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Solubilized Rabbit Striatal A_{2a}-Adenosine Receptors: Stability and Antagonist Binding

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Abstract

The A_{2a}-adenosine binding subunit from rabbit striatal membranes was solubilized using 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate and was characterized using the antagonist radioligand [³H]8-[4-[[[2 - aminoethyl]amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine (XAC). The solubilized receptor was very stable, with 55% of the specific [³H]XAC binding remaining after storage for 15 days at 4°C. The dissociation constant (K_d) for binding of [³H]XAC to solubilized A₂ receptors was determined in saturation studies to be 4.0 nM, with a B_{max} of 600 fmol/mg protein. Xanthine inhibitors displaced the specific binding of the adenosine antagonist [³H]XAC (in the presence of 50 nM 8-cyclopentyl-1,3-dipropylxanthine) at 25°C, with K_i values consonant with the expected affinities at A_{2a} receptors. Binding of [³H]XAC (1 nM) or the adenosine agonist [³H]2-(carboxyethylphenylethylamino)adenosine-5'-N-ethyl carboxamide (5 nM) to A_{2a} receptors was diminished in the presence of 0.1 M Na⁺ in both membranes and solubilized preparations. Agonist binding was increased (by 280% for membranes and 180% for solubilized receptors), and antagonist binding was decreased in the presence of 10 mM Mg²⁺. Displacement of [³H]XAC by the agonist (*R*)-N⁶-phenylisopropyladenosine was biphasic, corresponding to high (IC₅₀ = 188 nM, R_H = 30%) and low (IC₅₀ = 9730 nM, R_L = 70%) affinity sites. Preincubation with 100 μM GTP (10 mM Mg²⁺) converted the high affinity binding to low affinity, suggesting that receptor and G-protein are dissociated by the guanine nucleotide. The solubilized receptor was more easily inactivated by exposure to the reducing agent dithiothreitol (IC₅₀ = 3 mM) than in membranes (IC₅₀ = 220 mM), suggesting increased accessibility of structurally essential disulfide bridges.

The biological actions of adenosine as a neuromodulator in the central and peripheral nervous systems (1) and as an autocrine regulator in the cardiac (2), renal (3), and other systems are mediated through A₁ and A₂ subtypes (4). A₂ receptors are further divided into those having high affinity (A_{2a}) and low affinity (A_{2b}) for adenosine derivatives (5). The hypotensive, platelet antiaggregatory, locomotor depressant (6) and other effects of A_{2a}-selective adenosine agonists have been described (review Ref. 4). The A_{2a} receptor was recently cloned from canine thyroid (7) and later rat (8) and human brain (9) cDNA libraries. The second messenger system usually associated with activation of A_{2a} receptors is stimulation of adenylate cyclase activity (5).

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A_{2a}-adenosine receptors in striatal membranes have been characterized using agonist (10, 11) and antagonist (12–15) radioligands, photoaffinity (11) and acylating probes (16), and a fluorescent label (17). Desensitization of A_{2a} receptors in a smooth muscle cell line (18) and *in vivo* (19) has been demonstrated. An adenosine receptor model (4), featuring seven transmembrane helices characteristic of G-protein-linked receptors, has been proposed based on structural analysis of the receptor sequence. This model predicts the location of a sodium regulatory site within the central ligand-binding cavity.

In this report we have detergent-solubilized A_{2a} receptors and shown the preparation to be very stable in binding to the antagonist radioligand [³H]XAC.² The solubilized receptor retains its radioligand-binding characteristics and G-protein association in the absence of guanine nucleotides. This study is intended as a preliminary step toward the isolation of the receptor using affinity chromatography, as has been carried out for A₁ receptors (20, 21).

MATERIALS AND METHODS

Materials

2-Chloroadenosine, XAC, CPX, and 8-SPT were obtained from Research Biochemicals, Inc. (Natick, MA). CGS 15943 was the gift of Dr. John Francis (CIBA Geigy, Summit, NJ). [³H]XAC and [³H]CGS 21680 were obtained from Dupont NEN (Boston, MA).

Preparation of striatal membranes

Striatal tissue was isolated by dissection from rabbit brains obtained frozen from Pel-Freeze Biologicals Co. (Rogers, Arkansas). Tissue was homogenized in the presence of protease inhibitors (5 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, 0.01 mg/ml soybean trypsin inhibitor, 5 µg/ml leupeptin, 1 µg/ml pepstatin A) in 20 vol of ice-cold 50 mM Tris–HCl (pH 7.4), using a Polytron (Kinematica, GmbH, Luzern, Switzerland) (14, 16).³ The membrane suspension was then centrifuged at 37,000*g* for 10 min at 4°C. The pellet was resuspended (20 mg tissue/ml) in the above buffer solution and preincubated at 30°C for 30 min with 3 IU/ml of adenosine deaminase, and the membranes were again homogenized and centrifuged. Finally the pellet was suspended in buffer (200 mg wet weight per milliliter) and stored frozen for no longer than 2 weeks at –70°C.

Solubilization

One gram (tissue wet weight) of membranes was thawed and suspended in a total volume of 20 ml of Tris–HCl at pH 7.4 containing 5 mM EDTA. After centrifugation, the pellet was resuspended in 7 ml of solubilization buffer (10 mM Tris–HCl with 1 mM EDTA and 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps, pH 7.4, at 4°C) with 3 U/ml of adenosine deaminase. This corresponded to a Chaps: protein mass ratio of

²Abbreviations used: CGS 15943, 9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-*c*]quinazolin-5-amine; CGS 21680, 2-(carboxyethylphenylethylamino)adenosine-5'-*N*-ethyl carboxamide; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CPX, 8-cyclopentyl-1,3-dipropylxanthine; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; GPP(NH)P, guanosine 5'-(β, γ)imidotriphosphate; NECA, 5'-*N*-ethylcarboxamidoadenosine; *R*-PIA, (*R*)-*N*⁶-phenylisopropyladenosine; 8-SPT, 8-sulfophenyltheophylline; Tris, tris(hydroxymethyl)amino-methane; XAC, 8-[4-[[[(2-aminoethyl)amino] carbonyl]methyl]oxy]-phenyl] - 1,3 - dipropylxanthine; XCC, 8- [4[(carboxymethyl)oxy]-phenyl]1,3-dipropylxanthine.

³A₂ receptors in membrane suspension tended to be denatured by excessive mechanical agitation.

approximately 2.5:1, which was used throughout unless noted. The suspension was mixed by gentle stirring at 0°C for 1 h and the membranes were centrifuged at 100,000g for 50 min in a Beckman L5–50 ultracentrifuge with a Beckman Type 50 Ti rotor. Aliquots of the supernatant containing solubilized receptors were removed carefully with a hand pipetman. The Chaps extraction of the pellet was repeated two consecutive times by the same procedure for the extraction times test (Table I).

Following solubilization of the receptor in the presence of dithiothreitol (DTT) the preparation was passed over small columns of Sephadex G-50 in disposable pipette tips fitted with porous polyethylene frits (26). Chaps (2 mM) in Tris–HCl at pH 7.4 was used to equilibrate the columns and to elute the receptor.

Following exposure to various chemical agents and solvents, the solubilized receptor was subjected to ultrafiltration using a 10 K molecular weight cutoff Microsep microconcentrator (Filtron Technology Corp., Northborough, MA), which was centrifuged at 10,000 rpm for 4 h.

Radioligand assays

The binding assay was performed as follows, unless otherwise indicated. Radioligand (1 nM for [³H]XAC; 5 nM for [³H]CGS 21680) and unlabeled displacer (if present) were added first, followed by membranes or solubilized receptor. Most experiments with [³H]XAC binding to solubilized receptors utilized 0.1 mg protein in each tube, which was equivalent to 10 mg original tissue wet weight. A minimum of 0.08 mg protein was required to optimize percentage specific binding. The final Chaps concentration in the binding assay with solubilized receptor was 0.08–0.1%. For [³H]CGS 21680 binding, 0.3 mg of protein was used. The incubation buffer consisted of 50 mM Tris–HCl at pH 7.4 for all ligands. [³H]XAC was diluted in 50 mM Tris–HCl containing 50 nM CPX, and [³H]CGS 21680 was diluted in 50 mM Tris–HCl with 10 mM MgCl₂. Nonspecific binding was defined as binding in the presence of 100 μM NECA for [³H]XAC and 20 μM 2-chloroadenosine for [³H]CGS 21680. All incubations were performed at 25° C in a volume of 0.5 ml, containing 3 U/ml of adenosine deaminase. The incubation times were: for [³H]XAC binding, 40 min for solubilized receptors and 60 min for membrane-bound receptor; for [³H]CGS 21680 binding, 90 min for both. Following incubation the reaction was terminated by vacuum filtration through a polyethylenimine-treated Whatman GF/B glass-fiber filter and washing (2×, 4 ml) with ice-cold 50 mM Tris–HCl containing 0.01% Chaps. Each filter disc was added to 4 ml of scintillation fluid, vortexed, and counted in a Packed 1900 TR liquid scintillation analyzer. Binding data for $n = 3$ is given as $\pm$ SD. Figures 1–6 show curves that are representative of multiple experiments.

The protein content of samples were determined using the bicinchoninic acid protein assay reagents (Pierce Chemical Co., Rockford, IL).

Data analysis

For saturation by both agonist and antagonist radioligands, $B_{\max}$ and K_d values were determined using the LIGAND computer program and Scatchard analysis program (Dr. P. Munson, Analytical Biostatistical Section, DCRT, NIH, Bethesda, MD), using a linear

regression formula. Competition curves were analyzed using Inplot (Graphpad, San Diego, CA). IC_{50} values in competition experiments were converted to K_i values using the Cheng–Prusoff equation (22).

RESULTS

Radioligand binding to rabbit striatal A_{2a} receptors was measured by a method described previously (14) for membrane binding. This method utilizes the antagonist [3H]XAC in the presence of 50 nM CPX in the medium to eliminate binding to A_1 -adenosine receptors, also occurring in striatal tissue. Although A_1 -selective in rat brain, XAC was found to have increased A_2 affinity in rabbit and human tissue (12, 15) and thus could be used as an A_2 radioligand in these species.

A_2 -adenosine receptors from human platelets (23, 24) were previously solubilized and detected with the agonist radioligand [3H]NECA. In this study, the A_{2a} -adenosine-binding subunit from rabbit striatal membranes was solubilized using 1% CHAPS. Varying the detergent:protein ratio, adding the reducing reagent DTT, and increasing the number of extractions were explored (Table I). DTT at 1 mM was previously found to improve the yield and stability of solubilized cardiac A_1 receptors (25). Repeated extractions of the pellet did not increase the yield of solubilized receptor. Approximately the same amount of binding activity as was solubilized during the first extraction persistently remained in the pellet following sequential extractions. There was no advantage to adding DTT or doubling the concentration of Chaps (initially 2.5:1, detergent:protein mass ratio). Use of 1% digitonin as detergent resulted in a roughly 5% lower total solubilization yield than with Chaps (data not shown). Adding Mg^{2+} (10 mM) did not improve the solubilization yield. The yield of the solubilized receptors did not change significantly within the pH range 6.5 to 7.6.

Passage of the solubilized preparation over a Sephadex G-50 column, which reduced the concentration of small solutes and Chaps (from 16 to 2 mM), prior to radioligand binding did not affect the [3H]XAC-binding activity. When the receptors were solubilized in the presence of DTT (1 mM), treatment on a Sephadex column was necessary to restore binding activity from 8.0 ± 0.7 to $69 \pm 7\%$ of control and to determine protein content. DTT (1 mM) has been added as an antioxidant to solubilized A_1 receptors (25), but this concentration of DTT proved to be detrimental for binding at solubilized A_{2a} receptors (see below).

The optimal conditions for the postincubation filtration in the [3H]XAC-binding assay of solubilized receptors were found to be presoaking of the filter in polyethyleneimine (0.3%) and the inclusion of Chaps (0.01%) in the washing buffer. These conditions, similar to those used for other solubilized receptors (26), were used for all subsequent experiments.

Time and temperature variables during the incubation with radioligand (Fig. 1) and during storage of the solubilized preparation (Fig. 2) were explored. During the incubation with [3H]XAC an initial rise in specific binding was observed on this time scale for both membrane-bound and solubilized preparations. Equilibrium was reached after approximately 40 min for membrane-bound and after 10 min for solubilized preparations. However, after an initial 20 min the effect of a temperature increase from 25 to 37°C was much greater for the

solubilized receptor than for the membrane-bound receptors. At 37°C the [³H]XAC-binding activity of the solubilized receptors decayed (Fig. 1), likely indicating receptor instability. The decreased thermostability of receptors upon solubilization has been noted previously (31). The [³H]XAC-binding activity of the membrane-bound receptors was the same at 37°C as at 25°C, and prolonged incubation after reaching a equilibrium resulted in no loss of activity.

After storage of the solubilized preparation for 15 days at 4°C (Fig. 2), 55% of the specific [³H]XAC binding remained. Thus, solubilized rabbit A₂ receptors, even in the absence of caffeine (see below), were even more stable to storage at 4°C than was reported for A₁ receptors (half-life of 5 days) (26). Curiously, there was an initial rise of 30% in the level of binding of [³H]XAC 1 day after solubilization.

Stabilization of the solubilized A_{2a} receptors in the presence of glycerol or caffeine was studied (data not shown), based on the report that receptor occupation with caffeine stabilized solubilized A₁ receptors (26). Adding glycerol (20%) to the medium appeared to increase specific [³H]XAC or [³H]CGS 21680 binding by 50–100%, measured both initially and following 5 days incubation of the receptor at 4°C. In the presence of 1 mM caffeine, the solubilized A₂ receptors were somewhat more stable over 5 days than were control preparations, but this required an additional step prior to assay, i.e., passage over a Sephadex G-50 column. For this reason, neither glycerol nor caffeine was used in subsequent experiments.

A representative saturation isotherm for specific [³H]XAC binding, in the presence of 50 nM CPX is shown in Fig. 3. The Scatchard plot of the data (Fig. 3B) is linear, indicating a homogeneous population of noncooperative binding sites. The amount of specific binding was approximately 70% of total binding at 1 nM [³H]XAC. The dissociation constant (K_d) for binding of [³H]XAC to solubilized A_{2a} receptors was determined to be 4.02 nM, with a binding capacity (B_{max}) of 600 fmol/mg protein. The K_d value for [³H]XAC binding to rabbit striatal A₂ receptors in membranes was previously found to be 3.80 nM with a B_{max} of 1230 fmol/mg protein (14). Thus, the affinity of [³H]XAC for solubilized and membrane-bound receptors is indistinguishable.

Xanthine inhibitors displaced the specific binding of the adenosine antagonist [³H]XAC (in the presence of 50 nM CPX) at 25°C, with K_i values consonant with the expected affinities at A_{2a} receptors (Fig. 4 and Table II). XAC itself was a very potent inhibitor of [³H]XAC binding with a K_i value of 7.6 nM, identical to the value in membranes (14). This value is also in good agreement with the K_d value derived from saturation experiments with [³H]XAC. CPX, a potent and highly A₁-selective xanthine, was a weak displacer of [³H]XAC binding, with a K_i value of 184 nM. There was close agreement in affinity between solubilized and membrane preparations for XAC, CPX, and XCC. CGS 15943 is a nonxanthine antagonist that is among the most potent ligands known for A_{2a} receptors (15). Differences in K_i values of several fold were noted for theophylline and CGS 15943. The curves for antagonists inhibiting binding to solubilized membranes were monophasic (Fig. 4) with slope factors approximately equal to 1.

The effects of added cations and guanine nucleotides (Table III) were very similar in membranes and in the solubilized preparation. Binding of [^3H]XAC (1 nM) or the adenosine agonist [^3H]CGS 21680 (5 nM) to $\text{A}_{2\text{a}}$ receptors was diminished in the presence of 0.1 M Na^+ in both membranes and solubilized preparations (Table III). Agonist binding was increased (by 280% for membranes and 180% for solubilized receptors) and antagonist binding was decreased in the presence of 10 mM Mg^{2+} . Displacement of [^3H]XAC by the agonist *R*-PIA was biphasic (Fig. 5), corresponding to high ($\text{IC}_{50} = 188$ nM, $R_{\text{H}} = 30\%$) and low ($\text{IC}_{50} = 9730$ nM, $R_{\text{L}} = 70\%$) affinity sites. The presence of 10 mM magnesium ions raised the control-binding levels for agonist radioligand several fold, but not for antagonist binding, which was somewhat diminished. In the presence of added Mg^{2+} , preincubation of the solubilized or membrane-bound $\text{A}_{2\text{a}}$ receptor with 100 μM GTP or GPP(NH)P converted the high affinity binding of *R*-PIA to low affinity, suggesting that receptor and G-protein are dissociated by the guanine nucleotide. In the absence of magnesium ions, guanine nucleotides did not have a major effect on either radioligand binding, consistent with previous observations (32). Raising the concentration of guanine nucleotides from 100 to 200 μM did not alter these findings.

The disulfide-reactive reagent dithiothreitol was examined as an inhibitor of radioligand binding in rabbit striatal membranes and in solubilized preparations. The effects of these reagent were examined by preincubation with the receptor at neutral pH followed immediately by [^3H]XAC binding, during which time the chemical agent was still present, and the results are shown in Fig. 6. The solubilized receptors were more easily inactivated by exposure to DTT ($\text{IC}_{50} = 3$ mM) than were membrane-bound receptors ($\text{IC}_{50} = 220$ mM), suggesting increased accessibility of structurally essential disulfide bridges (4). When the receptor was solubilized in the presence of 1 mM DTT most of the binding activity lost could be restored upon removal of DTT, suggesting that cleaved disulfide bridges may reform.

The stability of solubilized rabbit A_2 receptors to other chemical agents and organic solvents was examined (data not shown). A solubilized preparation was incubated with the agent (20 min, 25°C) and then the sample was ultrafiltered using a microconcentrator cartridge for centrifugation. Agents that completely denatured the binding activity included: 3 M guanidine hydrochloride, 0.5 M NH_4OH , Tris buffer adjusted to pH 3.0, and 9% acetonitrile. Agents that only partially denatured the binding activity included (percentage residual binding after treatment relative to control solubilized preparation): 9% ethanol (58%), 9% dimethylformamide (80%), 1 M hydroxylamine hydrochloride at pH 5.5 (53%), 20% DMSO (103%), 30% DMSO (84%), and Tris buffer adjusted to pH 10.0 (8%).

DISCUSSION

$\text{A}_{2\text{a}}$ receptors from rabbit striatum, solubilized with 1% Chaps, have been characterized using an antagonist radioligand, [^3H]XAC (Fig. 3). The binding was most effectively measured at 25°C. The appropriate order of potency for inhibition by xanthine derivatives at $\text{A}_{2\text{a}}$ receptors was observed (Fig. 4). Most of the xanthines were of identical affinity at membrane-bound and solubilized $\text{A}_{2\text{a}}$ receptors, but several appeared to be more potent in the solubilized preparation (Table II).

Solubilized preparations of A_{2a} receptors have proven to be relatively stable, perhaps more than solubilized A₁ receptors (26), to storage at 4°C and to a variety of chemical agents (notably, the solvent DMSO at concentrations as high as 30%). The initial rise in [³H]XAC binding 1 day after solubilization is unexplained, but may result from a more complete depletion of endogenous adenosine in the medium (27) by adenosine deaminase. The A_{2a} receptors were more sensitive to the reducing agent DTT (Fig. 6) than were either solubilized A₁ receptors (26) or membrane-bound rabbit A_{2a} receptors. In the model proposed by van Galen *et al.* (4), between one and four disulfide bridges are likely present in extracellular loops of A_{2a} receptors. The rat A_{2a} sequence contains more cysteinyl residues (13) than does the A₁ sequence (9). Thus, there is reason to expect that reducing agents might inactivate the receptor by opening structurally important disulfide bridges. The relative stability of membrane-bound A_{2a} receptors may be a result of these disulfide bridges being less accessible to DTT in the natural phospholipid bilayer than in the detergent micelles. Alternately, the phospholipid bilayer may hold intact an active conformation of the receptor, even if disulfide bridges are reduced.

Nanoff and Stiles have studied solubilized bovine striatal A_{2a} receptors using an agonist radioligand (28). In this study treatment of solubilized rabbit striatal receptors with GTP resulted in the conversion of high affinity agonist-binding sites to low affinity (Fig. 5). This presumably results from uncoupling of the receptor from the G-protein (29). We have confirmed expectations that magnesium ions (29) and sodium ions should have a significant effect on radioligand binding. Magnesium ions (10 mM) greatly enhanced agonist binding, and this enhancement was suppressed in the presence of guanine nucleotides. This suggests in the absence of magnesium there is a large fraction of the receptors in the low affinity state, and they are shifted to high affinity in the presence of magnesium. Magnesium ions (10 mM) somewhat diminished antagonist binding, an effect similar to that observed for [³H]XAC binding to A₁ receptors (30). As with A₁ receptors, sodium (100 mM) decreased agonist binding dramatically, and there was some decrease in antagonist binding (Table III). A putative sodium-binding site (associated with an aspartyl residue) on the receptor in the second transmembrane helical region has been proposed based on sequence analysis (4).

These conditions for solubilization are intended to aid in the purification of A_{2a} receptors by affinity chromatography. Agarose resins derivatized with the functionalized congener XAC, immobilized through its terminal amino group, have been useful in isolating A₁ receptors from various species (21, 22). This study suggests that such affinity supports may likewise be used for isolation of rabbit A_{2a} receptors (in the presence of a selective A₁ antagonist such as CPX), at which XAC has a particularly high affinity both in membranes (14) and in solubilized preparations. A similar high affinity would be expected for solubilized human A_{2a} receptors (15).

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FIG. 1.

Time course for [³H]XAC binding (1 nM, in the presence of 50 nM CPX) to membrane-bound receptors (closed symbols) and the detergent-solubilized receptors (open symbols) from rabbit striatum. The incubation was carried out in Tris-HCl, pH 7.4, at 25°C (circles) or 37°C (triangles) in the presence of adenosine deaminase. The final concentration of Chaps was 0.1%. Incubations were done as described under Materials and Methods except the time was varied.

FIG. 2.
Time dependence of the inactivation of [^3H]XAC binding (1 nM, in the presence of 50 nM CPX) to detergent-solubilized receptors from rabbit striatum incubated at 4°C in the presence of adenosine deaminase.

FIG. 3.

A representative saturation curve (A) and Scatchard plot (B) for the binding of [³H]XAC in the presence of 50 nM CPX to solubilized A₂-adenosine receptors from rabbit striatal membranes. The solubilized preparation was incubated with radioligand at 25°C for 45 min in Tris-HCl, pH 7.4, in the presence of adenosine deaminase. The protein content during incubation was 0.12 mg/ml.

FIG. 4.

Inhibition of binding of [³H]XAC to rabbit striatal A₂-adenosine receptors by various adenosine receptor ligands. The binding was carried out at 25°C for 60 min. [³H]XAC was present at a concentration of 1.0 nM in 0.05 M Tris-HCl at pH 7.4 in the presence of 3 IU/ml adenosine deaminase, 10 mM MgCl₂, and 50 nM CPX. (A) Comparison of membrane-bound (closed symbols) and solubilized (open symbols) receptors for 8-SPT (circles) and a nonxanthine antagonist, CGS 15943 (triangles). (B) Displacement of [³H]XAC binding by various xanthine antagonists at solubilized A₂ receptors (conditions as in A): XAC (◆), CPX (■), 8-SPT (●), theophylline (▲).

FIG. 5.

Representative inhibition curves for the displacement by *R*-PIA of [^3H]XAC (1 nM, in the presence of 50 nM CPX) binding to solubilized rabbit striatal A_2 -adenosine receptors. A 25-min preincubation with *R*-PIA, 50 nM CPX, and 10 mM Mg^{2+} at 25 °C in the absence (○) or presence (●) of 100 μM GTP was performed and followed immediately by the addition of radioligand and incubation for 20 min at 25°C in the presence of adenosine deaminase (3 IU/ml). Slope factors were 0.59 and 0.99 in the absence and presence of GTP, respectively. Analysis of the biphasic curve for displacement by *R*-PIA without GTP gave the following values: $\text{IC}_{50} = 188 \text{ nM}$, $R_{\text{H}} = 30\%$ and $\text{IC}_{50} = 9730 \text{ nM}$, $R_{\text{L}} = 70\%$. In the presence of GTP, only one affinity state was detected ($R_{\text{L}} = 100\%$), with an IC_{50} value of 9660 nM. Values are means of a typical experiment done in triplicate.

FIG. 6.

Effects of exposure of rabbit striatal membranes to the reducing agent dithiothreitol (DTT). Varying concentrations of DTT were added during a 20-min preincubation at 25°C in membrane-bound (○) and in solubilized (●) preparations that was followed immediately (without desalting) by [³H]XAC (1 nM) binding at A₂-adenosine receptors (in the presence of 50 nM CPX). The IC₅₀ values for inactivation by exposure to DTT were 3 mM in solubilized receptors and 220 mM in membranes.

TABLE I

Efficiency of Solubilization of Specific [^3H]XAC-Binding Sites of Rabbit Striatum Membranes by the Detergent Chaps^a

Treatment	% Protein solubilized	% Binding sites solubilized
Consecutive extractions		
Chaps (first)	38 ± 2.4 (6)	26 ± 3.6 (6)
Chaps (second)	5.0 ± 0.75 (6)	0
Chaps (third)	1.6 ± 0.72 (6)	0
Remaining in pellet	35 ± 0.5 (5)	24 ± 3.0 (4)
Single extractions		
Chaps + DTT (1 mM)	36 ± 1.4 (4)	17
Chaps (5:1)	37	23

^aThe solubilization procedures are as given under Materials and Methods. The detergent:protein ratio is 2.5:1, unless indicated. Values are means $\pm$ SD. The number of experiments, each performed in triplicate, is given in parentheses.

TABLE II

Comparison of K_i Values for [^3H]XAC Binding to Solubilized and Membrane-Bound $\text{A}_{2\text{a}}$ -Adenosine Receptors^a

Compound	K_i (nM)	
	Solubilized	Membrane-bound
CGS 15943	2.3 ± 0.09	9.2 ± 2.1
XAC	7.6 ± 3.42	7.5 ± 1.5
CPX	184 ± 76	129 ± 12
XCC	128 ± 4	196 ± 31
8-SPT	2450 ± 240	5780 ± 740
Theophylline	13000 ± 980	38250 ± 240

^aSolubilized and membranes-bound A_2 -adenosine receptors were incubated with 1 nM [^3H]XAC at 25°C for 90 min in the presence of 50 nM CPX as described before. At least seven concentrations of the each antagonist were used for competition. K_i values $\pm$ SD are shown for three separate experiments, each performed in triplicate.

TABLE III

Effect of Guanine Nucleotides and Cations on [^3H]XAC or [^3H]CGS 21680 Binding in Membrane-Bound or Solubilized A_{2a} Receptors^a

Agent	% of control			
	Membrane-bound		Solubilized	
	XAC	CGS 21680	XAC	CGS 21680
Mg ²⁺	64 ± 2.0	380 ± 30	83 ± 5.5	280 ± 28
Na ⁺	55 ± 5.0	20 ± 3.3	52 ± 1.5	18 ± 3.1
GTP	89 ± 0.0	86 ± 10	98 ± 14	86 ± 2.1
GTP (200 μM)	93 ± 1.8	77 ± 3.4	98 ± 2.5	87 ± 1.0
GTP + Mg ²⁺	75 ± 0.9	137 ± 18	76 ± 3.2	153 ± 18
GTP + Na ⁺	63 ± 2.0	27 ± 5.7	56 ± 5.7	23 ± 4.0
GPP(NH)P	92 ± 6.8	90 ± 2.5	88 ± 4.9	84 ± 3.7
GPP(NH)P (200 μM)	89 ± 2.5	82 ± 2.0	88 ± 4.1	81 ± 1.0
GPP(NH)P + Mg ²⁺	64 ± 1.5	126 ± 18	75 ± 10	147 ± 7.0
GPP(NH)P + Na ⁺	55 ± 1.0	19 ± 3.3	47 ± 3.9	16 ± 3.7

^aExperimental protocols are as given under Materials and Methods. Guanine nucleotides and cations were present only during the radioligand incubation (no preincubation). GTP and GPP(NH)P concentrations were 100 μM, or as indicated in parentheses. Mg²⁺ indicates treatment with 10 mM MgCl₂, and Na⁺ indicates treatment with 100 mM NaCl. Values are means ± SD for at least three separate experiments, each performed in triplicate.