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 $\alpha$-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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It is well known that ligand-receptor-G protein interactions are strongly influenced by anions, proteins and MgCl₂. Studies indicate that MgCl₂ influences both agonist and antagonist binding at A₁ and A₂₅ receptors[7-9]. There are a number of contrasting results, with MgCl₂ reported to increase[10] and decrease[11,12] agonist binding at adenosine receptors. In addition to modulation by MgCl₂, guanine nucleotides also influence binding to adenosine receptors. For many GPCRs, including the A₁ 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 A₁ and A₂₅ 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[18,19]. Unlike agonists, antagonists at A₁ and A₂₅ 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 A₂₅ antagonists, [³H]SCH58261[20] and [³H]KF17837S[19], appear consistent with this hypothesis. However, for the A₁ receptor this may not be the case as a variety of different effects have been reported[15,18,20,24].

The contrasting effects of MgCl₂ and guanine nucleotides on radioligand binding to adenosine A₁ receptors still require investigation. In this study we systematically examined the effect of MgCl₂ and the non-hydrolysable GTP analogue, 5'-guanylylimidodiphosphate [Gpp(NH)p] on both antagonist [³H]-8-cyclopentyl-1,3-dipropylxanthine ([³H]DPCPX) and agonist [³H]-2-chloro-N⁶-cyclopentyladenosine ([³H]CCPA)[25] binding to adenosine A₁ receptors, in an attempt to clarify these discrepancies.

MATERIALS AND METHODS

Materials [³H]DPCPX and [³H]CCPA were obtained from New England Nuclear, Stevenage, UK. 2-Chloroadenosine (CADO), 2-chloro-N⁶-cyclopentyladenosine (CCPA), 2-p-(2-carboxyethyl)-phenylamino-5'-N-carboxamidoadenosine (CGS21680), N⁶-cyclohexyladenosine (CHA), N⁶-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(-)-N⁶-(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 (MeSO), 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 10000g for 10 min, and the resulting supernatant centrifuged at 17000g for 20 min. The synaptosomal/mitochondrial P₂ pellet was lysed with 30 vol of ice-cold water for 30 min, then centrifuged at 48000g for 10 min. The membrane pellet was resuspended in 30 vol of 50 mmol/L Tris-HCl buffer (pH 7.4), centrifuged at 48000g for 10 min, resuspended in 5 vol of 50 mmol/L Tris-HCl buffer (pH 7.4), and stored at -20 ºC. Protein content was determined as described previously[26].

[³H]DPCPX radioligand binding assay [³H]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 48000g, 4 ºC 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 MeSO 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), 190 µL of MeSO or Gpp(NH)p or buffer, 100 µL of 1 nmol/L [³H]DPCPX and 500 µL of cortical membrane suspension (10-20 µg). Non-specific binding was determined in the presence of 10 µmol/L R(-)-N⁶-(2-phenylisopropyl)adenosine (R-PIA). Test compounds were prepared by serial dilution in MeSO; the final assay concentration of 1 % MeSO did not affect [³H]ligand binding in either assay (see below). Samples were incubated at 25 ºC 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.

\[ [^3H]CCPA \text{ radioligand binding assay} \]

\[ [^3H]CCPA \text{ (NEN; } 30 \text{ Ci}/\text{mmol}=2.9 \text{ PBq/mol)} \text{ binding was carried out as described for } [^3H]\text{DPCPX binding, with the following modifications. The final assay concentration of } [^3H]CCPA \text{ was } 0.2 \text{ nmol/L, the amount of P}_2 \text{ rat cortical membrane suspension was } 20-40 \mu g \text{ and the incubation period } 120 \text{ min.} \]

**Data analysis**

Data were analysed using an iterative, non-linear least square curve fitting program (SigmaPlot; Jandel, USA.) to a one site logistic model; \( Y = \frac{M \times IC_{50}}{IC_{50}}/(1 + IC_{50}/Y) + B \), 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, \( K_p \) and the binding site density, \( B_{\text{max}} \) were calculated using the equations; \( K_p = IC_{50}/[\text{ligand}] \) and \( B_{\text{max}} = (M \times IC_{50})/[\text{ligand}] \), respectively. For other test compounds, \( K \) values were calculated using the Cheng Prusoff approximation \[\{[^{3}H]\text{ligands, as these concentrations are almost maximally effective and have been used routinely in the literature}^{7,11,21} \]. When the effects of these single concentrations of MgCl\(_2\) and Gpp(NH)p were used to directly compare the binding site affinity (\( K_p \)) and density (\( B_{\text{max}} \)) for both \([^3H]\text{ligands, all comparisons were made within individual experiments.} \)

Studies using \([^3H]\text{DPCPX (0.1 nmol/L} \) and increasing concentrations of unlabelled DPCPX gave a binding site affinity (\( K_p \)) of (0.35±0.04) nmol/L (nH=0.91±0.08) and a binding site density (\( B_{\text{max}} \)) of (2.00±0.22) nmol·g\(^{-1}\) (protein) (n=9). In the presence of 10 mmol/L MgCl\(_2\), the \( K_p \) was unaltered at (0.42±0.05) nmol/L (nH=0.91±0.09), whereas there was a significant decrease in \( B_{\text{max}} \) to (1.28±0.19) nmol·g\(^{-1}\) (protein) (P<0.05, in a t-test). The \( K_p \), in the absence of Gpp(NH)p was (0.25±0.01) nmol/L (nH=0.98±0.03) and the \( B_{\text{max}} \) was (1.17±0.07) nmol·g\(^{-1}\) (protein) (n=15), whereas in the presence of 10 µmol/L Gpp(NH)p the \( K_p \) was unchanged [(0.26±0.02) nmol/L (nH=0.99±0.03)] and the \( B_{\text{max}} \) 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 \([^3H]\text{DPCPX binding sites in the absence and presence of 10 mmol/L MgCl}_{2} \) or \( 10 \mu \text{mol/L Gpp(NH)p} \)**

Antagonists and agonists exhibited the typical adenosine \( A_1 \) receptor pharmacological profile for \([^3H]\text{DPCPX binding sites in the absence and presence of 10 mmol/L MgCl}_{2} \). 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

using a Dunnett’s test.

**RESULTS**

**Effect of MgCl\(_2\) & Gpp(NH)p on \([^3H]\text{DPCPX binding to rat cerebral cortical membranes} \)**

MgCl\(_2\) produced a concentration-dependent decrease in \([^3H]\text{DPCPX binding, with a maximal reduction of 44%}\)

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 \([^3H]\)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 (\( K_p \)) and density (\( B_{\text{max}} \)) for both \([^3H]\)ligands, all comparisons were made within individual experiments.

Studies using \([^3H]\text{DPCPX (0.1 nmol/L} \) and increasing concentrations of unlabelled DPCPX gave a binding site affinity (\( K_p \)) of (0.35±0.04) nmol/L (nH=0.91±0.08) and a binding site density (\( B_{\text{max}} \)) of (2.00±0.22) nmol·g\(^{-1}\) (protein) (n=9). In the presence of 10 mmol/L MgCl\(_2\), the \( K_p \) was unaltered at (0.42±0.05) nmol/L (nH=0.91±0.09), whereas there was a significant decrease in \( B_{\text{max}} \) to (1.28±0.19) nmol·g\(^{-1}\) (protein) (P<0.05, in a t-test). The \( K_p \), in the absence of Gpp(NH)p was (0.25±0.01) nmol/L (nH=0.98±0.03) and the \( B_{\text{max}} \) was (1.17±0.07) nmol·g\(^{-1}\) (protein) (n=15), whereas in the presence of 10 µmol/L Gpp(NH)p the \( K_p \) was unchanged [(0.26±0.02) nmol/L (nH=0.99±0.03)] and the \( B_{\text{max}} \) 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 \([^3H]\text{DPCPX binding sites in the absence and presence of 10 mmol/L MgCl}_{2} \) or \( 10 \mu \text{mol/L Gpp(NH)p} \)**

Antagonists and agonists exhibited the typical adenosine \( A_1 \) receptor pharmacological profile for \([^3H]\text{DPCPX binding sites in the absence and presence of 10 mmol/L MgCl}_{2} \). 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
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 $[^3H]$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, 

The pharmacological profile of the compounds for $[^3H]$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 $[^3H]$DPCPX binding sites in rat cortical membranes. $n=3$ independent experiments. Mean±SEM. $^bP<0.05$ vs control.

<table>
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<th>Control</th>
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<tr>
<td></td>
<td>$K$/nmol·L$^{-1}$</td>
<td>Hill slope</td>
<td>$K$/nmol·L$^{-1}$</td>
<td>Hill slope</td>
</tr>
<tr>
<td><strong>Antagonists</strong></td>
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<td></td>
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<tr>
<td>DPCPX</td>
<td>0.35±0.04</td>
<td>0.91±0.08</td>
<td>0.42±0.05</td>
<td>0.91±0.09</td>
</tr>
<tr>
<td>CGS15943</td>
<td>1.35±0.24</td>
<td>0.98±0.03</td>
<td>1.57±0.11</td>
<td>1.09±0.05</td>
</tr>
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<td>CPT</td>
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<td>1.01±0.05</td>
<td>8.41±0.63</td>
<td>1.04±0.08</td>
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<tr>
<td>DPX</td>
<td>33.1±2.01</td>
<td>1.03±0.03</td>
<td>36.3±2.19</td>
<td>1.10±0.02</td>
</tr>
<tr>
<td>8-PT</td>
<td>45.0±1.37</td>
<td>1.07±0.03</td>
<td>49.3±5.61</td>
<td>1.13±0.04</td>
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<td><strong>Agonists</strong></td>
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<tr>
<td>CCPA</td>
<td>3.39±0.40</td>
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<td>1.69±0.07$^b$</td>
<td>0.64±0.06</td>
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<tr>
<td>CPA</td>
<td>4.02±0.59</td>
<td>0.63±0.02</td>
<td>1.92±0.32</td>
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<tr>
<td>R-PIA</td>
<td>4.56±0.28</td>
<td>0.59±0.02</td>
<td>3.13±0.57$^b$</td>
<td>0.65±0.03</td>
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<tr>
<td>CHA</td>
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<td>NECA</td>
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<td>14.6±2.51$^b$</td>
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<tr>
<td>CADO</td>
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<td>CGS21680</td>
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<td>2670±430$^b$</td>
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Statistical analyses were made using a $t$-test.
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\text{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\text{H}\)DPCPX binding sites** Shallow Hill slopes (nH~0.6) for agonist inhibition of \[^3\text{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\text{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\text{H}\)DPCPX binding assay (Tab 2)
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 [³H]DPCPX binding sites in rat cortical membranes. n=3 independent experiments. Mean±SEM. *P<0.05 vs control.

<table>
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<th>R-PIA</th>
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<td>K_i /nmol·L⁻¹</td>
<td>Hill slope</td>
</tr>
<tr>
<td>Control</td>
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<td>0.66±0.04</td>
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<tr>
<td>Gpp(NH)p</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+1 µmol·L⁻¹</td>
<td>7.96±1.92ᵇ</td>
<td>0.66±0.01</td>
</tr>
<tr>
<td>+10 µmol·L⁻¹</td>
<td>15.3±1.07ᵇ</td>
<td>0.67±0.01</td>
</tr>
<tr>
<td>+100 µmol·L⁻¹</td>
<td>20.9±1.92ᵇ</td>
<td>0.71±0.03</td>
</tr>
</tbody>
</table>

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 MgCl₂ & Gpp(NH)p on [³H]CCPA binding to rat cerebral cortical membranes

Initial experiments with [³H]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 [³H]DPCPX, MgCl₂ produced a significant concentration-dependent increase in [³H]CCPA binding with a maximal increase of 72 % at 1 mmol/L MgCl₂ (Fig 1A), and Gpp(NH)p produced a significant concentration-dependent decrease in [³H]CCPA binding, up to 85 % at 1 mmol/L Gpp(NH)p (Fig 1B). The effects of 10 mmol/L MgCl₂ and 10 µmol/L Gpp(NH)p

Fig 3. Inhibition of [³H]DPCPX binding to rat cortical P₂ membranes by CCPA and R-PIA in the absence or presence of increasing concentrations of Gpp(NH)p. P₂ membranes were incubated with [³H]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; Kᵢ 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 \(\mu\)mol/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 \(\mu\)mol/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**

<p>| 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. (^{a}P&lt;0.05) vs control. |</p>
<table>
<thead>
<tr>
<th>Antagonists</th>
<th>Control</th>
<th>Hill slope</th>
<th>MgCl(_2), 10 mmol·L(^{-1})</th>
<th>Hill slope</th>
<th>K(_i), Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPCPX</td>
<td>0.36±0.04</td>
<td>0.96±0.07</td>
<td>0.43±0.11</td>
<td>0.95±0.20</td>
<td>1.19</td>
</tr>
<tr>
<td>CGS15943</td>
<td>2.31±0.31</td>
<td>0.98±0.03</td>
<td>3.00±0.48</td>
<td>0.92±0.09</td>
<td>1.30</td>
</tr>
<tr>
<td>CPT</td>
<td>8.92±0.90</td>
<td>1.02±0.05</td>
<td>9.87±1.72</td>
<td>0.98±0.04</td>
<td>1.11</td>
</tr>
<tr>
<td>DPX</td>
<td>37.6±3.52</td>
<td>0.93±0.03</td>
<td>44.9±4.66</td>
<td>0.88±0.07</td>
<td>1.19</td>
</tr>
<tr>
<td>8-PT</td>
<td>64.0±5.72</td>
<td>1.01±0.04</td>
<td>91.1±10.5(b)</td>
<td>1.07±0.09</td>
<td>1.42</td>
</tr>
<tr>
<td>Agonists</td>
<td>Control</td>
<td>Hill slope</td>
<td>MgCl(_2), 10 mmol·L(^{-1})</td>
<td>Hill slope</td>
<td>K(_i), Ratio</td>
</tr>
<tr>
<td>CCPA</td>
<td>0.47±0.03</td>
<td>1.01±0.04</td>
<td>0.30±0.06(b)</td>
<td>0.90±0.08</td>
<td>0.64</td>
</tr>
<tr>
<td>CPA</td>
<td>0.46±0.10</td>
<td>0.92±0.08</td>
<td>0.28±0.07</td>
<td>0.80±0.08</td>
<td>0.61</td>
</tr>
<tr>
<td>R-PIA</td>
<td>0.91±0.12</td>
<td>0.94±0.08</td>
<td>0.73±0.18</td>
<td>0.86±0.08</td>
<td>0.80</td>
</tr>
<tr>
<td>CHA</td>
<td>1.31±0.22</td>
<td>0.92±0.04</td>
<td>0.60±0.08</td>
<td>0.91±0.02</td>
<td>0.46</td>
</tr>
<tr>
<td>NECA</td>
<td>4.24±0.41</td>
<td>0.83±0.04</td>
<td>1.95±0.14(b)</td>
<td>0.87±0.03</td>
<td>0.46</td>
</tr>
<tr>
<td>CADO</td>
<td>3.69±0.40</td>
<td>0.86±0.05</td>
<td>1.02±0.32(b)</td>
<td>0.82±0.11</td>
<td>0.28</td>
</tr>
<tr>
<td>CGS21680</td>
<td>2300±320</td>
<td>0.86±0.06</td>
<td>220±29.3(b)</td>
<td>0.78±0.01</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Statistical analyses were made using a t-test.
Gpp(NH)p on $[^3]$HCCPA binding sites  Hill slopes for agonist inhibition of $[^3]$HCCPA binding were closer to unity than those observed for $[^3]$HDPCPX. The increase in agonist affinity on addition of MgCl$_2$ to the $[^3]$HCCPA binding assay (Tab 5) was investigated further using 20 drug concentrations. Despite sufficient levels of $[^3]$HCCPA 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 $[^3]$HCCPA binding assay (Tab 6) was studied by altering the concentration of Gpp(NH)p (1-100 µmol/L). As for $[^3]$HDPCPX 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 $[^3]$HCCPA binding with increasing concentrations of Gpp(NH)p it was not possible to use the two-site model.

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

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

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Gpp(NH)p 10 µmol·L$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K/nmol·L$^{-1}$</td>
<td>Hill slope</td>
</tr>
<tr>
<td>Antagonists</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPCPX</td>
<td>0.34±0.07</td>
<td>0.94±0.12</td>
</tr>
<tr>
<td>CGS15943</td>
<td>2.08±0.20</td>
<td>0.94±0.03</td>
</tr>
<tr>
<td>CPT</td>
<td>7.18±0.89</td>
<td>0.96±0.09</td>
</tr>
<tr>
<td>DPX</td>
<td>39.1±4.81</td>
<td>0.92±0.06</td>
</tr>
<tr>
<td>8-PT</td>
<td>60.5±3.65</td>
<td>1.01±0.11</td>
</tr>
<tr>
<td>Agonists</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCPA</td>
<td>0.41±0.04</td>
<td>0.86±0.03</td>
</tr>
<tr>
<td>CPA</td>
<td>0.33±0.21</td>
<td>0.74±0.15</td>
</tr>
<tr>
<td>R-PIA</td>
<td>1.10±0.13</td>
<td>0.82±0.11</td>
</tr>
<tr>
<td>CHA</td>
<td>1.07±0.31</td>
<td>0.87±0.06</td>
</tr>
<tr>
<td>NECA</td>
<td>4.24±0.41</td>
<td>0.83±0.04</td>
</tr>
<tr>
<td>CADO</td>
<td>3.69±0.40</td>
<td>0.86±0.05</td>
</tr>
<tr>
<td>CGS21680</td>
<td>2300±320</td>
<td>0.86±0.06</td>
</tr>
</tbody>
</table>

Statistical analyses were made using a t-test.

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

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

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 [3H]DPCPX and agonists (Tab 3). The
K_i value of CCPA for the high affinity sites in the
[3H]DPCPX binding assay (1 nmol/L; Tab 3) is also
similar to the K_i of [3H]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 systemati-
cally and separately examined the effect of MgCl_2
and Gpp(NH)p on [3H]antagonist and [3H]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 [3H]ligand binding to A_1 recep-
tors 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 recep-
tors[12]. At physiological expression levels agonists
discriminate between these two states, whereas antago-
nists recognize both states with equal affinity; the situ-
ation may be different when receptors are over-ex-
pressed[28]. The reduction in [3H]DPCPX binding pro-
duced by MgCl_2 in these studies using native tissue is
therefore complex, as the K_i of [3H]DPCPX would re-
quire to increase 2-fold to account for the 40% reduc-
tion 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 de-
crease 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 adenos-
ine 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 modu-
lar 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, con-
sistent with a recent study[12]. Under control conditions,
the proportion of these states and their 30-fold differ-
ence in affinity is similar in studies across species, mem-
branes and cloned receptors[12,21].

K_0 and B_max values for [3H]CCPA binding to corti-
cal membranes agree well with previous data[25]. The
K_0 of [3H]CCPA and the K_i value for R-PIA, were simi-
lar to their high affinity values (K_0) for inhibiting
[3H]DPCPX binding. Moreover, the [3H]CCPA B_max is
65% of the [3H]DPCPX B_max, identical to the propor-
tion of [3H]DPCPX binding sites in the high affinity state,
indicating that [3H]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 [3H]DPCPX binding,
this is consistent with the calculation that at the con-
centration of [3H]CCPA (0.2 nmol/L) used in this study,
the high affinity state of A_1 receptors was 98% of its bind-
ing would be to the high affinity site. This is supported by the inability to resolve
[3H]CCPA (+MgCl_2) using a two-site model.

MgCl_2 increased [3H]CCPA binding, consistent with data for other A_1 [3H]agonists and GPCRs[13,7]. The
increased [3H]CCPA binding by MgCl_2 was due to an increase in B_max (consistent with the propor-
tional increase in high affinity state labelling seen for
[3H]DPCPX), and also to a small but genuine (near 2-
fold) increase in ligand affinity. Antagonist affinities
were similar to those for [3H]DPCPX, and were unaf-
ected by MgCl_2. However, agonist affinities were 5-
17-fold higher and Hill slopes were near unity, consist-
tent with [3H]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
[3H]CCPA itself. The reason why this increase in affin-
ity of agonists for the high affinity site was not de-
tected for CCPA and R-PIA using [3H]DPCPX, is pre-
sumably 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 [3H]ligand binding to A_1 recep-
tors 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\textsuperscript{[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\textsuperscript{[20,30]}. However the magnitude of increase varies, reflecting species differences and assay conditions\textsuperscript{[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\textsuperscript{[32]} or a preference for the uncoupled form of the receptor\textsuperscript{[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\textsuperscript{[22]}.

The 5-fold decrease in agonist affinity for \(^{3}H\)DPCPX binding by Gpp(NH)p is characteristic of adenosine and other GPCRs\textsuperscript{[13,31]}, 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\textsuperscript{[27]}, data that have been confirmed recently using human brain autoradiography\textsuperscript{[209]}. Although again, as in many previous studies\textsuperscript{[12,15,23]}, interpretation of the effects of GTP on \(^{3}H\)DPCPX binding to human A\textsubscript{1} receptors in this recent study\textsuperscript{[20]}, is complicated by the presence of 1 mmol/L MgCl\textsubscript{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\textsubscript{2}. This was consistent with its reduction in the \(B_{\text{max}}\) 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\textsubscript{1} receptor and G-protein\textsuperscript{[28,29]}, contrasting with other GPCRs\textsuperscript{[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\textsubscript{1} receptors by MgCl\textsubscript{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\textsuperscript{[12,20,27,34]}. In this study we systematically and separately examined the effects of MgCl\textsubscript{2} and Gpp(NH)p on the binding of an antagonist and agonist ligand selective for A\textsubscript{1} receptors, using identical experimental conditions. The effects of MgCl\textsubscript{2} and Gpp(NH)p on agonist affinities were similar for both the antagonist and agonist radioligand. In addition, MgCl\textsubscript{2} and Gpp(NH)p had little effect on antagonist affinities in either assay, despite some modulation of \(^{3}H\)DPCPX binding. In conclusion, the effects observed with MgCl\textsubscript{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 \(^{3}H\)DPCPX binding by magnesium and Gpp(NH)p still appear complicated.

**REFERENCES**

7. Goodman RR, Cooper MJ, Gavish M, Snyder SH. Guanine nucleotide and cation regulation of the binding of \(^{3}H\)cyclohexyladenosine and \(^{3}H\)diethylphosphorylthioadenosine to adenosine A\textsubscript{1} receptors in brain membranes. Mol Pharmacol 1982; 21: 329-35.
8. Olah ME, Stiles GL. Agonists and antagonists recognise different but overlapping populations of A\textsubscript{1} adenosine receptors: modulation of receptor number by MgCl\textsubscript{2}, solubilisation and guanine nucleotides. J Neurochem 1990; 55: 1432-38.
9. Mazzone MR, Martini C, Lucacchini A. Regulation of agonist binding to A\textsubscript{2a} adenosine receptors: effects of guanine nucleotides (GDP[S] and GTP[S]) and Mg\textsuperscript{2+} ion. Biochim Biophys Acta 1993; 1220: 76-84.
10. Johansson B, Parkinson FE, Fredholm BB. Effects of monovalent and divalent ions on the binding of the adenosine analogue.


