Comparison of effects of $MgCl_2$ and Gpp(NH)p on antagonist and agonist radioligand binding to adenosine A_1 receptors

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KEY WORDS purinergic P_1 receptors; radioligand assay; magnesium chloride; guanylyl imidodiphosphate; brain

ABSTRACT

AIM: To investigate modulation of antagonist and agonist binding to adenosine A_1 receptors by $MgCl_2$ and 5´-guanylimidodiphosphate (Gpp(NH)p) using rat brain membranes and the A_1 antagonist [^{3}H]-8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX) and the A_1 agonist [^{3}H]-2-chloro-N^6-cyclopentyladenosine ([^{3}H]CCPA). **METHODS:** Parallel saturation and inhibition studies were performed using well-characterised radioligand binding assays and a Brandel Cell Harvester. **RESULTS:** $MgCl_2$ produced a concentration-dependent decrease (44 %), whereas Gpp(NH)p increased [^{3}H]DPCPX binding (19 %). In [^{3}H]DPCPX competition studies, agonist affinity was 1.5-14.6-fold higher and 4.6-10-fold lower in the presence of 10 mmol/L $MgCl_2$ and 10 μmol/L Gpp(NH)p respectively; antagonist affinity was unaffected. The decrease in agonist affinity with increasing Gpp(NH)p concentrations was due to a reduction in the proportion of binding to the high affinity receptor state. In contrast to [^{3}H]DPCPX, $MgCl_2$ produced a concentration-dependent increase (72 %) and Gpp(NH)p a decrease (85 %) in [^{3}H]CCPA binding. Using [^{3}H]CCPA, agonist affinities were 5-17-fold higher than those for [^{3}H]DPCPX, consistent with binding only to the high affinity receptor state. Agonist affinity was 1.3-10.5-fold higher and 2.4-4.7-fold lower on adding $MgCl_2$ or Gpp(NH)p respectively; antagonist affinities were as for [^{3}H]DPCPX. **CONCLUSION:** The inconsistencies surrounding the effects of $MgCl_2$ and guanine nucleotides on radioligand binding to adenosine A_1 receptors were systematically examined. The effects of $MgCl_2$ and Gpp(NH)p on agonist binding to A_1 receptors are consistent with their roles in stimulating GTP-hydrolysis at the G-protein α-subunit and in blocking formation of the high affinity agonist-receptor-G protein complex.

INTRODUCTION

The four adenosine receptors identified to date, termed A_1, A_{2A}, A_{2B} and A_3[1,2] are all G protein coupled and fit the structural motif typical of G protein-coupled receptors (GPCRs)[3,4]. In common with other GPCRs, A_1 receptors have glycosylation sites on the second extracellular loop and residues important for sodium regulation, disulphide bond formation and palmitoylation[1].

On activation, adenosine receptors display a number of characteristics associated with G protein-mediated transmembrane coupling[3,5,6]. Two areas, which have been the focus of a number of contradictory studies, are the magnesium and guanine nucleotide dependence of adenosine agonist and antagonist binding.

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Received 2002-11-01 Accepted 2003-03-24

It is well known that ligand-receptor-G protein interactions are strongly influenced by anions, proteins and $MgCl_2$[3]. Studies indicate that $MgCl_2$ influences both agonist and antagonist binding at A_1 and A_{2A} receptors[7-9]. There are a number of contrasting results, with $MgCl_2$ reported to increase[10] and decrease[11,12] agonist binding at adenosine receptors. In addition to modulation by $MgCl_2$, guanine nucleotides also influence binding to adenosine receptors. For many GPCRs, including the A_1 receptor, high and low affinity states exist for agonist binding[4,13,14], and guanine nucleotides uncouple the G protein from the receptor-G protein complex, resulting in a predominantly low affinity agonist state[14,15]. The finding that high affinity agonist binding for A_1 and A_{2A} receptors is not completely abolished by guanine nucleotides, or by receptor solubilisation[16,17], is indicative of both receptors forming a tight association with their G proteins[4,17]. Unlike agonists, antagonists at A_1 and A_{2A} receptors reportedly recognize coupled and uncoupled states of the receptor with equal affinity[13,18,19]. If this premise is true, guanine nucleotides should not modulate antagonist binding. Studies with the A_{2A} antagonists, [^{3}H]SCH58261[20] and [^{3}H]KF17837S[19], appear consistent with this hypothesis. However, for the A_1 receptor this may not be the case as a variety of different effects have been reported[15,18,20-24].

The contrasting effects of $MgCl_2$ and guanine nucleotides on radioligand binding to adenosine A_1 receptors still require investigation. In this study we systematically examined the effect of $MgCl_2$ and the non-hydrolysable GTP analogue, 5'-guanylyl-imidodi-phosphate [Gpp(NH)p] on both antagonist [^{3}H]-8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX) and agonist [^{3}H]-2-chloro-N^6-cyclopentyladenosine ([^{3}H]CCPA)[25] binding to adenosine A_1 receptors, in an attempt to clarify these discrepancies.

MATERIALS AND METHODS

Materials [^{3}H]DPCPX and [^{3}H]CCPA were obtained from New England Nuclear, Stevenage, UK. 2-Chloroadenosine (CADO), 2-chloro-N^6-cyclopentyladenosine (CCPA), 2-*p*-(2-carboxyethyl)-phenylamino-5'-*N*-carboxamidoadenosine (CGS21680), N^6-cyclohexyladenosine (CHA), N^6-cyclopentyladenosine (CPA), 8-cyclopentyl-1,3-dimethylxanthine (CPT), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 8-phenyl-1,3-diethylxanthine (DPX), 5'-*N*-ethylcarboxamido-adenosine (NECA), *R*(-)N^6-(2-phenylisopropyl)adenosine (*R*-PIA), and 8-phenyl-1,3-dimethylxanthine (8-PT) were obtained from Sigma RBI, Poole, UK. 9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine (CGS15943) was a generous gift from Ciba-Geigy. Adenosine deaminase Type III (ADA), dimethylsulphoxide (Me_2SO), Gpp(NH)p, and other chemicals were from Sigma, Poole, UK.

Membrane preparation Male Sprague-Dawley rats (200-400 g; Charles-River, Margate, UK) were killed by cervical dislocation and membranes prepared as described previously[26]. Briefly, brains were removed and immediately placed in ice-cold saline, before dissection of the cortex. Tissues were homogenized in 15 volumes (vol) of 0.32 mol/L sucrose using a glass/Teflon homogenizer, the homogenate centrifuged at 1000×*g* for 10 min, and the resulting supernatant centrifuged at 17 000×*g* for 20 min. The synaptosomal/mitochondrial P_2 pellet was lysed with 30 vol of ice-cold water for 30 min, then centrifuged at 48 000×*g* for 10 min. The membrane pellet was resuspended in 30 vol of 50 mmol /L Tris-HCl buffer (pH 7.4), centrifuged at 48 000×*g* for 10 min, resuspended in 5 vol of 50 mmol/L Tris-HCl buffer (pH 7.4), and stored at -20 ° C. Protein content was determined as described previously[26].

[^{3}H]DPCPX radioligand binding assay [^{3}H]DPCPX (NEN; 98.1 Ci/mmol=3.6 PBq/mol) binding was carried out as described previously[27]. Briefly, frozen cortical membranes were thawed, resuspended in 30 vol of 50 mmol/L Tris-HCl buffer (pH 7.4), then centrifuged at 48 000×*g,* 4 ° C for 10 min. The pellet was resuspended in 200 vol of 50 mmol/L Tris-HCl buffer (pH 7.4) and kept on ice prior to use in the assay. The binding assay consisted of 10 μL of Me_2SO or test drug, 100 μL of adenosine deaminase (ADA; 1 kU/L; the concentration used was sufficient to remove all endogenous adenosine present in these membrane preparations; data not shown), 190 μL of 50 mmol/L Tris-HCl buffer (pH 7.4), 100 μL of $MgCl_2$ or Gpp(NH)p or buffer, 100 μL of 1 nmol/L [^{3}H]DPCPX and 500 μL of cortical membrane suspension (10-20 μg). Non-specific binding was determined in the presence of 10 μmol/L *R*(-)N^6-(2-phenylisopropyl)adenosine (R-PIA). Test compounds were prepared by serial dilution in Me_2SO; the final assay concentration of 1 % Me_2SO did not affect [^{3}H]ligand binding in either assay (see below). Samples were incubated at 25 ° C for 20 min, then binding was terminated by filtration onto glass fibre filters (GF/B, Whatman) using a Brandel cell harvester, followed by three washes (3 mL) with 50 mmol/L Tris-HCl buffer (pH 7.4). Filter disks were transferred to scintillation

vials, 100 μL of formic acid was added, followed 10 min later by 4 mL Emulsifier Safe™ scintillation fluid. Vials were left overnight then radioactivity was determined in a Packard 2500TR liquid scintillation counter using automatic quench correction.

[^{3}H]CCPA radioligand binding assay [^{3}H]CCPA (NEN; 30 Ci/mmol=2.9 PBq/mol) binding was carried out as described for [^{3}H]DPCPX binding, with the following modifications. The final assay concentration of [^{3}H]CCPA was 0.2 nmol/L, the amount of P_2 rat cortical membrane suspension was 20-40 μg and the incubation period 120 min.

Data analysis Data were analysed using an iterative, non-linear least square curve fitting program (SigmaPlot; Jandel, USA.) to a one site logistic model; $Y=[M\times IC_{50}{}^{P}/(I^{P}+IC_{50}{}^{P})]+B$, where P is the Hill coefficient and Y is bound ligand in the presence of inhibitor concentration, I; M and B are specific binding in the absence of inhibitor and non-specific binding respectively. Estimates of M and B were within 10 % of experimentally determined values. When the inhibitor was the unlabelled form of the radioligand, the binding site affinity, K_D and the binding site density, B_{max} were calculated using the equations; $K_D=IC_{50}$-[ligand] and $B_{max}=(M\times IC_{50})/$[ligand], respectively. For other test compounds, K_i values were calculated using the Cheng Prusoff approximation[27]; $K_i=IC_{50}/\{1+([ligand]/K_D)\}$. Data were routinely analysed using the one site logistic model. In addition, for some agonists, inhibition of [^{3}H]ligand binding was examined in the absence and presence of increasing concentrations of $MgCl_2$ or Gpp(NH)p and data were analysed using a 2-site hyperbolic model. To determine the relative proportions of the high and low affinity states for these agonists, 20 different concentrations of competing drug (instead of 10 duplicate concentrations), were added across an extended range using 3 concentrations per log cycle. Data were fitted to the following equation; $Y_T=Y_1+Y_2=[(M_1\times IC_{50(1)})/(IC_{50(1)}+I)]+[(M_2\times IC_{50(2)})/(IC_{50(2)}+I)]$, where Y_1 and Y_2 represent binding to the high and low affinity states respectively, at inhibitor concentration, I. Statistical comparisons were performed using commercially available software (JMP 3.2; SAS Institute Inc, USA). Prior to statistical analysis by ANOVA, box plots were inspected to ensure a normal symmetrical distribution of data, and the homogeneity of variance was confirmed to be within acceptable limits. Thereafter, ANOVA was used to demonstrate significant differences, with further *post-hoc* analysis-performed using a Dunnett's test.

RESULTS

Effect of $MgCl_2$ & Gpp(NH)p on [^{3}H]DPCPX binding to rat cerebral cortical membranes $MgCl_2$ produced a concentration-dependent decrease in [^{3}H]DPCPX binding, with a maximal reduction of 44 % at 10 mmol/L $MgCl_2$ (Fig 1A), whereas Gpp(NH)p produced a small but significant concentration-dependent increase, plateauing at concentrations above 3 μmol/L (Fig 1B). Consequently, the effects of 10 mmol/L $MgCl_2$ and 10 μmol/L Gpp(NH)p were used to examine the effect upon adenosine antagonist and agonist affinity for both [^{3}H]ligands, as these concentrations are almost maximally effective and have been used routinely in the literature[7,11,21]. When the effects of these single concentrations of $MgCl_2$ and Gpp(NH)p were used to directly compare the binding site affinity (K_D) and density (B_{max}) for both [^{3}H]ligands, all comparisons were made within individual experiments.

Studies using [^{3}H]DPCPX (0.1 nmol/L) and increasing concentrations of unlabelled DPCPX gave a binding site affinity (K_D) of (0.35±0.04) nmol/L (nH=0.91±0.08) and a binding site density (B_{max}) of (2.00±0.22) nmol· g^{-1} (protein) (n=9). In the presence of 10 mmol/L $MgCl_2$, the K_D was unaltered at (0.42± 0.05) nmol/L (nH=0.91±0.09), whereas there was a significant decrease in B_{max} to (1.28±0.19) nmol· g^{-1} (protein) (P<0.05, in a t-test). The K_D in the absence of Gpp(NH)p was (0.25±0.01) nmol/L (nH=0.98±0.03) and the B_{max} was (1.17±0.07) nmol· g^{-1} (protein) (n=15), whereas in the presence of 10 μmol/L Gpp(NH)p the K_D was unchanged [(0.26±0.02) nmol/L (nH=0.99± 0.03)] and the B_{max} significantly increased to (1.47± 0.12) nmol· g^{-1} (protein) (P<0.05, in a t-test).

Affinity of adenosine receptor antagonists and agonists for rat brain [^{3}H]DPCPX binding sites in the absence and presence of 10 mmol/L $MgCl_2$ or 10 μmol/L Gpp(NH)p Antagonists and agonists exhibited the typical adenosine A_1 receptor pharmacological profile for [^{3}H]DPCPX binding sites in the absence and presence of 10 mmol/L $MgCl_2$. For antagonists the rank order of potency was: DPCPX>CGS15943 >CPT> DPX>8-PT and for agonists was: CCPA=CPA≥ R-PIA> CHA>NECA=CADO>CGS21680 (Tab 1). Antagonist affinity was not significantly different in the presence of $MgCl_2$, as shown for DPCPX (Tab 1, Fig 2A), with Hill slopes close to unity for all antagonists under both

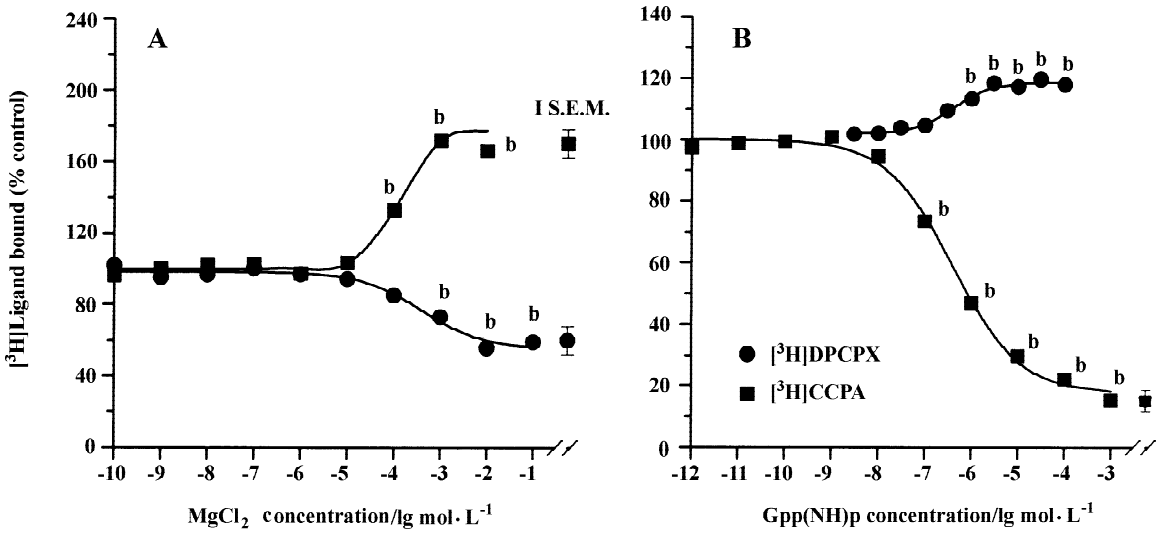

Fig 1. Concentration dependence of (A) $MgCl_2$ and (B) Gpp(NH)p on [^{3}H]DPCPX and [^{3}H]CCPA binding to rat brain membranes. P_2 membranes were incubated with either [^{3}H]ligand in buffer containing increasing concentrations of $MgCl_2$ and Gpp(NH)p. Binding was terminated after varying incubation times by filtration using a Brandel Cell Harvester. Statistical analyses were made using a one-way ANOVA followed by a Dunnett's test. *n*=3 independent experiments. Mean±SEM. $^bP<0.05$ *vs* control.

conditions. In contrast, agonists showed a significant 2-fold increase in affinity, as shown for CCPA (Tab 1, Fig 2A), with the exception of CGS21680 which showed a greater than 10-fold increase in affinity. Hill slopes were approximately 0.6 for all agonists under both conditions.

The pharmacological profile of the compounds for [^{3}H]DPCPX binding sites in the presence of 10 μmol/L Gpp(NH)p (Tab 2) was as observed above. Antagonist affinity was not significantly different in the presence of Gpp(NH)p, whereas agonists showed a significant 5-fold decrease in affinity with the exception of CADO,

Tab 1. Effect of 10 mmol/L $MgCl_2$ on adenosine receptor antagonist and agonist affinity for [^{3}H]DPCPX binding sites in rat cortical membranes. *n*=3 independent experiments. Mean±SEM. $^bP<0.05$ *vs* control.

	Control		$MgCl_2$ 10 mmol· L^{-1}		
	K_i/nmol· L^{-1}	Hill slope	K_i/nmol· L^{-1}	Hill slope	K_i Ratio
Antagonists					
DPCPX	0.35±0.04	0.91±0.08	0.42±0.05	0.91±0.09	1.20
CGS15943	1.35±0.24	0.98±0.03	1.57±0.11	1.09±0.05	1.16
CPT	6.49±0.87	1.01±0.05	8.41±0.63	1.04±0.08	1.30
DPX	33.1±2.01	1.05±0.03	36.3±2.19	1.10±0.02	1.10
8-PT	45.0±1.37	1.07±0.03	49.3±5.61	1.13±0.04	1.10
Agonists					
CCPA	3.39±0.40	0.65±0.02	1.69±0.07^b	0.64±0.06	0.50
CPA	4.02±0.59	0.63±0.02	1.92±0.32	0.65±0.05	0.48
R-PIA	4.56±0.28	0.59±0.02	3.13±0.57^b	0.65±0.03	0.69
CHA	7.16±1.26	0.62±0.06	4.27±0.56	0.67±0.13	0.60
NECA	39.1±7.84	0.58±0.06	14.6±2.51^b	0.59±0.03	0.37
CADO	41.0±4.51	0.60±0.02	16.4±3.38^b	0.58±0.04	0.40
CGS21680	39000±9400	0.65±0.04	2670±430^b	0.56±0.02	0.07

Statistical analyses were made using a *t*-test.

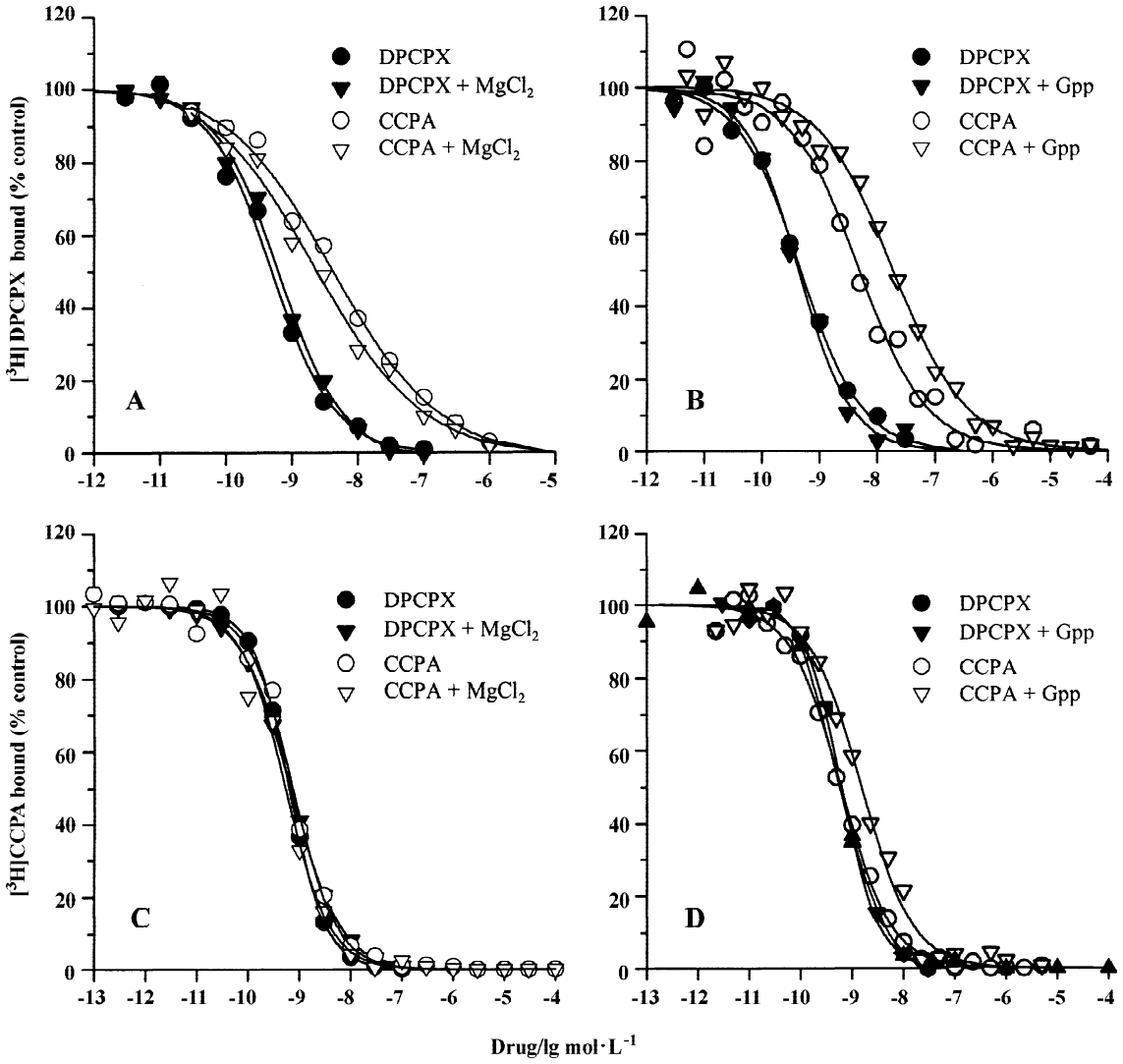

Fig 2. Inhibition of [^{3}H]DPCPX (A & B) and [^{3}H]CCPA (C & D) binding to rat brain membranes by adenosine receptor antagonists and agonists. P_2 membranes were incubated with [^{3}H]DPCPX (0.1 nmol/L) or [^{3}H]CCPA (0.2 nmol/L) in 50 mmol/L Tris-HCl buffer (pH 7.4) containing competing ligands and 0.1 kU/L ADA, in the absence or presence of 10 mmol/L $MgCl_2$ or 10 mmol/L Gpp(NH)p. Binding was terminated (20 min, [^{3}H]DPCPX; 120 min, [^{3}H]CCPA) by filtration using a Brandel Cell Harvester. Data shown are representative competition curves from a single experiment; K_D/K_i and B_{max} values were determined from at least three independent experiments.

which decreased more than 10-fold (Tab 2, Fig 2B). Hill slopes remained close to unity for all antagonists and again approximately 0.6 for agonists in the absence and presence of Gpp(NH)p. As Gpp(NH)p was supplied as a sodium salt, it was possible that the effects observed were due to the presence of Na^+ ions. NaCl at concentrations up to 1 mmol/L, did not affect [^{3}H]DPCPX (0.1 nmol/L) binding and had no effect on agonist affinity (data not shown).

Two-site modelling of the effects of $MgCl_2$ and Gpp(NH)p on [^{3}H]DPCPX binding sites Shallow Hill slopes (nH~0.6) for agonist inhibition of [^{3}H]DPCPX binding is indicative of the presence of high and low affinity sites, a typical feature of GPCRs. The increase in apparent agonist affinity caused by $MgCl_2$ in the [^{3}H]DPCPX binding assay (Tab 1) was examined further with two agonists, CCPA and R-PIA using 20 drug concentrations (3 concentrations per log cycle) to increase precision when using a two site model. Magnesium increased the proportion of the high affinity state labelled, with no alteration in affinity for the high and low states (Tab 3).

The apparent decrease in agonist affinity caused by Gpp(NH)p in the [^{3}H]DPCPX binding assay (Tab 2)

Tab 2. Effect of 10 μmol/L Gpp(NH)p on adenosine receptor antagonist and agonist affinity for [^{3}H]DPCPX binding sites in rat cortical membranes. *n*=3 independent experiments. Mean±SEM. ^{b}*P*<0.05 *vs* control.

	Control		Gpp(NH)p 10 μmol· L^{-1}		
	K_i/nmol· L^{-1}	Hill slope	K_i/nmol· L^{-1}	Hill slope	K_i Ratio
Antagonists					
DPCPX	0.25±0.01	0.98±0.03	0.26±0.02	0.99±0.03	1.04
CGS15943	1.35±0.24	0.98±0.03	1.27±0.06	0.99±0.01	0.94
CPT	3.61±0.66	0.96±0.13	4.60±0.29	1.05±0.09	1.27
DPX	41.0±4.52	1.09±0.03	45.7±7.11	1.12±0.03	1.11
8-PT	49.3±1.84	1.02±0.07	44.7±1.61	1.01±0.08	0.91
Agonists					
CCPA	3.18±0.22	0.66±0.04	15.3±1.07^b	0.67±0.01	4.81
CPA	4.33±0.68	0.61±0.06	21.8±2.50^b	0.71±0.05	5.03
R-PIA	5.13±0.35	0.59±0.03	28.4±3.67^b	0.70±0.02^b	5.54
CHA	8.34±0.66	0.62±0.09	48.0±3.18^b	0.72±0.03	5.76
NECA	43.3±13.0	0.59±0.12	257±30.3^b	0.67±0.07	5.94
CADO	52.2±2.15	0.59±0.04	526±71.3^b	0.76±0.05	10.0
CGS21680	16650±2750	0.65±0.01	76000±11000^b	0.74±0.07	4.56

Statistical analyses were made using a *t*-test.

Tab 3. Effect of $MgCl_2$ and increasing Gpp(NH)p concentrations on adenosine receptor agonist affinity for [^{3}H]DPCPX binding sites in rat cortical membranes: two-site model. *n*=3 independent experiments. Mean±SEM. ^{b}*P*<0.05 *vs* control.

		Y_1/nmol· g^{-1}	K_H/nmol· L^{-1}	Y_2/nmol· g^{-1}	K_L/nmol· L^{-1}	% Y_1
CCPA	Control	0.28±0.02	1.20±0.35	0.15±0.03	31.5±7.8	65.1
	$MgCl_2$					
	+10 mmol· L^{-1}	0.23±0.07	0.88±0.13	0.07±0.01	47.7±9.7	76.6
	Gpp(NH)p					
	+1 μmol· L^{-1}	0.29±0.05	2.30±0.82	0.24±0.07	61.4±21.5	54.7
	+10 μmol· L^{-1}	0.20±0.03	2.02±0.87	0.40±0.08^b	44.4±10.9	33.3
	+100 μmol· L^{-1}	0.13±0.01^b	1.22±0.77	0.48±0.04^b	39.0±2.4	21.3
R-PIA	Control	0.28±0.02	1.63±0.48	0.14±0.01	86.8±28.4	66.6
	$MgCl_2$					
	+10 mmol L^{-1}	0.30±0.02	1.81±0.32	0.10±0.01	109±24.7	75.0
	Gpp(NH)p					
	+1 μmol L^{-1}	0.29±0.04	3.92±0.93	0.25±0.02	74.5±12.0	53.7
	+10 μmol L^{-1}	0.23±0.08	2.71±2.09	0.34±0.06^b	99.2±36.0	40.4
	+100 μmol L^{-1}	0.14±0.05	3.95±2.05	0.42±0.02^b	85.0±18.2	25.0

Data for CCPA and R-PIA were fitted to a two-site model as described in the methods. Y_1 and Y_2 are the percentage of ligand bound to the high and low affinity states respectively. K_H and K_L are the respective K_i values. Statistical differences between control and $MgCl_2$ and/or Gpp(NH)p treated membranes, were determined using a one way ANOVA.

was also examined further for CCPA and R-PIA. Increasing concentrations of Gpp(NH)p (1-100 μmol/L) produced a concentration-dependent reduction in the apparent affinity of the agonists (Tab 4). This was accompanied by a small increase in Hill slope that attained significance for R-PIA. CADO, which appeared

Tab 4. Effect of increasing Gpp(NH)p concentrations on adenosine receptor agonist affinity for [^{3}H]DPCPX binding sites in rat cortical membranes. *n*=3 independent experiments. Mean±SEM. ^{b}*P*<0.05 *vs* control.

	CCPA			R-PIA		
	K_i/nmol· L^{-1}	Hill slope	K_i Ratio	K_i/nmol· L^{-1}	Hill slope	K_i Ratio
Control	3.18±0.22	0.66±0.04	-	5.13±0.35	0.59±0.03	-
Gpp(NH)p						
+1 μmol· L^{-1}	7.96±1.92^b	0.66±0.01	2.50	14.6±1.16^b	0.69±0.03^b	2.85
+10 μmol· L^{-1}	15.3±1.07^b	0.67±0.01	4.81	28.4±3.67^b	0.70±0.02^b	5.54
+100 μmol· L^{-1}	20.9±1.92^b	0.71±0.03	6.57	43.6±8.00^b	0.77±0.03^b	8.50

Statistical analyses were made using a *t*-test.

more sensitive to Gpp(NH)p (Tab 2), behaved in a similar manner to CCPA and R-PIA (data not shown). In addition, a similar shift in affinity for CCPA and R-PIA in the presence of Gpp(NH)p was seen in human, mouse, and guinea pig membranes (data not shown). When these data for CCPA and R-PIA were fitted to a two-site hyperbolic model (Fig 3), it was clear that the apparent decrease in agonist affinity on addition of Gpp (NH)p is due to a decrease in the proportion of the high affinity state labelled, with no significant alteration in the affinity of either state (Tab 3).

Effect of $MgCl_2$ & Gpp(NH)p on [^{3}H]CCPA binding to rat cerebral cortical membranes Initial experiments with [^{3}H]CCPA (0.2 nmol/L) indicate that binding to rat cerebral cortical membranes (25 ° C) was at equilibrium by 120 min; in good agreement with the initial characterisation of this ligand by Klotz *et al*[22] (data not shown).

In contrast to the effects described for the antagonist ligand [^{3}H]DPCPX, $MgCl_2$ produced a significant concentration-dependent increase in [^{3}H]CCPA binding with a maximal increase of 72 % at 1 mmol/L $MgCl_2$ (Fig 1A), and Gpp(NH)p produced a significant concentration-dependent decrease in [^{3}H]CCPA binding, up to 85 % at 1 mmol/L Gpp(NH)p (Fig 1B). The effects of 10 mmol/L $MgCl_2$ and 10 μmol/L Gpp(NH)p

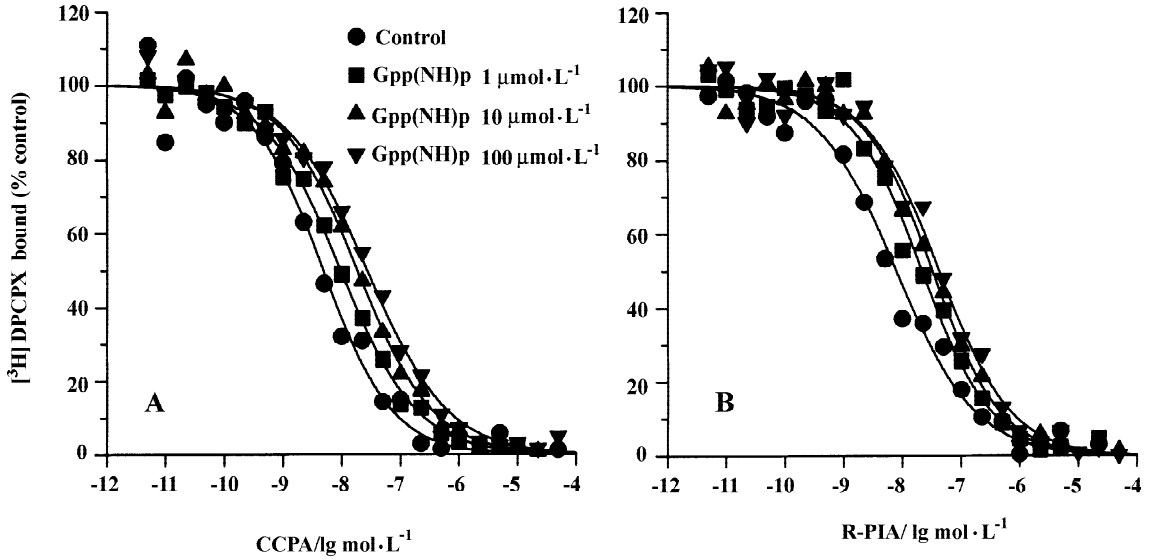

Fig 3. Inhibition of [^{3}H]DPCPX binding to rat cortical P_2 membranes by CCPA and R-PIA in the absence or presence of increasing concentrations of Gpp(NH)p. P_2 membranes were incubated with [^{3}H]DPCPX (0.1 nmol/L) in 50 mmol/L Tris-HCl buffer (pH 7.4) containing competing drug and 0.1 kU/L ADA. Binding was terminated after 20 min by filtration using a Brandel Cell Harvester. Data shown are representative competition curves from a single experiment; K_i values were determined from at least three independent experiments.

on the affinity of adenosine antagonists and agonists for [^{3}H]CCPA binding sites were examined to allow direct comparison with [^{3}H]DPCPX.

Studies using [^{3}H]CCPA (0.2 nmol/L) and increasing concentrations of unlabelled CCPA gave a K_D of (0.52±0.02) nmol/L (nH=1.03 ± 0.05) and a B_{max} of (1.21±0.01) nmol· g^{-1} (protein) (n=3). Addition of 10 mmol/L $MgCl_2$ produced a decrease in K_D to (0.31± 0.04) nmol/L (nH=1.01±0.02) and an increase in B_{max} to (1.42±0.04) nmol· g^{-1} (protein).

In the absence of Gpp(NH)p studies gave a K_D of (0.41±0.04) nmol/L (nH=0.86±0.03) and B_{max} of (1.31± 0.36) nmol· g^{-1} (protein) (n=3), whereas in the presence of 10 μmol/L Gpp(NH)p there was a significant increase in K_D to (0.98±0.15) nmol/L (nH=0.80±0.10) and reduction in B_{max} to (0.66 ± 0.22) nmol· g^{-1} (protein) (P<0.05, in a t-test).

Affinity of adenosine receptor antagonists and agonists for rat brain [^{3}H]CCPA binding sites in the absence and presence of 10 mmol/L $MgCl_2$ or 10 μmol/L Gpp(NH)p The pharmacological profile of [^{3}H] CCPA binding sites was the same as [^{3}H]DPCPX binding sites in the absence and presence of 10 mmol/L $MgCl_2$. For antagonists the rank order of potency was: DPCPX>CGS15943>CPT>DPX>8-PT and for agonists was: CCPA=CPA≥R-PIA≥CHA>NECA=CADO> CGS21680 (Tab 5). Antagonist affinity was generally unaffected by addition of $MgCl_2$ as shown for DPCPX (Fig 2C), although there was a significant reduction for 8-PT (Tab 5). Hill slopes were close to unity for all antagonists under both conditions and antagonist affinities were similar to values obtained for [^{3}H]DPCPX binding sites (Tab 1). Agonist affinity, despite the same rank order of potency, was 5-17-fold higher when compared with [^{3}H]DPCPX binding (Tab 1). In addition, Hill slopes for agonists were near unity in [^{3}H]CCPA binding studies, in contrast to data from [^{3}H]DPCPX binding (nH=0.6). For agonists $MgCl_2$ caused a further approximate 2-fold increase in affinity, with the exception of CGS21680, which showed a 10-fold increase (Tab 5). Hill slopes for agonists were unaffected by $MgCl_2$.

The antagonist and agonist pharmacological profile of [^{3}H]CCPA binding sites was unaltered by Gpp(NH)p (Tab 6). As with [^{3}H]DPCPX binding, antagonist affinity was unaffected by Gpp(NH)p and Hill slopes remained close to unity (Fig 2D). Agonists showed a generally significant 2-5-fold decrease in affinity in the presence of Gpp(NH)p, with the majority of Hill slopes again close to unity in [^{3}H]CCPA binding studies (Fig 2D).

Two-site modelling of the effects of $MgCl_2$ and

Tab 5. Effect of 10 mmol/L $MgCl_2$ on adenosine receptor antagonist and agonist affinity for [^{3}H]CCPA binding sites in rat cortical membranes. n=3 independent experiments. Mean±SEM. ^bP<0.05 *vs* control.

	Control		$MgCl_2$ 10 mmol· L^{-1}		
	K_i/nmol· L^{-1}	Hill slope	K_i/nmol· L^{-1}	Hill slope	K_i Ratio
Antagonists					
DPCPX	0.36±0.04	0.96±0.07	0.43±0.11	0.95±0.20	1.19
CGS15943	2.31±0.31	0.98±0.03	3.00±0.48	0.92±0.09	1.30
CPT	8.92±0.90	1.02±0.05	9.87±1.72	0.98±0.04	1.11
DPX	37.6±3.52	0.93±0.03	44.9±4.66	0.88±0.07	1.19
8-PT	64.0±5.72	1.01±0.04	91.1±10.5^b	1.07±0.09	1.42
Agonists					
CCPA	0.47±0.03	1.01±0.04	0.30±0.06^b	0.90±0.08	0.64
CPA	0.46±0.10	0.92±0.08	0.28±0.07	0.80±0.08	0.61
R-PIA	0.91±0.12	0.94±0.08	0.73±0.18	0.86±0.08	0.80
CHA	1.31±0.22	0.92±0.04	0.60±0.08	0.91±0.02	0.46
NECA	4.24±0.41	0.83±0.04	1.95±0.14^b	0.87±0.03	0.46
CADO	3.69±0.40	0.86±0.05	1.02±0.32^b	0.82±0.11	0.28
CGS21680	2300±320	0.86±0.06	220±29.3^b	0.78±0.01	0.10

Statistical analyses were made using a t-test.

Gpp(NH)p on [^{3}H]CCPA binding sites Hill slopes for agonist inhibition of [^{3}H]CCPA binding were closer to unity than those observed for [^{3}H]DPCPX. The increase in agonist affinity on addition of $MgCl_2$ to the [^{3}H]CCPA binding assay (Tab 5) was investigated further using 20 drug concentrations. Despite sufficient levels of [^{3}H]CCPA binding in the presence of 10 mmol/L $MgCl_2$, these data could not be resolved into two states.

In addition, the decrease in agonist affinity on addition of Gpp(NH)p to the [^{3}H]CCPA binding assay (Tab 6) was studied by altering the concentration of Gpp(NH)p (1-100 μmol/L). As for [^{3}H]DPCPX binding, there was a concentration-dependent reduction in agonist affinity in the presence of Gpp(NH)p, with little alteration in Hill slope (Tab 7). Due to the reduction in [^{3}H]CCPA binding with increasing concentrations of Gpp(NH)p it was not possible to use the two-site model.

Data supporting the hypothesis that [^{3}H]CCPA predominantly labels the high affinity state of the receptor come from competition studies when [^{3}H]CCPA and [^{3}H]DPCPX binding were examined in parallel. In these studies the B_{max} for [^{3}H]DPCPX was (2.09±0.38) nmol· g^{-1} (protein) (n=3) and for [^{3}H]CCPA, (1.21± 0.01) nmol· g^{-1} (protein). The B_{max} of [^{3}H]CCPA was approximately 60 % of [^{3}H]DPCPX. These data are

Tab 6. Effect of 10 μmol/L Gpp(NH)p on adenosine receptor antagonist and agonist affinity for [^{3}H]CCPA binding sites in rat cortical membranes. n=3 independent experiments. Mean±SEM. ^bP<0.05 *vs* control.

	Control		Gpp(NH)p 10 μmol· L^{-1}		
	K_i/nmol· L^{-1}	Hill slope	K_i/nmol· L^{-1}	Hill slope	K_i ratio
Antagonists					
DPCPX	0.34±0.07	0.94±0.12	0.33±0.06	1.19±0.15	0.97
CGS15943	2.08±0.20	0.94±0.03	1.58±0.05	0.84±0.02^b	0.76
CPT	7.18±0.89	0.96±0.09	7.29±2.07	0.85±0.20	1.02
DPX	39.1±4.81	0.92±0.06	28.8±1.23	1.15±0.15	0.74
8-PT	60.5±3.65	1.01±0.11	42.7±3.81^b	1.23±0.06	0.71
Agonists					
CCPA	0.41±0.04	0.86±0.03	0.98±0.15	0.80±0.09	2.39
CPA	0.33±0.21	0.74±0.15	1.17±0.38	0.81±0.10	3.55
R-PIA	1.10±0.13	0.82±0.11	4.57±1.87	1.05±0.22	4.15
CHA	1.07±0.31	0.87±0.06	3.11±0.59^b	0.80±0.10	2.91
NECA	4.24±0.41	0.83±0.04	13.9±1.07^b	0.84±0.08	3.28
CADO	3.69±0.40	0.86±0.05	17.2±0.34^b	0.65±0.08	4.66
CGS21680	2300±320	0.86±0.06	9330±1030^b	0.78±0.03	4.06

Statistical analyses were made using a *t*-test.

Tab 7. Effect of increasing Gpp(NH)p concentrations on adenosine receptor agonist affinity for [^{3}H]CCPA binding sites in rat cortical membranes. n=3 independent experiments. Mean±SEM. ^bP<0.05 *vs* control.

	CCPA			R-PIA		
	K_i/nmol· L^{-1}	Hill slope	K_i Ratio	K_i/nmol· L^{-1}	Hill slope	K_i Ratio
Control	0.41±0.04	0.86±0.03	-	1.10±0.13	0.82±0.11	-
Gpp(NH)p						
+1 μmol· L^{-1}	0.64±0.07	0.79±0.03	1.56	1.96±0.53	0.81±0.09	1.78
+10 μmol· L^{-1}	0.98±0.15	0.80±0.09	2.39	4.57±1.87	1.05±0.22	4.15
+100 μmol· L^{-1}	0.80±0.10	0.78±0.03^b	1.95	3.09±0.66	0.90±0.04	2.81

Statistical analyses were made using a *t*-test.

consistent with the proportion of receptors (65 %) deemed to be in the high affinity state under control conditions using [^{3}H]DPCPX and agonists (Tab 3). The K_i value of CCPA for the high affinity sites in the [^{3}H]DPCPX binding assay (1 nmol/L; Tab 3) is also similar to the K_D of [^{3}H]CCPA (0.47 nmol/L).

DISCUSSION

Previous studies contrasting the effects of $MgCl_2$ and guanine nucleotides on binding to adenosine A_1 receptors have been inconsistent. This study systematically and separately examined the effect of $MgCl_2$ and Gpp(NH)p on [^{3}H]antagonist and [^{3}H]agonist binding to A_1 receptors, using tissues from the same species, with matching temperatures and pH, thereby negating some factors responsible for the variability.

Modulation of [^{3}H]ligand binding to A_1 receptors by $MgCl_2$ For some GPCRs, including adenosine receptors, two independent, thermodynamically distinct, high and low affinity states exist for agonist binding, corresponding to G-protein-coupled and uncoupled receptors[12]. At physiological expression levels agonists discriminate between these two states, whereas antagonists recognize both states with equal affinity; the situation may be different when receptors are over-expressed[28]. The reduction in [^{3}H]DPCPX binding produced by $MgCl_2$ in these studies using native tissue is therefore complex, as the K_D of [^{3}H]DPCPX would require to increase 2-fold to account for the 40 % reduction in binding. However, parallel incubations with $MgCl_2$ gave almost identical affinities for DPCPX (and other antagonists), consistent with some data[7], but contrasting with a recent study that did show a decrease in both DPCPX affinity and B_{max} in the presence of $MgCl_2$[12]. It is therefore possible that other factors may be involved, including promoting the high-affinity state, not preferred by antagonists[11,28], or the presence of endogenous adenosine[20,28,29]. We feel the latter is unlikely, as concentration response curves for ADA had shown clearly that this concentration was sufficient to remove all the endogenous adenosine present in our membrane preparations (data not shown) and others have used similar concentrations[12,22,30,31]; we cannot however totally rule out any contribution from adenosine present in ' cryptic' pools[32]. $MgCl_2$ produced a 2-fold increase (10-fold for the A_{2a} selective CGS21680) in agonist affinities, consistent with magnesium' s modulatory role on GPCRs[3,12]. Two-state modelling for CCPA and R-PIA indicated that the increase in apparent affinity by $MgCl_2$, was due to a proportional increase in high affinity receptor state labelling (65 % to 75 %), with no change in agonist affinity for either state, consistent with a recent study[12]. Under control conditions, the proportion of these states and their 30-fold difference in affinity is similar in studies across species, membranes and cloned receptors[12, 21].

K_D and B_{max} values for [^{3}H]CCPA binding to cortical membranes agree well with previous data[25]. The K_D of [^{3}H]CCPA and the K_i value for R-PIA, were similar to their high affinity values (K_h) for inhibiting [^{3}H]DPCPX binding. Moreover, the [^{3}H]CCPA B_{max} is 65 % of the [^{3}H]DPCPX B_{max}, identical to the proportion of [^{3}H]DPCPX binding sites in the high affinity state, indicating that [^{3}H]CCPA labels, exclusively, the high affinity state of A_1 receptors[13,14]. Allowing for the 30-fold difference in affinity of CCPA for the two states, and their relative proportions in [^{3}H]DPCPX binding, this is consistent with the calculation that at the concentration of [^{3}H]CCPA (0.2 nmol/L) used in this study, at least 98 % of its binding would be to the high affinity site. This is supported by the inability to resolve [^{3}H]CCPA (±$MgCl_2$) using a two-site model.

$MgCl_2$ increased [^{3}H]CCPA binding, consistent with data for other A_1 [^{3}H]agonists and GPCRs[3,7]. The increased [^{3}H]CCPA binding by $MgCl_2$ was due to an increase in B_{max} (consistent with the proportional increase in high affinity state labelling seen for [^{3}H]DPCPX), and also to a small but genuine (near 2-fold) increase in ligand affinity. Antagonist affinities were similar to those for [^{3}H]DPCPX, and were unaffected by $MgCl_2$. However, agonist affinities were 5-17-fold higher and Hill slopes were near unity, consistent with [^{3}H]CCPA labelling the high affinity site. $MgCl_2$, in addition, produced a further 2-fold increase in agonist affinity (10-fold for CGS21680) as seen for [^{3}H]CCPA itself. The reason why this increase in affinity of agonists for the high affinity site was not detected for CCPA and R-PIA using [^{3}H]DPCPX, is presumably because it was masked by the presence/change in proportion of the low affinity sites and was outwith the resolution of the two-site model.

Modulation of [^{3}H]ligand binding to A_1 receptors by Gpp(NH)p If antagonists recognize G-protein-coupled and uncoupled receptors with equal affinity, guanine nucleotides should not modulate antagonist binding. However for adenosine receptors guanine nucleotides produce many effects, including increased

binding to membranes and cloned receptors[15,18,20,28,31]. In addition, inhibition of [^{3}H]DPCPX binding by agonists shows that the radioligand recognizes both receptor states. The increase in [^{3}H]DPCPX binding by Gpp(NH)p, with no alteration in affinity is consistent with other data[20,30]. However the magnitude of increase varies, reflecting species differences and assay conditions[11,15,20,23]. The 20 % increase in [^{3}H]DPCPX binding observed here, would be explained by a small but genuine increase in affinity (0.25 to 0.19 nmol/L), rather than as mentioned earlier, cryptic pools of adenosine[32] or a preference for the uncoupled form of the receptor[28]. The affinity of the other antagonists was also unaffected by Gpp(NH)p, consistent with kinetic data in this (data not shown) and other studies[22].

The 5-fold decrease in agonist affinity for [^{3}H]DPCPX binding by Gpp(NH)p is characteristic of adenosine and other GPCRs[3,31], being associated with slight increases in Hill slope, which did not reach unity. We have shown previously that this decrease in affinity is similar for rat, mouse, guinea pig and human brain membranes[27], data that have been confirmed recently using human brain autoradiography[20]. Although again, as in many previous studies[12,15,24] interpretation of the effects of GTP on [^{3}H]DPCPX binding to human A_1 receptors in this recent study[20], is complicated by the presence of 1 mmol/L $MgCl_2$ in the buffer, which have the opposite effect. Using [^{3}H]DPCPX, Gpp(NH)p caused a concentration-dependent decrease in the proportion of the high affinity agonist sites, with no detectable alteration in agonist affinity of either state, essentially opposite to that of $MgCl_2$. This was consistent with its reduction in the B_{max} for [^{3}H]CCPA, which only labels high affinity sites. The inability of Gpp(NH)p to completely shift to the low agonist affinity state reflects tight coupling between the A_1 receptor and G-protein[28,29], contrasting with other GPCRs[33]. Gpp(NH)p also produced a small but genuine decrease in agonist affinity as shown using [^{3}H]CCPA, with a 2-4-fold decrease in the K_D value, and K_i values of other agonists. As with [^{3}H]DPCPX, Gpp(NH)p did not alter antagonist affinity using [^{3}H]CCPA.

Previous studies examining modulation of binding to A_1 receptors by $MgCl_2$ and guanine nucleotides produced inconsistent results. Factors accounting for this variability have included, the use of tissue from different species, membrane structure, chelators, alterations in pH and temperature and the simultaneous addition of guanine nucleotides and magnesium[12,20,27,34]. In this study we systematically and separately examined the effects of $MgCl_2$ and Gpp(NH)p on the binding of an antagonist and agonist ligand selective for A_1 receptors, using identical experimental conditions. The effects of $MgCl_2$ and Gpp(NH)p on agonist affinities were similar for both the antagonist and agonist radioligand. In addition, $MgCl_2$ and Gpp(NH)p had little effect on antagonist affinities in either assay, despite some modulation of [^{3}H]antagonist binding. In conclusion, the effects observed with $MgCl_2$ and Gpp(NH)p in modulating agonist binding are consistent, when examined separately, with their respective roles in stimulating GTP-hydrolysis at the α-subunit of the G-protein and in blocking the formation of the high affinity agonist-receptor-G protein complex. However, the reasons for the modulation of [^{3}H]antagonist binding by magnesium and Gpp(NH)p still appear complicated.

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