1}

Figure 4 Specific [^3H]-1,3-dipropyl-8-cyclopentylxanthine ([^3H]-DPCPX) binding to frog brain membranes in the presence of 8 mM MgCl_2 ($\blacksquare$), and in the presence of 100 μM GTP ($\blacktriangle$). Specific [^3H]-DPCPX binding was determined by subtracting nonspecific binding from the total binding. Nonspecific binding was determined in the presence of 10 mM theophylline, and at the K_d value for [^3H]-DPCPX was $41 \pm 3\%$ of the total binding in the presence of Mg^{2+} , and of $72.2 \pm 2.1\%$ of the total binding, in the presence of GTP. For each curve, the points correspond to one representative experiment performed in triplicate, out of three experiments with similar results. Inset: Scatchard plot of the specific [^3H]-DPCPX binding. Binding parameters (K_d and B_{max} values) for the experiment showed in the figure were obtained by computer-assisted nonlinear curve fitting and were 12 nM and 0.253 ± 0.103 pmol mg^{-1} of protein in the presence of Mg^{2+} , and 77 nM and 0.264 ± 0.059 pmol mg^{-1} of protein in the presence of GTP. B, bound (pmol mg^{-1} protein) (i.e. specific binding); F, free [^3H]-DPCPX in the incubation medium (nM) after occupation of the binding sites.

Table 1 Binding parameters for [³H]-1,3-dipropyl-8-cyclopentylxanthine ([³H]-DPCPX) to frog brain membranes

	K_d (nM)	B_{max} (pmol mg ⁻¹ prot)
Control ($n = 4$)	43.8 (27–63)	0.238 ± 0.016
Mg ²⁺ (8 mM, $n = 3$)	14.0 (10–21) ^a	0.261 ± 0.018 ^c
GTP (100 μM, $n = 3$)	65.6 (54–83) ^b	0.266 ± 0.026 ^c

K_d and B_{max} (means ± s.e.mean) values were determined with the LIGAND computer-assisted non-linear curve-fitting programme (Munson & Rodbard, 1980). In parentheses are shown the confidence limits of the means of the K_d values obtained from the individual experiments to allow statistical comparison.

^aStatistically different from the control ($P < 0.05$; Student's t test).

^bStatistically different from the K_d value obtained in the presence of Mg²⁺ ($P < 0.05$; Student's t test).

^cNot statistically different from the control ($P > 0.05$; Student's t test).

of protein, in the presence of Mg²⁺, $n = 3$) when R-PIA (30 μM) instead of theophylline (10 mM) was used to define nonspecific binding. When R-PIA was used instead of theophylline, nonspecific [³H]-DPCPX binding in the presence of Mg²⁺ (8 mM) was 51 ± 3% of the total binding at the K_d value for [³H]-DPCPX; this nonspecific binding was not significantly different ($P > 0.05$) from that obtained with theophylline (10 mM).

One [³H]-R-PIA binding experiment was performed in the presence of 100 μM-GTP using concentrations of [³H]-R-PIA up to 200 nM. With concentrations below 10 nM, no measurable specific [³H]-R-PIA binding was observed, and with higher concentrations of [³H]-R-PIA (i.e. 200 nM) the saturation was not yet achieved. This made impossible an accurate determination of the K_d value for [³H]-R-PIA binding in the presence of GTP. Though the K_d of [³H]-R-PIA in the presence of GTP could not be determined by this method, the need of the high concentrations of [³H]-R-PIA to obtain a measurable specific binding in the presence of GTP suggests that GTP affected mainly the affinity of the agonist for the binding sites.

Because Mg²⁺ increased the affinity of [³H]-DPCPX for the binding sites, we performed concentration-inhibition curves for adenosine agonists and antagonists versus [³H]-DPCPX (15 nM) binding in the presence of 8 mM MgCl₂. The inhibition of [³H]-DPCPX binding showed the following order of potencies (K_i): CPA (0.8 nM) > R-PIA (3.2 nM) ≈ NECA (4.8 nM) > S-PIA (19.8 nM) >> CGS 21680 (1749 nM), for the agonists (Figure 5, and Table 2), and XAC (12.7 nM) ≈ DPCPX (13.7 nM) >> XCC (2047 nM) > PACPX (> 10000 nM), for the antagonists (Figure 6 and Table 2). Nonspecific binding determined in the presence of 30 μM-R-PIA was about 40% of the total binding. Computer analysis revealed that all the inhibition binding curves (except for PACPX) were best described by one site binding model. The Hofstee plots for all these compounds were linear, and the Hill coefficients were near 1.0, a result also suggesting the presence of only one binding site (see Table 2). Problems with the solubility of PACPX made impossible its use in concentrations higher than 10⁻⁴ M; thus, complete concentration-inhibition curves for PACPX and determination of the Hill coefficient could not be obtained.

Discussion

The results obtained in the present work show that the frog brain membranes possess a high affinity binding site for [³H]-DPCPX, whose affinity is different either from the A₁ (K_i ≤ 1 nM, Bruns *et al.*, 1987) or the A₂ (K_d = 340 nM, Lohse

Figure 5 Concentration-inhibition curves for adenosine agonists versus specific binding of [³H]-1,3-dipropyl-8-cyclopentylxanthine ([³H]-DPCPX, 15 nM) to frog brain membranes: (●) CPA, (■) R-PIA, (▲) NECA, (▼) S-PIA and (◆) CGS 21680. For abbreviations, see text. Incubations were performed, as described in Methods, in the presence of 8 mM Mg²⁺. For each curve, the points correspond to one representative experiment out of two to four with similar results. K_i values for each agonist are in Table 2. Curves were generated using the best fit parameters from the computer LIGAND programme.

Figure 6 Concentration-inhibition curves for adenosine antagonists versus specific binding of [³H]-1,3-dipropyl-8-cyclopentylxanthine ([³H]-DPCPX, 15 nM) to frog brain membranes: (■) XAC, (●) DPCPX, (▲) XCC, and (◆) PACPX. For abbreviations, see text. Incubations were performed, as described in Methods, in the presence of 8 mM Mg²⁺. For each curve, the points correspond to one representative experiment out of three with similar results. K_i values for each antagonist are in Table 2. Curves were generated using the best fit parameters from the computer LIGAND programme.

et al., 1987) adenosine receptors obtained in rat brain membranes. The K_d value now obtained for the binding of [³H]-DPCPX to the frog brain membranes in the absence of added Mg²⁺ or GTP (i.e. control conditions, K_d = 43.8 nM) suggests that this site could be equivalent to the receptor detected in the functional studies in the frog neuromuscular

Table 2 K_i values for the inhibition of [3 H]-1,3-dipropyl-8-cyclopentylxanthine ([3 H]-DPCPX) binding (15 nM) to frog brain membranes by agonists and antagonists of adenosine receptors

	n	K_i (nM)	n_H
Agonists			
CPA	2	0.8	1.00 ± 0.02
R-PIA	4	3.2	1.00 ± 0.02
NECA	2	4.8	1.03 ± 0.03
S-PIA	3	19.8	1.10 ± 0.15
CGS 21680	3	1749	1.08 ± 0.07
Antagonists			
XAC	3	12.7	0.99 ± 0.07
DPCPX	3	13.7	0.99 ± 0.06
XCC	3	2047	1.01 ± 0.05
PACPX ^a	3	>10000	—

Membranes were incubated with 15 nM [3 H]-DPCPX, for 2 h, at 25°C, in the presence of 8 mM Mg^{2+} , and competitors in a range of 1 nM to 300 μ M, as described in Methods. K_i values were determined by the computer LIGAND programme, assuming a K_d value for [3 H]-DPCPX binding of 14.0 nM. In all cases the one site-model fitted the data better than the two site model ($P < 0.05$). n represents the number of experiments; n_H is the Hill coefficient (means $\pm$ s.d.). For abbreviations, see text.

^aProblems with the solubilization of PACPX made impossible the accurate determination of the K_i of PACPX, as well as of its n_H .

junction where the K_i value obtained for DPCPX was 35 nM (Sebastião & Ribeiro, 1989).

[3 H]-DPCPX binding to the frog brain membranes was displaced by adenosine analogues with the rank order of potency CPA ($K_i = 0.8$ nM) > R-PIA (3.2 nM) $\geq$ NECA (4.8 nM) > S-PIA (19.8 nM) >> CGS 21680 (1.7 μ M), which, except for the near equipotency between NECA and R-PIA and the small (≈ 6) stereoselectivity for the PIA isomers, is not markedly different from what one could expect for the rank order of potency for binding to A_1 adenosine receptors to rat brain membranes (see Table 3, and references therein).

Several adenosine receptor antagonists were used to perform concentration-inhibition curves vs [3 H]-DPCPX binding in the presence of Mg^{2+} . The rank order of potency of the 1,3,8-substituted xanthine derivatives was XAC ($K_i = 13$ nM) $\approx$ DPCPX (14 nM) >> XCC (2 μ M) > PACPX (> 10 μ M). This profile conforms with the data obtained for the adenosine receptor mediating the adenosine-induced pre-synaptic inhibition of neuromuscular transmission in the innervated frog sartorius muscle, where XAC ($K_i = 23$ nM) $\geq$ DPCPX (35 nM) >> XCC (1.9 μ M) $\geq$ PACPX (2.3 μ M) (Sebastião & Ribeiro, 1989). The order of antagonist potency found in the present work is different from that described either for the A_1 or the A_2 adenosine receptors (see Table 3). The main differences related to the A_1 receptor are, besides the low potency of DPCPX, the very low potency of PACPX and XCC.

The finding that the agonist affinities, in contrast to the antagonist affinities for the adenosine receptor in frog brain membranes did not exhibit great differences from the agonist affinities reported for the A_1 adenosine receptor might indicate that the adenosine receptor in the frog brain membranes is a variety of the A_1 adenosine receptor. Indeed, species differences between ligand affinities are more intense for adenosine receptor antagonists, than for adenosine receptor agonists (Ukena *et al.*, 1986; Klotz *et al.*, 1991). For example, sheep, bovine (Klotz *et al.*, 1991) and calf (Ukena *et al.*, 1986) brains have A_1 adenosine receptors highly sensitive to DPCPX ($K_d = 0.2$ nM), whereas guinea-pig brain has an A_1 adenosine receptor with a relatively low affinity for DPCPX ($K_d = 2$ nM; Klotz *et al.*, 1991; 5 nM; Ukena *et al.*, 1986). If the adenosine receptor in the frog brain membranes

Table 3 Comparison of the activity of various agonists and antagonists for the A_1 and A_2 adenosine receptor of rat brain membranes with that for the adenosine receptor of frog brain membranes

	K_i (nM)		
	Frog brain membranes	A_1	A_2
Agonists			
CPA	0.8	0.6 ^a	462 ^a
R-PIA	3.2	1.17 ^a	124 ^a
NECA	4.8	6.3 ^a	10.3 ^a
S-PIA	19.8	49.3 ^a	1820 ^a
CGS 21680	1749	2600 ^b	15 ^b
Antagonists			
XAC	12.7	4 ^b	44 ^b
DPCPX	13.7 (43.8)*	0.5 ^c	340 ^c
XCC	2047	42 ^a	1500 ^a
PACPX	> 10000	2.5 ^a	91.9 ^a

Mean K_i values for the activity of adenosine ligands in the A_1 [3 H]-CHA and in the A_2 [3 H]-NECA (+ 50 nM CPA) binding assays were obtained from ^aBruns *et al.* (1986), ^bJarvis *et al.* (1989), and ^cBruns *et al.* (1987). Agonist and antagonist K_i values for the adenosine receptor in the frog brain membranes were obtained by inhibition of [3 H]-DPCPX binding (15 nM), in the presence of 8 mM Mg^{2+} , which increased the affinity of the binding of the antagonist, DPCPX.

*The K_d value for [3 H]-DPCPX in conditions similar to that used in the studies for the A_1 and A_2 receptor is shown in parentheses.

belongs to the A_1 subtype it has thus an even lower affinity for DPCPX, with a K_d value of 43.8 nM, when we used experimental conditions (i.e. absence of added Mg^{2+} or GTP) similar to those used by Ukena *et al.* (1986) or Klotz *et al.* (1991). Thus, considering that the adenosine receptor in the frog brain membranes belongs to the A_1 subtype, one has to assume that one of the more selective antagonists for this receptor, DPCPX, has more than a 200 fold difference in its affinity for the A_1 receptor according to the animal species used. This issue becomes even more complicated when in the same animals species, the rat, some A_1 adenosine receptors have subnanomolar affinities for DPCPX (Bruns *et al.*, 1987), whereas other non A_2 -DPCPX sensitive receptors have considerably lower affinity, i.e. intermediate affinity between the typical A_1 and the A_2 adenosine receptor subtypes for this xanthine (Alexander *et al.*, 1989; Oliveira *et al.*, 1991).

Modulation by Mg^{2+} and GTP of the [3 H]-R-PIA binding showed that, as occurs with the A_1 -adenosine receptors from rat brain (Perez-Reyes *et al.*, 1987), or rat adipocytes (Ukena *et al.*, 1984), GTP decreased and Mg^{2+} increased specific binding of this agonist to the frog brain membranes. The [3 H]-R-PIA saturation binding studies indicate that the effects of Mg^{2+} and GTP are probably on the affinity of the agonist for the binding site. In the solubilized rat brain membranes, the non- A_1 non- A_2 binding site for [3 H]-R-PIA is also enhanced by Mg^{2+} and inhibited by GTP (Oliveira *et al.*, 1991). It thus appears that the binding of agonists to the non- A_1 non- A_2 adenosine receptor is influenced by GTP and Mg^{2+} in the same manner as the binding of agonists to the A_1 adenosine receptor. There is a consensus that this is a general characteristic of receptors coupled to G-proteins (Gilman, 1987).

Another point of interest in the present results is that the K_d value of this newly detected binding site for [3 H]-DPCPX was increased by GTP. High Mg^{2+} decreased the K_d for [3 H]-DPCPX, an effect common in adenosine agonist binding, but usually not seen in the adenosine antagonist binding. Thus, DPCPX seems to discriminate between two conformations of the adenosine receptor in frog brain membranes, the high affinity form induced by Mg^{2+} , and the low

affinity form induced by GTP, suggesting that a guanine nucleotide binding protein regulates the currently described adenosine antagonist binding in the same manner as it regulates adenosine agonist binding in other situations. This differs from the [3 H]-DPCPX binding to the A_1 adenosine receptor (Weber *et al.*, 1990; Klotz *et al.*, 1990). In some receptor systems, e.g. D_2 -dopamine (De Lean *et al.*, 1982), muscarinic (Burgisser *et al.*, 1982) and opioid (Simon *et al.*, 1984) receptors, it has been found that the antagonists could discriminate between two different conformations of the receptor molecule, with GTP increasing and Mg^{2+} decreasing antagonist affinity. This contrasts with what was found in the present work, where GTP decreased and Mg^{2+} increased antagonist binding. Whether this behaviour of DPCPX means that this xanthine has some agonist properties needs to be elucidated in functional studies. However, in the same animal species, at the frog neuromuscular junction, DPCPX was a consistent competitive adenosine receptor antagonist (Sebastião & Ribeiro, 1989). The same occurs at the rat neuromuscular junction and the rat hippocampus (Sebastião *et al.*, 1990).

In solubilized rat brain preparations, the K_d for the second

high affinity site for [3 H]-DPCPX ($K_d = 11-14$ nM), in the presence of Mg^{2+} (Oliveira *et al.*, 1991), is similar to the K_d value (14 nM) for [3 H]-DPCPX binding to the frog brain membranes also obtained in the presence of Mg^{2+} . Although the GTP-sensitivity of the second binding site for [3 H]-DPCPX in rat brain is not known, the similarity between its K_d value and the K_d value of the [3 H]-DPCPX binding site in frog brain membranes, in similar conditions (i.e. in the presence of Mg^{2+}), might suggest that the binding site similar to that now detected in frog brain membranes is also present in a mammalian preparation.

In summary, besides functional studies establishing the existence of a non- A_1 non- A_2 adenosine receptor at the frog motor nerve terminals (Ribeiro & Sebastião, 1985; Sebastião & Ribeiro, 1989), the present work suggests that the frog brain also possesses this adenosine receptor. This new entity behaves as a receptor G-protein complex, where G-protein coupling regulates its affinity for the agonist, R-PIA, as well as for the antagonist, DPCPX.

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