Biochem. J. (1991) 275, 651–656 (Printed in Great Britain)

Interactions of purified bovine brain A_1-adenosine receptors with G-proteins

Reciprocal modulation of agonist and antagonist binding

Michael FREISSMUTH,* Edgar SELZER and Wolfgang SCHÜTZ
Institute of Pharmacology, University of Vienna, Waehringer Strasse 13a, A-1090 Vienna, Austria

The bovine brain A_1-adenosine receptor was purified 8000-fold by affinity chromatography on xanthine-amine-congener (XAC)–Sepharose. Addition of a 120-fold molar excess of a purified bovine brain G-protein preparation ($G_{o,i}$ a mixture of G_o and G_i, containing predominantly G_o) decreases the $B_{max.}$ of the binding of the antagonist radioligand [^{3}H]XAC to the receptor. This decease is observed not only after insertion into phospholipid vesicles but also in detergent solution, and is reversed by GTP analogues. In the presence of $G_{o,i}$, about 20 and 40 % of the receptors display guanine-nucleotide-sensitive high-affinity binding of the agonist radioligand (−)-N^6-3-([^{125}I]iodo-4-hydroxyphenylisopropyl)adenosine after reconstitution into lipid vesicles and in detergent solution, respectively. The ability of $G_{o,i}$ to enhance agonist binding and decrease antagonist binding is concentration-dependent, with a half-maximal effect occurring at ~ 10-fold molar excess of G-proteins over A_1-adenosine receptors. In the presence of the receptor, the rate of guanosine 5′-[γ-[^{35}S]thio]triphosphate (GTP[^{35}S]) binding to $G_{o,i}$ is accelerated. This rate is further enhanced if the receptor is activated by the agonist (−)(*R*)-N^6-phenylisopropyladenosine, whereas the antagonist XAC decreases the association rate of GTP[^{35}S] to levels observed in the absence of receptor. These results show (1) that detergent removal is not a prerequisite for the observation of coupling between the A_1-adenosine receptor and $G_{o,i}$, and (2) that the regulatory effect of G-proteins on antagonist binding to the A_1-adenosine receptor can be reconstituted by using purified components.

INTRODUCTION

Adenosine receptors belong to the family of G-protein-coupled receptors and are divided into two major classes: A_2-adenosine receptors mediate stimulation of adenylate cyclase, presumably via activation of G_s, whereas A_1-adenosine receptors are linked to transmembrane signalling pathways controlled by the G_i/G_o group, such as inhibition of adenylate cyclase and regulation of ion-channel activity. The coupling between the A_1-adenosine receptor and the yet-unidentified G-protein appears to be very tight: in membranes, high concentrations of guanine nucleotides are not able to induce a complete conversion of the agonist-specific high-affinity state of the receptor (reflecting the ternary complex between agonist, receptor and G-protein) into the low-affinity state [1–3], which is generally believed to represent the receptor dissociated from the G-protein [4]. In addition, upon solubilization by various detergents the A_1-adenosine receptor remains associated with the G-protein [2,3,5,6]. These properties appear unique when the A_1-adenosine receptor is compared with other G-protein-coupled receptors [2].

The A_1-adenosine receptor has recently been purified from rat brain and testis by using an affinity matrix based on the antagonist XAC [7,8]. Whereas it seems clear from these studies that the purified A_1-adenosine receptor displays low-affinity agonist and high-affinity antagonist binding, no information is available on the interaction of the purified receptor with G-proteins after reconstitution. Hence, using the recently described A_1-adenosine-receptor purification procedure and a purified bovine brain G-protein preparation, we investigated the direct coupling between receptor and G-proteins. The fact that G-protein-dependent regulation of A_1-adenosine-receptor binding is not lost upon solubilization prompted us to attempt a reconstitution of both entities without removal of detergent.

MATERIALS AND METHODS

Materials

Carrier-free Na^{125}I, GTP[^{35}S] and [^{3}H]XAC (170 Ci/mmol) were purchased from NEN (Boston, MA, U.S.A.). CHAPS, cholic acid and digitonin were obtained from Fluka (Buchs, Switzerland). Guanine nucleotides, (−)*R*-PIA and (+)*S*-PIA were from Boehringer (Mannheim, Germany). NECA and DPCPX were generously given by Byk Gulden Lomberg (Konstanz, Germany) and Gödecke A.G. (Berlin, Germany), respectively. XAC was obtained from RBI (Natick, MA, U.S.A.). Phosphatidylcholine (type XVE) was purchased from Sigma (St. Louis, MO, U.S.A.). Activated CH-Sepharose 4B, Sephadex S-200 and G-50 were from Pharmacia (Uppsala, Sweden), and Extracti-Gel was from Pierce (Rockford, IL, U.S.A.). ^{125}I-labelled and unlabelled IHPIA were prepared as described in ref. [9]. XAC was coupled to activated CH–Sepharose 4B at pH 9 as recommended by the manufacturer. The incorporation was determined with trace amounts of [^{3}H]XAC and amounted to about 3 μmol/ml of Sepharose; this affinity matrix is subsequently referred to as XAC–Sepharose.

Protein purification

Bovine brain membranes were prepared as described in [10] in the presence of a mixture of protease inhibitors (0.2 mM-phenylmethanesulphonyl fluoride, 0.02 mM-aprotinin, 0.1 mM-

Abbreviations used: G-proteins, guanine-nucleotide-binding regulatory proteins; G_s and G_i, originally defined as the G-proteins mediating respectively stimulation and inhibition of adenylate cyclase; G_o, a G-protein found in high concentrations in brain; PIA, N^6-(phenylisopropyl)-adenosine; IHPIA, (−)-N^6-3-(iodo-4-hyroxyphenylisopropyl)adenosine; NECA, 5′-(*N*-ethylcarboxamido)adenosine; 2-Cl-ADO, 2-chloroadenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; XAC, xanthine amine congener; p[NH]ppG, guanosine 5′-[βγ-imido]triphosphate; GTP[S], guanosine 5′-[γ-thio]triphosphate.

* To whom reprint requests should be addressed.

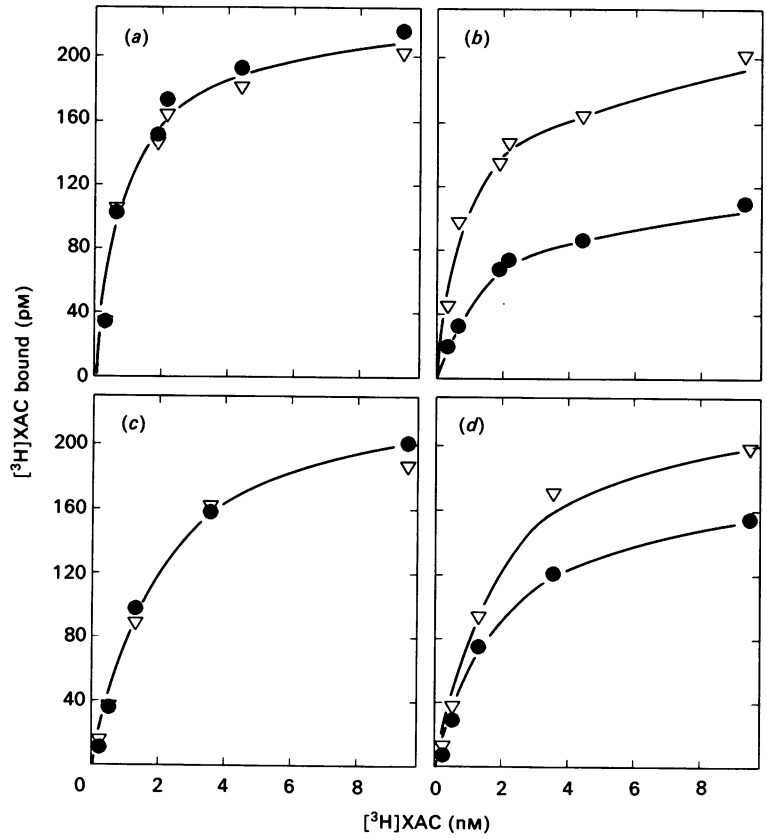

Fig. 1. Saturation isotherms for [^{3}H]XAC binding to the purified A_1-adenosine receptor preparation

(*a*), (*b*), The purified receptor preparation was incubated in the absence (*a*) or presence of 1.2 pmol of bovine brain $G_{o,i}$ (*b*) in 15 μl and held on ice for 15 min. (*c*), (*d*), The purified receptor preparation (1 pmol) was subjected to chromatography over an Extracti-Gel column in the absence (*c*) and presence (*d*) of 100 pmol of bovine brain $G_{o,i}$ as described in the Materials and methods section; 5 μl of the eluate (containing 0.56 pmol of $G_{o,i}$) was used per assay. Binding was initiated by the addition of 15 μl containing the appropriate amount of radioligand in the absence (●) and presence (▽) of p[NH]ppG (0.1 mM final concn.). Data points represent specific binding; non-specific binding amounted to 20% of total binding at the highest concentration of radioligand used. Specific binding of [^{3}H]XAC to the G-protein preparation in the absence of receptor remained undetectable up to the highest concentration of radioligand. The curves were obtained by fitting the data to a rectangular hyperbola. Data represent the means from duplicate determinations in a single experiment; replicates varied by less than 10%.

tosylphenylalanylchloromethane and 0.2 mM-tosyl-lysylchloromethane). Bovine brain G-proteins were purified as in [10]; the fractions containing pertussis-toxin substrates were pooled, no attempt being made to resolve G_i from G_o. Upon SDS/PAGE about 80% of the α-subunits in the final pool migrated with a molecular mass of 39 kDa, corresponding to $G_{o\alpha}$. This preparation is subsequently referred to as $G_{o,i}$.

The A_1-adenosine receptor was purified from bovine brain membranes as described by Nakata [7,8], with the following modifications: digitonin was replaced by CHAPS (25 mM) during membrane solubilization and by the combination of CHAPS (12.5 mM) and phosphatidylcholine (0.05%) in the subsequent chromatographic steps; glycerol (20%) was present in all buffers; the A_1-adenosine receptor bound to the affinity support was eluted in the presence of 20 mM-adenosine. After the first and second affinity chromatography, adenosine was removed by gel filtration over Sephadex S-200. Analysis of the purified preparation by SDS/PAGE revealed a predominant band at about 34 kDa (results not shown), which corresponds to the molecular mass of the cerebral A_1-adenosine receptor estimated by others [7,11]; minor contaminants are present at 32 and 57 kDa. A specific activity of 10.4 and 12.5 nmol of antagonist-binding sites/mg of protein was determined from saturation experiments using [^{3}H]XAC in two different preparations (theoretical value 29.4 nmol/mg); hence, assuming one binding site per molecule, the receptor is about 40% pure and a roughly 8000-fold purification was achieved in the modified chromatographic procedure.

Coupling of A_1-adenosine receptors and G-proteins in detergent solution

The concentrated receptor preparations were diluted appropriately (10–200-fold) in buffer A (50 mM-Tris/HCl, pH 8, 1 mM-EDTA, 5 mM-$MgCl_2$, 20% glycerol, 12.5 mM-CHAPS, 0.05% phosphatidylcholine); the purified bovine brain G-protein fraction was desalted over Sephadex G-50 equilibrated in buffer B (buffer A without phosphatidylcholine). A 5 μl portion of an appropriate dilution or buffer B was added to 10 μl of diluted receptor, and the mixture was kept on ice for 15 min. This preincubation period was judged to be sufficient to promote interaction between receptor and G-proteins, since preincubations for up to 1 h did not result in further increases in agonist binding.

Reconstitution of A_1-adenosine receptors and G-proteins into phospholipid vesicles

Total lipids were prepared from grey matter as described in [12]. A 1.6 mg portion of lipid was dried under argon and suspended by sonication in 0.2 ml of buffer C (20 mM-Tris/HCl, pH 8, 1 mM-EDTA, 2 mM-$MgCl_2$, 150 mM-NaCl) containing

3.3 mM-CHAPS. The purified A_1-adenosine receptor preparation was diluted in buffer A. A 40 μl portion of this dilution (about 1 pmol of receptor) was mixed with either 10 μl of buffer B or 10 μl of buffer B containing 3–100 pmol of $G_{o,i}$; 40 μl of lipid suspension was added and the volume was adjusted to 200 μl with buffer A. The mixture was kept on ice for 1 h and passed over an Extracti-Gel column (0.5 ml bed volume), which had been pretreated with BSA. The column was eluted with 0.5 ml of buffer containing 20 mM-Tris/HCl (pH 8), 1 mM-EDTA, 2 mM-$MgCl_2$ and 40 mM-NaCl. The recovery of receptor and G-proteins varied between 40 and 60% in individual experiments. If the A_1-adenosine receptor preparation was passed over the Extracti-Gel column in the absence of the lipid suspension, no binding activity was recovered in the eluate.

Radioligand binding assays

For binding experiments in detergent solution, the binding reaction was started by the addition of 15 μl of buffer D (50 mM-Tris/HCl, pH 8, 1 mM-EDTA, 5 mM-$MgCl_2$) containing either [^{3}H]XAC or [^{125}I]HPIA, 0.06 units of adenosine deaminase and, where applicable, competing ligands; the final concentrations of the radioligands are indicated in the legends to Fig. 1–3 and Table 1. Competition experiments with adenosine were carried out in the absence of adenosine deaminase, which did not affect the binding of both [^{125}I]HPIA and [^{3}H]XAC. After 1 h ([^{3}H]XAC binding) or 90 min ([^{125}I]HPIA binding) at 20 °C, 10 μl of a solution containing 30% poly(ethylene glycol) and 0.1 mg of BSA/ml was added and the incubation was terminated by vacuum filtration over polyethylenimine-treated glass-fibre filters. The filters were washed with 10 ml of ice-cold buffer containing 20 mM-Tris/HCl (pH 8), 5 mM-$MgCl_2$ and 100 mM-NaCl. Non-specific binding was defined in the presence of 0.5 mM-theophylline and was comparable with the filter blank. Preliminary experiment verified that equilibrium conditions were met.

Binding experiments with the eluate from the Extracti-Gel column were carried out under similar conditions, except that poly(ethylene glycol) and BSA were not added to the samples and pretreatment of the glass-fibre filters with polyethyleneimine was omitted.

For kinetic experiments, binding of GTP[^{35}S] to $G_{o,i}$ was carried out in the incubation medium described above in the absence and presence of the purified A_1-adenosine receptor preparation. At the time points indicated in Fig. 4, the reaction was quenched by the addition of 2 ml of ice-cold buffer containing 20 mM-Tris/HCl (pH 8), 5 mM-$MgCl_2$, 100 mM-NaCl and 0.1 mM-GTP. Bound and free nucleotide were separated by filtration over BA85 nitrocellulose filters.

In order to determine the molar amount of G-proteins used in both A_1-adenosine-receptor binding experiments and in kinetic experiments, appropriate dilutions were added to a final volume of 50 μl containing 50 mM-NaHepes (pH 7.6), 1 mM-EDTA, 10 mM-$MgSO_4$, 0.1% Lubrol PX and 1 μM-GTP[^{35}S] (sp. radioactivity 10000 c.p.m./pmol). Incubation was for 2 h at 20 °C.

Protein was determined by a modified Amido Black method [13]. Experiments were performed in duplicate. Each experiment was repeated at least twice.

RESULTS

Binding characteristics of the purified receptor

Saturation binding isotherms of [^{3}H]XAC to purified preparations were monophasic, the observed K_D (1.3±0.4 nM; $n = 4$) being slightly higher than in membranes (0.5±0.2 nM; $n = 3$); whereas GTP and its hydrolysis-resistant analogues increase the maximum number of binding sites ($B_{max.}$) in membranes and solubilized preparations (see [3,8,14]), no guanine-nucleotide-dependent effect was observed with the purified receptor (Fig. 1*a*). Upon addition of a 120-fold molar excess of purified bovine brain $G_{o,i}$, the $B_{max.}$ of [^{3}H]XAC binding was decreased by about 40%, with no measurable change in affinity (K_D = 1.5±0.3 and 1.4±0.2 nM respectively); $n = 3$; the apparent decrease in detectable receptors was reversed in the presence of p[NH]ppG (Fig. 1*b*) and GTP[S] (results not shown). If the A_1-adenosine receptor is inserted into phospholipid vesicles by using an Extracti-Gel column, the binding characteristics of [^{3}H]XAC remain essentially unchanged (K_D = 2.0±0.1 nM; $n = 3$) and insensitive to regulation by

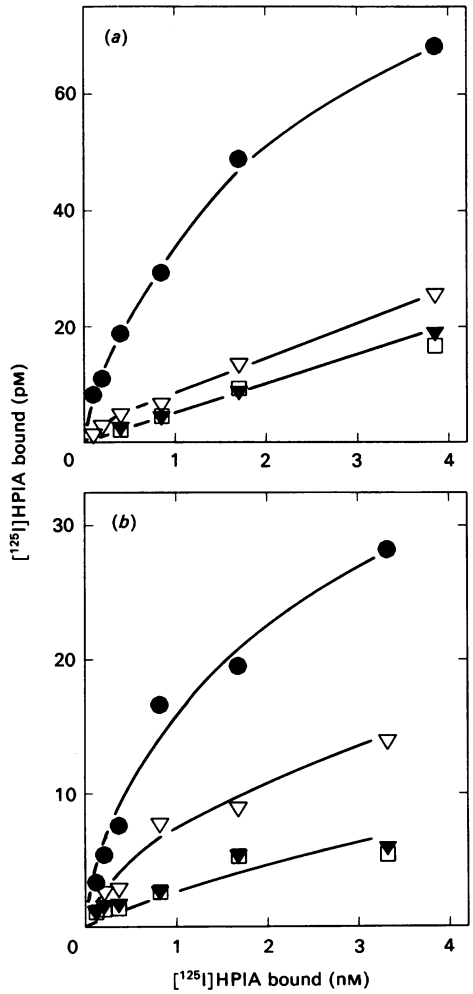

Fig. 2. Saturation isotherms for [^{125}I]HPIA binding to the purified A_1-adenosine receptor preparation

(*a*) A_1-adenosine receptors (5.3 fmol) were incubated with (●, ▽) or without (▼, □) 0.76 pmol of bovine brain $G_{o,i}$ as described in the legend to Fig. 1. (*b*) A 100 μl portion of Extracti-Gel eluate from Fig. 1, containing (●, ▽) or lacking (▼, □) $G_{o,i}$, was diluted with 75 μl of elution buffer; 5 μl of this dilution was used per assay. The binding reaction was carried out in the absence (●, □) and presence (▽, ▼) of 0.1 mM-GTP[S] as described in the Materials and methods section. Data points represent specific binding; non-specific binding at the highest radioligand concentration was about 10% of total binding. No specific binding was detected in $G_{o,i}$ in the absence of receptor. The continuous curves in the presence of $G_{o,i}$ were drawn by using the parameters derived from a non-linear least-squares fit to the equation $y = M[x/(x+k)]+px$. Shown are the means of duplicate determinations in a single experiment; replicates varied by less than 10%.

Table 1. Affinities of adenosine-receptor ligands for competing with [^{3}H]XAC and [^{125}I]HPIA binding to purified A_1-adenosine receptors

A_1-adenosine receptors (10 fmol) were incubated in the presence of 2 nM-[^{3}H]XAC or 0.4 nM-[^{125}I]HPIA and 7–8 logarithmically spaced concentrations of competitor. For experiments with [^{125}I]HPIA, the receptors were preincubated with 1 pmol of $G_{o,i}$ as described in the Materials and methods section. IC_{50} values (concn. giving 50% inhibition) were calculated from a non-linear least-squares fit to a monophasic inhibition curve and converted into K_i values by using the Cheng–Prusoff approximation. Shown are means ± S.E.M. from three determinations (XAC competition) or mean and range from two experiments (IHPIA competition) performed on different preparations.

	K_i (nM)	
	[^{3}H]XAC binding	[^{125}I]HPIA binding
Agonist		
(*R*)-PIA	35 ± 5	0.8 (0.6–1)
(*S*)-PIA	1082 ± 88	18 (15–21)
NECA	3530 ± 1250	26 (22–30)
2-Cl-ADO	1557 ± 428	10 (7–13)
Adenosine	636 ± 56	2 (1.7–2.3)
Antagonist		
DPCPX	1 ± 0.2	2 (1.7–2.3)
XAC	1 ± 0.5	2 (1.5–2.5)
Theophylline	3930 ± 440	7700 (5700–9700)

p[NH]ppG (Fig. 1*c*). Co-reconstitution of the receptor with a 110-fold molar excess of $G_{o,i}$ results in a 20% decrease in $B_{max.}$. This effect is again abolished by p[NH]ppG (Fig. 1*d*).

Conversely, in the absence of added G-proteins, binding of the agonist radioligand [^{125}I]HPIA to the purified preparation was of apparently low affinity and insensitive to guanine nucleotides; addition of $G_{o,i}$ promoted high-affinity [^{125}I]IHPIA binding, and GTP[S] decreased agonist binding almost completely to that observed in the absence of G-proteins (Fig. 2*a*). Binding of [^{125}I]HPIA was clearly not saturable over the concentration range used in the experiment. However, the difference in binding observed in the presence and absence of G-proteins yields a rectangular hyperbola with a K_D of 1.0 ± 0.3 nM ($n = 3$), and a $B_{max.}$-value corresponding to about 40% of the total receptor population determined in parallel experiments using [^{3}H]XAC. This range varied between 25 and 50% in several experiments with different preparations, and this ratio was not increased by adding higher amounts of G-proteins (see below).

In order to evaluate the component of the [^{125}I]HPIA binding curve which was not saturable over the useful concentration range, the specific radioactivity of the radioligand was progressively diluted with unlabelled IHPIA to cover a concentration range of 0.2–300 nM: transformation of the data yields a curved Scatchard plot with a 7:13 ratio of high- and low-affinity sites (K_D = 1.2 ± 0.4 nM and 72 ± 15 nM respectively; $n = 3$).

Analogous results were obtained in control experiments where detergent removal was achieved by the Extracti-Gel procedure. After co-insertion of the A_1-adenosine receptor and of a 110-fold molar excess of $G_{o,i}$ into lipid vesicles, the binding of [^{125}I]HPIA clearly displays a component of high affinity (K_D = 1.2 ± 0.2 nM; $n = 3$), which is sensitive to modulation by guanine nucleotides (Fig. 2*b*). However, the $B_{max.}$ value of this high-affinity component accounts for only about 20% of the receptors labelled by [^{3}H]XAC in the presence of 0.1 mM-p[NH]ppG.

In competition experiments, agonists displace [^{3}H]XAC binding to the receptor in the absence of $G_{o,i}$ with low affinity; if the receptor is labelled with [^{125}I]HPIA in the presence of $G_{o,i}$, the affinity of agonists is increased 40–200-fold. No affinity shift is observed for the antagonists (Table 1). The pharmacological profile is typical for A_1-adenosine-receptor binding.

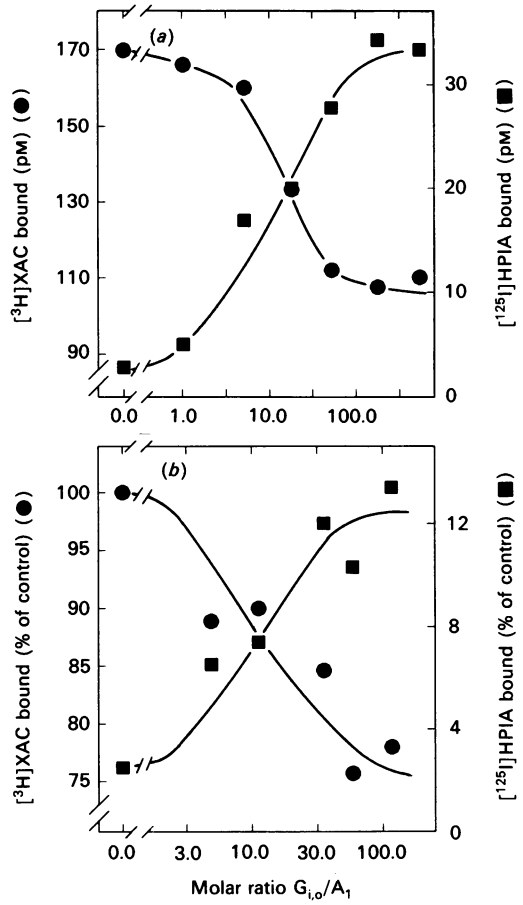

Fig. 3. Modulation of [^{3}H]XAC and [^{125}I]HPIA binding by bovine brain $G_{o,i}$

(*a*) A_1-adenosine receptors (7 fmol) were incubated with the indicated amounts of bovine brain $G_{o,i}$ as described in the legend to Fig. 1. (*b*) A_1-adenosine receptors (1 pmol) were mixed with 3–100 pmol of $G_{o,i}$ and passed over Extracti-Gel columns as described in the Materials and methods section; 5 μl of the eluate was used per assay. In order to account for varying recoveries, the binding at each point is expressed as a percentage of control, which is the binding of [^{3}H]XAC in the presence of 0.1 mM-p[NH]ppG. The binding reaction was carried out in the presence of 1 nM-[^{125}I]HPIA (■) or 6 nM-[^{3}H]XAC (●). Specific binding of either radioligand to $G_{o,i}$ was undetectable at the highest concentration of G-protein used. Data are means from duplicate determinations in a single experiment; replicate variation was less than 10%.

G-protein-dependence of agonist and antagonist binding

When the purified A_1-adenosine receptor preparation is incubated with increasing concentrations of $G_{o,i}$, binding of [^{125}I]HPIA is enhanced, whereas that of [^{3}H]XAC is decreased in a concentration-dependent manner; as shown in Fig. 3, both effects are seen over a similar concentration range of $G_{o,i}$. The half-maximal effect occurs at a roughly 10-fold molar excess of $G_{o,i}$, irrespective of whether the experiment is carried out in detergent solution (Fig. 3*a*) or following co-reconstitution of receptor with increasing amounts of G-protein into lipid vesicles (Fig. 3*b*). A maximum enhancement of [^{125}I]HPIA binding is observed at a 100–120-fold molar excess of G-protein; as

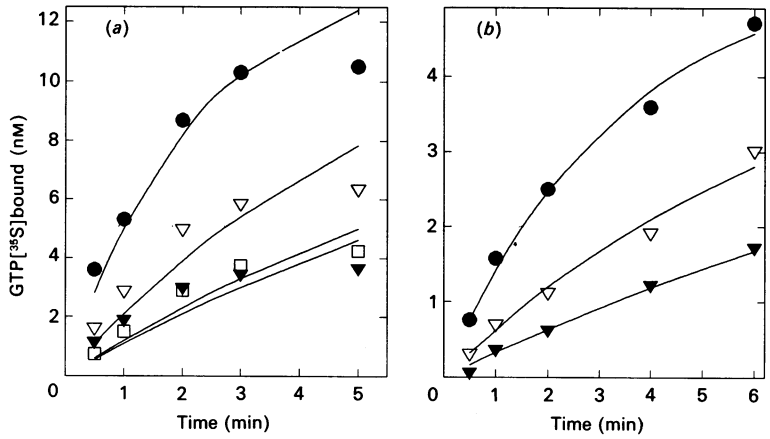

Fig. 4. Time course of GTP[^{35}S] binding to bovine brain $G_{o,i}$

(*a*) Bovine brain $G_{o,i}$ (8 pmol) was incubated with 0.6 pmol of A_1-adenosine receptor (●, ▼, ▽) or appropriate buffer (□) in the presence of 0.7 unit of adenosine deaminase in a final volume of 120 μl on ice for 15 min. Binding was initiated by the addition of 240 μl of a prewarmed solution (20 °C) containing buffer C/buffer A (3:1, v/v), 0.72 unit of adenosine deaminase, 7.5 μM-*R*-PIA (●) or 7.5 μM-XAC (▼), and 0.3 μM-GTP[^{35}S] (sp. radioactivity 100 c.p.m./fmol); at the time points indicated, 30 μl samples were withdrawn and processed as described in the Materials and methods section. Binding of GTP[^{35}S] to the purified A_1-adenosine receptor preparation was undetectable in the absence of added G-proteins. (*b*) A 120 μl portion of Extracti-Gel eluate (containing 0.12 pmol of A_1-adenosine receptor and 3.53 pmol of $G_{o,i}$) was mixed with an equal volume of prewarmed solution (20 °C) containing buffer C, 0.72 unit of adenosine deaminase, 0.2 μM-GTP[^{35}S] (sp. radioactivity 190 c.p.m./fmol), 10 μM-*R*-PIA (●), 10 μM-XAC (▼), or no adenosine-receptor ligand (▽). At the time points indicated, 20 μl portions were withdrawn and processed as described in the Materials and methods section. Shown are the means of duplicate determinations in a single experiment with a replicate variation of less than 10%.

mentioned above, only a fraction of the total receptor population displayed high-affinity agonist binding. When the experiments were performed in detergent solution, this fraction amounted to $39 \pm 5\%$ ($n = 5$). Following detergent removal and insertion into phospholipid vesicles, $20 \pm 3\%$ ($n = 3$) of the total receptors (determined by [^{3}H]XAC binding in the presence of p[NH]ppG) were capable of binding [^{125}I]HPIA with high affinity.

Kinetics of GTP[S] binding

In G-protein-controlled pathways, dissociation of pre-bound GDP from the G-protein oligomer represents the rate-limiting step in the activation of the transmembrane signalling cascade. The agonist-bound receptor catalyses guanine-nucleotide exchange by dramatically increasing the GDP dissociation rate [15,16]. Hence the interaction between receptor and G-protein can also be demonstrated by an alteration in the rate of GTP[^{35}S] binding to $G_{o,i}$ following the addition of the purified receptor (Fig. 4). In detergent solution, the addition of the A_1-adenosine receptor in the absence of any agonist results in an enhancement of the overall rate for GTP[S] binding from 0.08 ± 0.01 min^{-1} to 0.15 ± 0.03 min^{-1} (see ▽ in Fig. 4*a*); activation of the receptor by the agonist *R*-PIA produces an additional stimulation of the GTP[S] binding rate ($k = 0.44 \pm 0.09$ min^{-1}). Conversely, upon addition of the antagonist XAC, the rate of guanine nucleotide binding to $G_{o,i}$ in the presence of receptor (▼ in Fig. 4*a*) is decreased to the rate observed in the absence of A_1-adenosine receptors ($k = 0.07 \pm 0.01$ min^{-1}). Analogous findings were obtained after co-insertion of receptor and $G_{o,i}$ into phospholipid vesicles, namely an antagonist-induced decrease in and an agonist-mediated stimulation of the GTP[S] binding rate when compared with the effect of the unliganded receptor (Fig. 4*b*). The association rate in the absence of any receptor ligand was 0.12 ± 0.01 min^{-1}, and was 0.06 ± 0.01 min^{-1} and 0.3 ± 0.05 min^{-1} in the presence of XAC and *R*-PIA respectively. The modest difference between the rates observed in detergent solution and following co-insertion into phospholipid vesicles is most likely attributable to the different molar ratios of receptor and G-protein.

DISCUSSION

The present study demonstrates the reconstitution of A_1-adenosine receptors and G-proteins without removal of detergent. This approach was successful presumably due to the tight coupling between A_1-adenosine receptor and G-protein, and hence it was not mandatory to employ the standard procedure for co-insertion of receptor and G-protein oligomer into phospholipid vesicles, first worked out for the β-adrenergic receptor [17]. Comparison of the interaction between A_1-adenosine receptor and $G_{o,i}$ in detergent solution and after insertion into phospholipid vesicles indicates that the essential characteristics of the coupling process can be observed in the presence of detergent, namely the G-protein-promoted high-affinity binding of agonists and the receptor-mediated stimulation of guanine-nucleotide exchange. It is unlikely that the residual contaminants, which were present in the receptor preparations, influence the interaction between receptor and G-protein, since their relative proportion varied in individual preparations without affecting the observations. In addition, GTP[S] binding to the receptor preparation alone was undetectable, and high-affinity agonist binding was strictly dependent on the addition of G-protein.

One of the tenets generally accepted for the model of signal transduction by G-protein-coupled receptors states that only agonists but not antagonists can discriminate between two affinity states of the receptor. However, several reports indicate that under appropriate assay conditions the binding of antagonists to the β-adrenergic receptor [18,19] and to receptors which are known to be coupled to the G_o/G_i group, such as the D_2-dopamine [20], muscarinic [21] and A_1-adenosine [3,14] receptors, is modulated by hydrolysis-resistant GTP analogues. While this work was in progress, Nakata [8] reported that the guanine-nucleotide-dependent increase in antagonist binding was lost upon purification of the testicular A_1-adenosine receptor. We show here that this regulatory phenomenon can be reconstituted in a concentration-dependent manner by the addition of G-proteins to the purified A_1-adenosine receptor. Over a similar

concentration range of $G_{o,i}$, a reciprocal effect is observed for agonists, namely a G-protein-dependent appearance of high-affinity binding. However, the relation between these two findings appears to be complex. There is no affinity shift for antagonists either after addition of G-proteins to the purified receptor or in membranes after addition of a guanine nucleotide, which produces a roughly 30% increase in the $B_{max.}$ for [^{3}H]XAC binding. This seems to conflict with the ability of $G_{o,i}$ to decrease antagonist binding to the purified receptor. Based on a detailed kinetic analysis, Leung *et al.* [22] have recently demonstrated that a simple two-affinity-state model for the membrane-bound A_1-adenosine receptor was not compatible with the experimental data. The alternative models proposed assume at least three different affinity states of the receptor for agonist and antagonist binding, and are not symmetrical with respect to competition experiments using agonist or antagonist radioligands. According to these models, the antagonists induce a destabilization of the receptor–G-protein complex. Hence, in the competition of an antagonist for labelled agonist, the K_i observed under equilibrium conditions will approach the affinity of the antagonist for the free receptor.

If the β-adrenergic receptor and G_s are co-reconstituted into phospholipid vesicles, the basal rate of association of GTP[S] is decreased by the β-adrenergic antagonist propranolol, suggesting that in the absence of any activation by agonist the receptor is still capable of fruitful interaction with G_s [23–25]. In the present study, we obtained an analogous finding with the A_1-adenosine receptor, where a significant enhancement of the basal rate of guanine nucleotide exchange by the unliganded receptor can directly be demonstrated. This stimulation of the rate of GTP[S] binding is blocked by an antagonist. Taken together, both the antagonist-mediated decrease in the association rate of GTP[S] and the G-protein-dependent decrease in antagonist binding suggest that antagonists not only bind to the receptor but also induce a conformational change which favours uncoupling of the receptor from the G-protein. Hence, antagonists apparently possess a reverse intrinsic activity.

Although in membranes derived from various tissues about 80% of the total A_1-adenosine receptor population binds agonists with high affinity [1,3,14], only about 40% of the purified receptors were capable of binding [^{125}I]HPIA with high affinity when assayed in detergent solution. It is not clear whether this incomplete transition is attributable to alterations of the receptor protein during the purification or to the lack of as-yet unidentified component(s). On the other hand, the proportion of receptors that bind [^{125}I]HPIA with high affinity (20%) was lower after removal of detergent. This may be attributable to limitations imposed by the orientation of receptor and G-proteins after insertion into the phospholipid vesicles.

An apparently large molar excess of G-protein is needed to observe the appearance of high-affinity agonist binding. A molar excess of comparable magnitude was found to be necessary to reconstitute high-affinity guanine-nucleotide-sensitive binding of agonists to the bovine brain muscarinic receptor [26]. However, the ratios required are not unreasonably high, since bovine brain membranes contain a high concentration of G-proteins of the G_o/G_i group [10], which translates into a more than 300-fold molar excess over A_1-adenosine or muscarinic receptors. In addition, G-protein preparations purified from mammalian sources represent a mixture of several individual molecular species [15,16]. Hence the apparent affinity of the receptor for G-proteins may have been underestimated, if A_1-adenosine receptors selectively couple only to certain G-protein oligomers. Since individual G-protein α-subunits have been expressed in *Escherichia coli* and purified to homogeneity [27–29], it will be interesting to determine to what extent the A_1-adenosine receptor preferentially interacts with one of the known subtypes of α_o or α_i subunits.

We thank Ms. E. Tuisl for expert graphical work. This work was supported by grants from the 'Fonds zur Förderung der wissenschaftlichen Forschung' (P 6825 and 7622) and a grant from the 'Medizinisch-wissenschaftlicher Fonds des Bürgermeisters der Bundeshauptstadt Wien'.

REFERENCES

1. Lohse, M. J., Lenschow, V. & Schwabe, U. (1984) Mol. Pharmacol. **26**, 1–9
2. Stiles, G. L. (1985) J. Biol. Chem. **260**, 6728–6732
3. Ströher, M., Nanoff, C. & Schütz, W. (1989) Naunyn-Schmiedeberg's Arch. Pharmcol. **340**, 87–92
4. Lefkowitz, R. J., Stadel, J. M. & Caron, M. (1983) Annu. Rev. Biochem. **52**, 159–186
5. Klotz, K. N., Lohse, M. J. & Schwabe, U. (1986) J. Neurochem. **46**, 1528–1534
6. Yeung, S.-M. H., Perez-Reyes, E. & Cooper, D. M. F. (1987) Biochem. J. **248**, 635–642
7. Nakata, H. (1989) J. Biol. Chem. **264**, 16545–16551
8. Nakata, H. (1990) J. Biol. Chem. **265**, 671–677
9. Linden, J. (1984) Mol. Pharmacol. **26**, 414–423
10. Sternweis, P. C. & Robishaw, J. D. (1984) J. Biol. Chem. **259**, 13806–13813
11. Munshi, R. & Linden, J. (1989) J. Biol. Chem. **264**, 14853–14859
12. Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) J. Biol. Chem. **226**, 497–509
13. Kaplan, R. S. & Pedersen, P. L. (1985) Anal. Biochem. **150**, 97–104
14. Ramkumar, V. & Stiles, G. L. (1988) J. Pharmacol. Exp. Ther. **246**, 1194–1200
15. Gilman, A. G. (1987) Annu. Rev. Biochem. **56**, 615–649
16. Freissmuth, M., Casey, P. J. & Gilman, A. G. (1989) FASEB J. **3**, 2125–2131
17. Pedersen, S. E. & Ross, E. M. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 7228–7232
18. Wolfe, B. B. & Harden, T. K. (1981) J. Cyclic Nucleotide Res. **7**, 303–312
19. Lang, P. H. & Lemmer, B. (1985) J. Cyclic Nucleotide Res. **10**, 341–360
20. DeLean, A., Kilpatrick, B. F. & Caron, M. G. (1982) Endocrinology (Baltimore) **110**, 1064–1066
21. Bürgisser, E., DeLean, A. & Lefkowitz, R. J. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 1732–1736
22. Leung, E., Jacobson, K. A. & Green, R. D. (1990) Mol. Pharmacol. **38**, 72–83
23. Asano, T., Brandt, D. R., Pedersen, S. E. & Ross, E. M. (1985) Adv. Cyclic Nucleotide Res. **19**, 47–56
24. Cerione, R. A., Codina, J., Benovic, J. L., Lefkowitz, R. J., Birnbaumer, L. & Caron, M. G. (1984) Biochemistry **23**, 4519–4523
25. Cerione, R. A., Codina, J., Benovic, J. L., Lefkowitz, R. J., Caron, M. G., Gierschick, P., Somers, R., Spiegel, A. M., Codina, J. & Birnbaumer, L. (1985) J. Biol. Chem. **260**, 1493–1500
26. Florio, V. A. & Sternweis, P. C. (1985) J. Biol. Chem. **260**, 3477–3483
27. Graziano, M. P., Freissmuth, M. & Gilman, A. G. (1989) J. Biol. Chem. **264**, 21907–21914
28. Casey, P. J., Fong, H. K. W., Simon, M. I. & Gilman, A. G. (1990) J. Biol. Chem. **265**, 2383–2390
29. Linder, M. E., Ewald, D. A., Miller, R. J. & Gilman, A. G. (1990) J. Biol. Chem. **265**, 8243–8251

Received 12 November 1990/4 January 1991; accepted 16 January 1991