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Role of cysteine residues of rat A_{2a} adenosine receptors in agonist binding

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Abstract

In the present study, we investigated the role of disulfide bridges and sulfhydryl groups in A_{2a} adenosine receptor binding of the agonist 2-*p*-(2-carboxyethyl)phenylethylamino)-5′-*N*-ethylcarboxamidoadenosine (CGS 21680). To evaluate the presence of essential disulfide bridges, rat striatal membranes were incubated with [^{3}H]CGS 21680 in the presence of dithiothreitol and binding of the agonist to membranes was measured. The amount of [^{3}H]CGS 21680 which specifically bound, decreased progressively upon pretreatment of membranes with increasing concentrations of dithiothreitol. Pretreatment of rat striatal membranes with 12.5 mM dithiothreitol for 15 min at 25°C resulted in a 2-fold decrease of A_{2a} adenosine receptor affinity for [^{3}H]CGS 21680, and a reduction in the maximal number of binding sites. The presence of agonist or antagonist ligands protected the A_{2a} adenosine receptor sites from the effect of dithiothreitol. We also examined the susceptibility of A_{2a} adenosine receptors to inactivation by the sulfhydryl alkylating reagent, *N*-ethylmaleimide. When rat striatal membranes were pretreated with *N*-ethylmaleimide for 30 minutes at 37°C, a decrease in specific [^{3}H]CGS 21680 binding was observed. Pretreatment of membranes with 1 mM *N*-ethylmaleimide also resulted in a 2-fold reduction of A_{2a} adenosine receptor affinity for [^{3}H]CGS 21680, as well as a slight decrease in the maximal number of binding sites. Neither agonist nor antagonist ligands were effective in protecting the receptor sites from inactivation by *N*-ethylmaleimide. In contrast, addition of 100 μM guanosine-5′-*O*-(3-thiotriphosphate) or 5′-guanylylimidodiphosphate were both effective in protecting the receptor sites from inactivation by *N*-ethylmaleimide. This protective effect was significant but not complete. Our data suggest that disulfide bridges play a role in the stuctural integrity of the A_{2a} adenosine receptor, furthermore, reduced sulfhydryl groups appear to be important but we do not yet know if they are on the receptor or on the $G_{s\alpha}$ subunit.

Keywords: A_{2a} adenosine receptor; Cysteine residue; Chemical modification

Abbreviations: G_i, G protein that inhibits adenylyl cyclase; G_s, G protein that stimulates adenylyl cyclase; CGS 21680, 2-*p*-(2-carboxyethyl)phenylethylamino)-5′-*N*-ethylcarboxamidoadenosine; *m*-DITC-APEC, *m*-phenylenediisothiocyanate-2-[(2 aminoethylamino)carbonylethylphenylethylamino]-5′-*N*-ethylcarboxamidoadenosine; PAPA-APEC, 2[4-(2-{2-[(4-aminophenyl)methyl-carbonyl-amino]ethylaminocarbonyl}ethyl)phenyl]ethylamino-5′-*N*-ethylcarboxamidoadenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; Gpp(NH)p, 5′-guanylylimidodiphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GDP, guanosine 5′-*O*-diphosphate; GMP, guanosine 5′-*O*-monophosphate; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; XAC, xanthine amine congener (8-[4-[[[[(2-aminoethyl)amino)]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine); CHA, N^6-cyclohexyladenosine

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1. Introduction

Adenosine modulates a variety of physiological functions both in the central and peripheral nervous system via the activation of cell surface receptors [1,2]. Pharmacological studies and molecular cloning techniques have allowed the identification of four adenosine receptor subtypes, termed A_1 [3–6], A_{2a} [7–9], A_{2b} [10,11] and A_3 [12–14]. These subtypes, expressed either in native tissues or through recombinant techniques in mammalian cell lines, are distinguished by their binding affinities for various agonist and antagonist ligands. To date, all adenosine receptors thus far sequenced, belong to a superfamily of heptahelical transmembrane receptors which are coupled to a guanine nucleotide-binding regulatory protein (G protein). G-protein-coupled receptors elicit cellular responses via the activation or inhibition of distinct signaling pathways. Whereas, A_1 [15,16] and A_3 [17] adenosine receptors are coupled to G_i proteins, A_{2a} and A_{2b} adenosine receptors activate the adenylyl cyclase system through an interaction with G_s [18]. Similar to other G-protein-coupled receptors, adenosine receptors have the characteristic secondary structure of seven α-helical membrane spanning domains connected by three extra and three intracellular loops, respectively.

On the basis of conformational characteristics of ligands and the involvement of histidine residues in agonist and antagonist bindings as deduced by chemical modification and mutagenesis studies [6,19–21], three-dimensional models of A_1 [22,23], A_{2a} [23,24], and A_3 [25,26] adenosine receptors have been constructed using the atomic coordinates of the bacterial protein bacteriorhodopsin. The three-dimensional model structure of the A_{2a} adenosine receptor indicates that besides two histidine residues located in helical domains VI and VII, various hydrophilic and hydrophobic residues are important in accommodating the agonist ligands. In particular, the hydrophobic C_2-substituents of selective A_{2a} agonists (e.g., 2-*p*-(2-carboxyethyl)phenylethylamino)-5′-*N*-ethylcarboxamidoadenosine (CGS 21680) and 2[4-(2-{2-[(4-aminophenyl) methylcarbonylamino]ethylaminocarbonyl}ethyl)phenyl]ethylamino-5′-*N*-ethylcarboxamidoadenosine (PAPA-APEC)) would fit in the lipophilic environment, created by Ile^{132}, Phe^{177}, and Cys^{249} on the A_{2a} receptor [24]. A favorable interaction of the carboxyl-terminus of CGS 21680 with Cys^{249} of the A_{2a} receptor has also been suggested [24].

That Cys residues are important to ligand binding is supported by experimental data [21] indicating that the treatment of rabbit striatal membranes with an A_{2a}-selective site-directed affinity label, *m*-phenylenediisothiocyanate-2-[(2-aminoethylamino) carbonylethylphenylethylamino]-5′-*N*-ethylcarboxamidoadenosine (*m*-DITC-APEC), decreases specific binding of [^{3}H]CGS 21680 and [^{3}H]xanthine amine congener (8-[4-[[[[(2-aminoethyl)amino)]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine) ([^{3}H]XAC). Sequence analysis of A_{2a} adenosine receptors from various species has shown that this receptor subtype is particularly rich in cysteine residues [27]. An example is the rat A_{2a} receptor which contains thirteen cysteine residues, six within the transmembrane spanning regions and seven in the extracellular loops [24]. Jacobson et al. [27] suggested the extracellular cysteine residues were involved in the formation of disulfide bridges that stabilize the receptor structure. Additionally, the existence of intramolecular disulfide bridges in rabbit A_{2a} adenosine receptors has been shown by the ability of reducing reagents to inhibit [^{3}H]XAC binding to striatal membranes [21].

G protein-coupled receptors have two highly conserved cysteine residues in the second and third extracellular loops [28]. In the β_2-adrenergic receptor, substitution of either of these cysteine residues with Val [29,30] by site directed mutagenesis resulted in a destabilization of the tertiary structure and alterations in the binding characteristics, suggesting the cysteine residues play a role in maintaining the active conformation of the receptor. However, data from a series of β_2-adrenergic receptor mutants in which the cysteines were substituted with Ala show that there is no disulfide bridge between the two conserved cysteines [31]. Using reconstituted phospholipid vesicles which contained purified β_1-adrenergic receptors and mammalian G_s, Pedersen and Ross [32] showed that treatment of vesicles with dithiothreitol (DTT) increases the rate of guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) binding to G_s, in both the presence and absence of β-adrenergic agonists. DTT appeared to be working on the β_1-adrenergic receptor itself, not on G_s [32,33], and it was suggested that DTT activates the β_1-adrenergic receptor by reducing disul-

fide bridges to free sulfhydryl groups. Furthermore, the sufhydryl reagent *N*-ethylmaleimide (NEM) causes an irreversible inactivation of DTT-reduced β_1-adrenergic receptors, although it has no inhibitory effect on the regulatory or ligand binding activities of the nonreduced receptor [34].

As rat A_{2a} adenosine receptors contain numerous cysteine residues in the extracellular and transmembrane domains, modification of their redox status may be an important mechanism for receptor activation and regulation. In the present study, we investigated the role of the cysteine residues in agonist binding. The presence of disulfide bridges was studied by treating rat striatal membranes with DTT and examining agonist binding. Treatment with DTT resulted in a 2-fold decrease of receptor affinity for the agonist, [^{3}H]CGS 21680, and a modest reduction in the maximal number of binding sites. Binding activity was protected when rat striatal membranes were exposed to DTT in the presence of agonist or antagonist ligands. Moreover the A_{2a} adenosine receptors from rat striatal membranes were sensitive to the thiol-alkylating reagent, NEM. Following treatment with NEM a decrease in the receptor affinity for agonists was observed, and the presence of agonist or antagonist ligands did not protect the receptor binding activity.

2. Materials and methods

[^{3}H]CGS 21680 (41.2 Ci/mmol) was obtained from Dupont-New England Nuclear (Boston, MA, USA). Adenosine deaminase, 5′-*N*-ethylcarboxamidoadenosine (NECA) and NEM were purchased from Sigma (St. Louis, MO, USA). Guanine nucleotides (guanosine 5′-*O*-diphosphate (GDP) and guanosine 5′-*O*-monophosphate (GMP)) and nonhydrolyzable analogs, guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) and 5′-guanylylimidodiphosphate (Gpp(NH)p), were purchased from Boeringher-Mannheim (Mannheim, Germany). CGS 21680 and XAC were obtained from Research Biochemicals (Natick, MA, USA). DTT was purchased from Calbiochem (La Jolla, CA, USA). Other agents and reagents were from standard commercial sources.

2.1. Membrane preparation

Striatal tissue was isolated by dissection from male Sprague-Dawley rat brains. Membranes were prepared essentially as previously described [35]. Briefly, striatal tissue was homogenized in 20 vol ice-cold buffer A (50 mM Tris-HCl pH 7.4, 1 mM EDTA) containing protease inhibitors (20 μg/ml soybean trypsin inhibitor, 200 μg/ml bacitracin, and 160 μg/ml benzamidine). The membrane homogenate was centrifuged at $48\,000 \times g$ for 10 min at 4°C. The resulting pellet was resuspended in ice-cold buffer B (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 10 mM $MgCl_2$) containing protease inhibitors (as above) and adenosine deaminase (2 IU/ml) to 50 mg/ml of original tissue weight, and the suspension was incubated at 37°C for 30 min. The membrane homogenate was recentrifuged and the final pellet was stored in aliquots at −80°C until the time of assay.

2.2. Membrane treatment with DTT

Striatal membranes were resuspended (1.5 mg/ml of membrane proteins) in buffer B and incubated with various concentrations of DTT (0 to 200 mM) at 25°C for 15 min. The reaction mixtures were then transferred to 1.5 ml microfuge tubes and centrifuged ($13\,000 \times g$) for 4 min at 4°C. The resulting pellets were resuspended in buffer B and recentrifuged for 4 min. This washing step was repeated twice. Adenosine deaminase (2 IU/ml) was present throughout the entire incubation period and washing steps. The final pellets were resuspended in buffer B containing adenosine deaminase (2 IU/ml) at a protein concentration of 1 mg/ml and used in the binding assays.

To evaluate the time course of ligand-binding site inactivation membrane suspensions were incubated with 12.5 mM DTT at 25°C for various times. For saturation and inhibition experiments, membrane suspensions were incubated with and without 12.5 mM DTT at 25°C for 15 min. The effect of agonists, antagonists, or guanine nucleotides on ligand binding site inactivation was evaluated by incubating membrane suspensions with and without these agents for 10 min at room temperature before the addition of DTT (12.5 mM final concentration).

2.3. Membrane treatment with NEM

Striatal membranes were resuspended (1.5 mg/ml of membrane proteins) in buffer B (pH 7.7) and incubated with increasing concentrations of NEM (0.5 to 25 mM) at 37°C for 30 min. At the end of the incubation the reactions were quenched by the addition of cysteine (10 mM final concentration) and the samples were centrifuged as described above. The final pellets were resuspended as described above and used in the binding assays.

In order to assess the time course of ligand-binding site inactivation, membrane suspensions were incubated with 1 mM NEM at 37°C for various times. For saturation experiments, membrane suspensions were incubated with and without 1 mM NEM at 37°C for 30 min. The effect of agonists, antagonists, or guanine nucleotides on ligand-binding site inactivation was evaluated by incubating membrane suspensions with and without these agents for 10 min at room temperature before the addition of NEM (1 mM final concentration).

2.4. Radioligand binding assay

Routine binding assays were performed as previously described [36]. Briefly, incubation was for 90 min at 25°C in glass tubes containing 0.5 ml of buffer B with striatal membranes (100 μg), 5 nM [^{3}H]CGS 21680, and adenosine deaminase (2 IU/ml). Binding reactions were terminated by filtration through Whatman GF/C filters under reduced pressure. Non specific binding was defined in the presence of 100 μM NECA.

For saturation studies, control membranes or membranes treated with chemical reagents (50 μg of proteins) were incubated in buffer B with 6–8 different concentrations of [^{3}H]CGS 21680 ranging from 1 to 90 nM. Competition studies were carried out by incubating control membranes and membranes treated with 12.5 mM DTT, in buffer B with 5 nM [^{3}H]CGS 21680 and up to 6–8 concentration NECA ranging from 1 nM to 1 μM. For studying the effect of GTPγS on [^{3}H]CGS 21680 binding to control and DTT (12.5 mM) treated membranes, 6–8 different concentrations of the nucleotide were added to the incubation buffer together with [^{3}H]CGS 21680 (5 nM). Protein concentrations were determined by the method of Lowry et al. [37], using bovine serum albumin as standard.

2.5. Data analysis

A non-linear multipurpose curve-fitting computer program (EBDA-LIGAND, Elsevier-Biosoft) [38] was used for analysis of binding data. A partial F test was used to determine if the binding data were best fit by a one- or two-site model. Saturation and displacement curves were also analyzed by computer program GRAFIT Version 3.0 (Erithacus Software, Staines, UK) and GraphPad Prism (Graph Pad Software, San Diego, CA, USA). The IC_{50} values were converted to K_i values by the Cheng and Prusoff equation [39]. The statistical significance of the differences between control and treated groups was evaluated by paired *t* test (Student's *t*-test) using the Instat computer program (Erithacus Software, Staines, UK).

3. Results

The effects of DTT, a disulfide-reactive reagent, on agonist binding to rat A_{2a} adenosine receptors was examined. Rat striatal membranes, prepared as described in Section 2, were preincubated with millimolar concentrations of DTT and washed before radioligand binding. The amount of specifically bound [^{3}H]CGS 21680 decreased progressively upon pretreatment of the membranes with increasing concentrations of DTT (Fig. 1A). The IC_{50} value was calculated to be 12.5 mM. The binding was nearly completely abolished by pretreatment of the membranes with 200 mM DTT for 15 min at 25°C. Under these conditions, the nonspecific binding of [^{3}H]CGS 21680 was not affected. The effect of DTT was time-dependent (Fig. 1B). The inhibition of specific [^{3}H]CGS 21680 binding increased for 25 min of preincubation, while the maximal effect was reached at 90 min. When the percent of residual binding was plotted on a logarithmic scale (Fig. 1B, inset), the decrease was not linearly related to the preincubation time. The biphasic nature of the plot suggested that at least two disulfide bridges were reduced by DTT.

In order to determine whether preincubation with DTT modified the affinity and/or the maximal number of A_{2a} binding sites for agonist, we performed

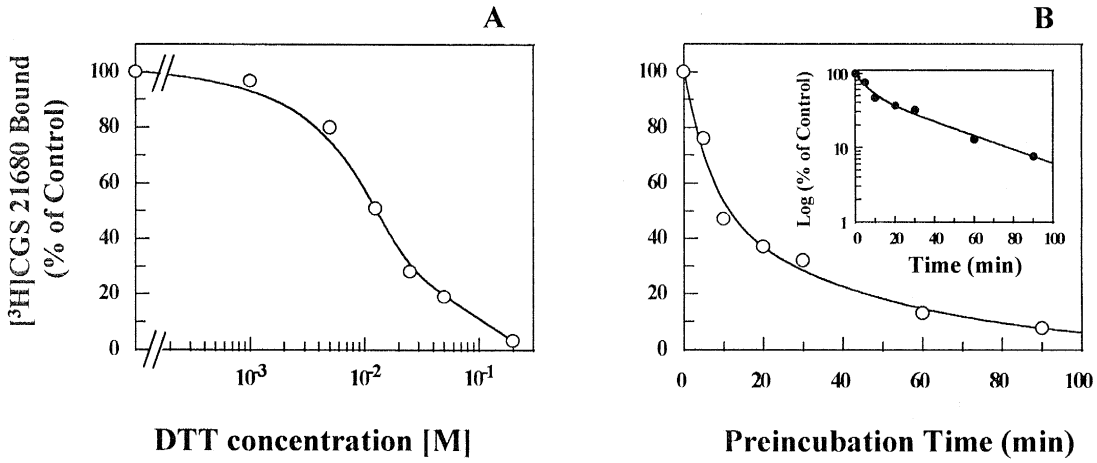

Fig. 1. Inactivation of A_{2a} adenosine receptors as a function of the concentration of DTT (A) and incubation time (B). (A) Membranes were preincubated with increasing concentrations of DTT (1 to 200 mM) for 15 min at 25°C, washed three times with buffer, and incubated with 5 nM [^{3}H]CGS 21680 as described in Section 2. Specific binding is presented as a function of the concentration of DTT during the preincubation. Control binding (240 fmol/mg protein) refers to binding of tracer to membranes pretreated with only buffer B. (B) Membranes were preincubated with 12.5 mM DTT for different periods of time at 25°C, washed three times with buffer, and incubated with 5 nM [^{3}H]CGS 21680 as described. Specific binding is presented as a function of the preincubation time. Control binding refers to binding of tracer to membranes pretreated with only buffer B for each indicated time. Data points are the mean of triplicate determinations with S.E. less than 10%. These results are representative of experiments conducted two additional times.

saturation experiments of [^{3}H]CGS 21680 binding using control and treated membranes. Specific binding of [^{3}H]CGS 21680 to control and DTT treated membranes was saturable (Fig. 2, left panel). Analysis of binding data using a nonlinear curve-fitting computer program (EBDA/LIGAND) revealed that the best fit obtained was for a one-site model under both experimental conditions (Fig. 2, right panel). The K_d value for [^{3}H]CGS 21680 binding to rat striatal A_{2a} adenosine receptors in control membranes was determined to be 11.7 ± 1.2 nM, while the maximal number of binding sites (B_{max}) was 818 ± 96 fmol/mg of protein. When 12.5 mM DTT was present during preincubation, analysis of binding

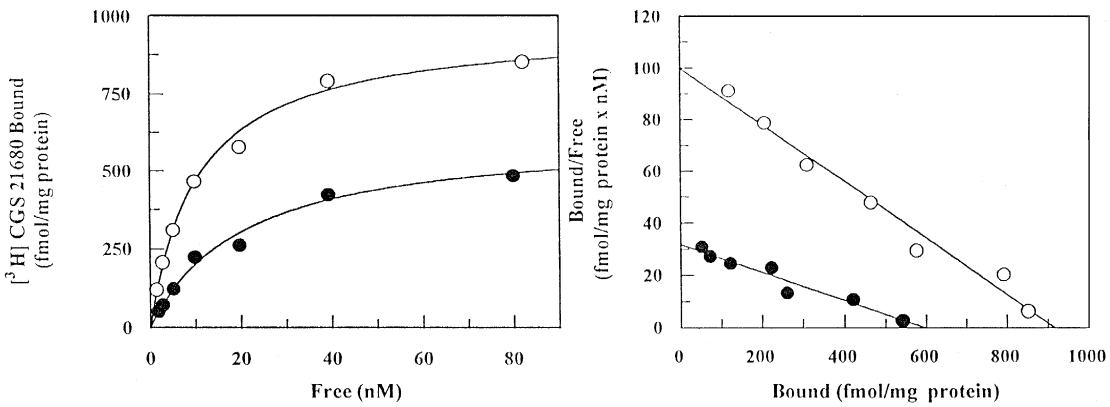

Fig. 2. Saturation curves (left panel) and derived Scatchard plots (right panel) of [^{3}H]CGS 21680 binding to A_{2a} adenosine receptors in native and DTT-treated membranes. Membranes were incubated with increasing concentrations of [^{3}H]CGS 21680 ranging from 0.5 to 90 nM. Saturation binding data were transformed using the EBDA/LIGAND and GRAFIT Version 3.0 computer programs. (○) Native membranes, preincubated in buffer B for 15 min at 25°C and washed; (●) membranes pretreated with 12.5 mM DTT for 15 min at 25°C and washed three times with buffer as described under Section 2. For native membranes, the K_d and B_{max} values were 9.2 nM and 917 fmol/mg protein, while for treated membranes the K_d and B_{max} values were 18.8 nM and 590 fmol/mg protein. Each point represents the mean of duplicate determinations. These results are representative of experiments conducted three additional times.

data (Fig. 2) indicated that the maximal number of binding sites and the affinity for the radioligand were diminished with K_d and B_{max} of 18.4 ± 0.5 nM and 451 ± 88 fmol/mg protein, respectively. The decline in the maximal number of binding sites showed no statistical significance, while the difference among K_d values was significant ($P < 0.05$).

We also examined the displacement of [^{3}H]CGS 21680 by NECA using membranes preincubated with either buffer or buffer containing 12.5 mM DTT. Preincubation of membranes with DTT resulted in a shift to the right of the displacement curve without changing the shape (data not shown). Non-linear regression analysis of GRAFIT Version 3.0 and GraphPad Prism computer programs were used to fit the dose-response curves and derive IC_{50} values. Both displacement curves showed slope values close to 1 and the best fits observed were for one-site models (GraphPad Prism computer program). For control membranes a K_i value of 17.3 nM was obtained, while after DTT treatment the K_i value was 44.2 nM, indicating a decrease of receptor affinity for NECA by approximately 2.5-fold.

Binding of agonist to A_{2a} adenosine receptors in rat striatal membranes is modulated by guanine nucleoside diphosphate and triphosphate [35,36]. To assess whether the treatment with DTT affected receptor G_s protein coupling, we investigated the inhibition of agonist binding by GTPγS using membranes treated either with buffer or buffer containing 12.5 mM DTT. The guanine nucleotide inhibited specific [^{3}H]CGS 21680 binding to both types of membranes in a dose-dependent manner. Both dose-response curves appeared to be biphasic (Fig. 3). For control membranes a similar result was noted in a previous study [35,36]. By using non-linear regression analysis (GraphPad Prism Version 2.1) of the inhibition data it was revealed that two receptor populations with different sensitivity to modulation by GTPγS binding to G_s proteins could be identified in control and treated membranes (Fig. 3).

In order to determine whether the effect of DTT was prevented by specific agonist and antagonist ligands, either NECA (780 nM) or XAC (2.9 μM) were added to the preincubation medium. Protection experiments were carried out using 12.5 mM DTT. In the absence of any agonist or antagonist, this concentration of DTT was determined to decrease the number of [^{3}H]CGS 21680 binding sites by 50% (see Fig. 1A). Under these conditions, both NECA and XAC were effective at preserving specific binding of [^{3}H]CGS 21680 to rat striatal membranes (Fig. 4). This protective effect was statistically significant but not complete (40% for NECA and 64% for XAC). These findings indicated that DTT inhibition was occurring either at the ligand binding site or at a site that was closely coupled conformationally to the ligand-binding event.

The effect of GTPγS (100 μM) on inactivation of [^{3}H]CGS 21680 binding sites by treatment with 12.5 mM DTT was also investigated. Both in the absence and presence of 780 nM NECA, the guanine nucleotide did not show any significant protective effect (Fig. 4). However, the presence of both NECA and GTPγS increased the inactivation of [^{3}H]CGS 21680 binding sites by DTT.

In addition to DTT, we also examined the susceptibility of A_{2a} adenosine receptors to inactivation by the sulfhydryl alkylating reagent, NEM. Pretreatment of rat striatal membranes with NEM for 30 min at 37°C resulted in a decrease (Fig. 5A) of specific

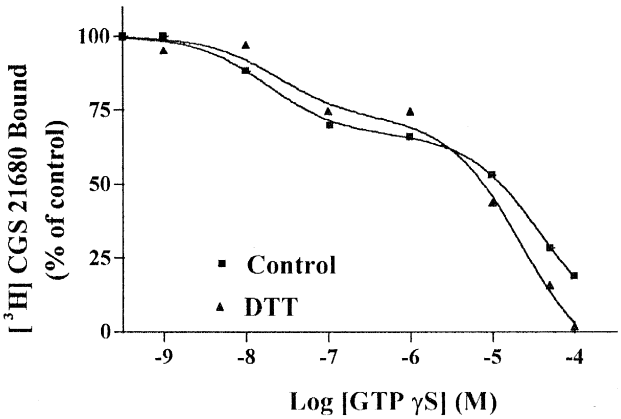

Fig. 3. Effect of GTPγS on [^{3}H]CGS 21680 binding to A_{2a} adenosine receptor in native and DTT-treated membranes. Membranes were preincubated in buffer B with and without 12.5 mM DTT for 15 min at 25°C. For binding assays, aliquots of membranes were incubated in buffer B (see Section 2) with [^{3}H]CGS 21680 (5 nM) and increasing concentrations of GTPγS. A non-linear regression analysis of GraphPad Prism computer program was used to fit the dose-response curve and derive IC_{50} values. For native membranes (■), IC_{50} values were: 18.2 nM and 37.2 μM. For DTT-treated membranes (▲), IC_{50} values were: 24.2 nM and 21.6 μM. Data points are the mean of triplicate determinations with S.E. less than 10%.

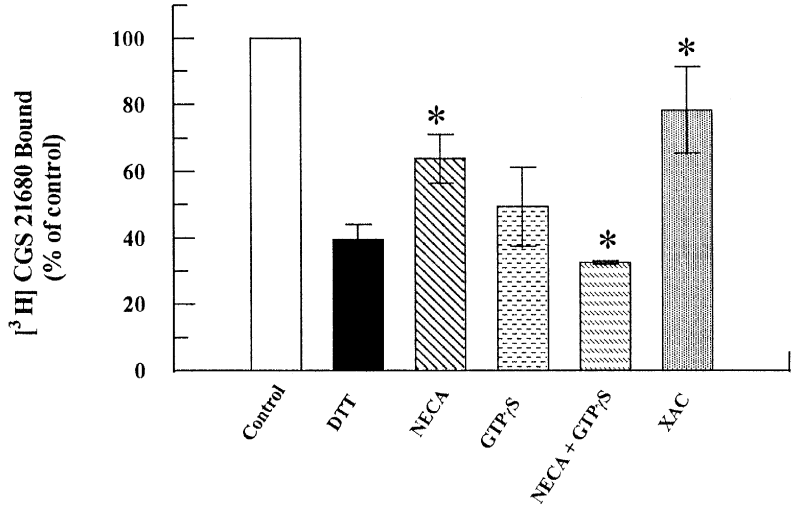

Fig. 4. Inhibition of [^{3}H]CGS 21680 binding to A_{2a} adenosine receptors by DTT and effects of ligands and guanine nucleotide. Membranes were preincubated with 12.5 mM DTT in buffer B for 15 min at 25°C in the presence and absence of the following compounds: 780 nM NECA; 2.9 μM XAC; 100 μM GTPγS; 780 nM NECA + 100 μM GTPγS. Controls were similarly preincubated in either buffer B or buffer B containing compounds but without DTT. Specific binding of [^{3}H]CGS 21680 (5 nM) is expressed as percent of controls. The values are the means ± S.E. of 4 to 6 separate experiments performed in duplicate. * $P < 0.05$: difference between values of DTT − and DTT plus compound-treated membranes is statistically significant, paired *t*-test.

[^{3}H]CGS 21680 binding. An inhibition of 40–50% was obtained at a NEM concentration of 1 mM. Specific [^{3}H]CGS 21680 binding never dropped below a certain level even when higher concentrations of NEM were used. When the pretreatment of membranes was performed with 1 mM NEM at 37°C at

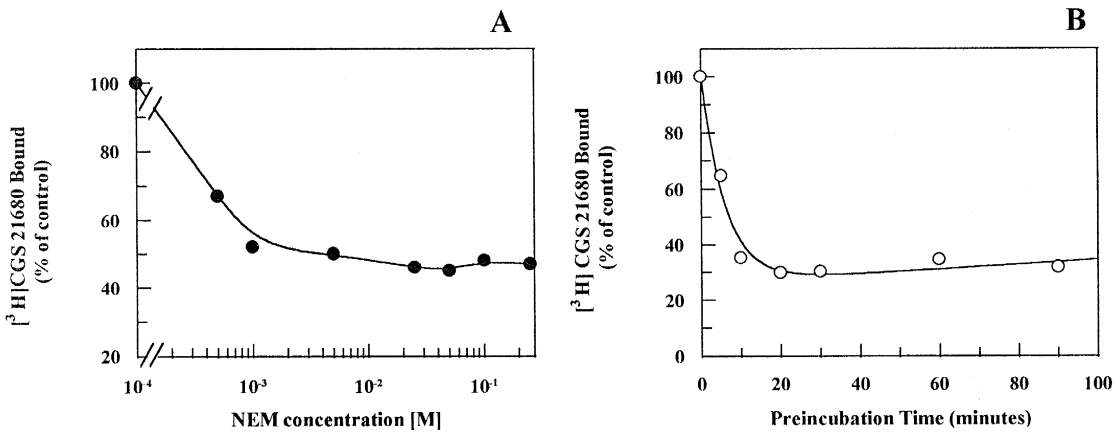

Fig. 5. Inactivation of A_{2a} adenosine receptors as a function of NEM concentration (A) and incubation time (B). (A) Membranes were preincubated with increasing concentrations of NEM (0.5 to 25 mM) for 30 min at 37°C, washed three times with buffer, and incubated with 5 nM [^{3}H]CGS 21680 as described in Section 2. Specific binding is presented as a function of the concentration of NEM during the preincubation. Control binding (215 fmol/mg protein) refers to binding of tracer to membranes pretreated with only buffer B. (B) Membranes were preincubated with 1 mM NEM for different periods of time at 25°C, washed three times with buffer, and incubated with 5 nM [^{3}H]CGS 21680 as described. Specific binding is presented as a function of the preincubation time. Control binding refers to binding of tracer to membranes pretreated with only buffer B for the indicated time. Data points are the mean of triplicate determinations with S.E. less than 10%. These results are representative of experiments conducted two additional times.

different time points, the binding activity decreased rapidly with increase in time (Fig. 5B). The maximal inhibition of specific [^{3}H]CGS 21680 binding was reached after 20 min of preincubation, and remained stable for 90 min. Pretreatment of rat striatal membranes with 12.5 mM DTT at 25°C for 25 min followed by incubation with 1 mM NEM at 37°C for 30 min increased the inhibition of specific binding by 40–50% (data not shown). The result indicated that a new pool of reduced sulfhydryl groups was available for NEM alkylation after treatment of membranes with DTT.

To assess whether pretreatment with NEM leads to a decrease of the number of binding sites and/or their affinity for [^{3}H]CGS 21680, rat striatal membranes were preincubated in either the presence or absence of 1 mM NEM at 37°C, washed, and then incubated with increasing concentrations of the radioligand. Specific binding of [^{3}H]CGS 21680 to control and treated membranes was saturable (Fig. 6, left panel). Analysis of binding data using a non-linear curve fitting computer program (EBDA/LIGAND) resulted in linear plots (Fig. 6, right panel), indicating the existence of a single class of binding sites in control and NEM treated membranes. However, pretreatment with NEM resulted in a decrease in the number of receptor sites and of their affinity for the radioligand. For control membranes, the derived K_d and B_{max} values were 15.0 ± 1.67 nM and 719 ± 13 fmol/mg protein, whereas for NEM treated membranes the K_d and B_{max} values were determined to be 33.6 ± 5.8 nM and 570 ± 32 fmol/mg protein, respectively. Statistical analysis showed that the observed differences among K_d or B_{max} values were not significant.

The influence of agonists, antagonists and guanine nucleotides on A_{2a} adenosine receptors NEM mediated inactivation was examined. Addition of agonist, either NECA (780 nM) or CGS 21680 (780 nM) before preincubation of membranes with 1 mM NEM did not protect the receptor sites from inactivation (Fig. 7, left panel). The antagonist, XAC (2.9 μM), was also ineffective in protecting the receptor sites (Fig. 7, left panel)). These results suggested that alkylation occurred at cysteine residues which were not in close proximity to the active site of the receptor. The addition of GTPγS (100 μM) or Gpp(NH)p (100 μM) effectively protected the receptor sites from inactivation (Fig. 7, right panel) with the protective effect being significant but not complete (58% for Gpp(NH)p and 57% for GTPγS). Gpp(NH)p and GTPγS prevented inactivation of A_{2a} adenosine receptors to a similar extent in the presence or absence of NECA (Fig. 7, left panel). The degree of protection by Gpp(NH)p plus NECA and GTPγS plus NECA was 50% and 58%, respectively. At a concen-

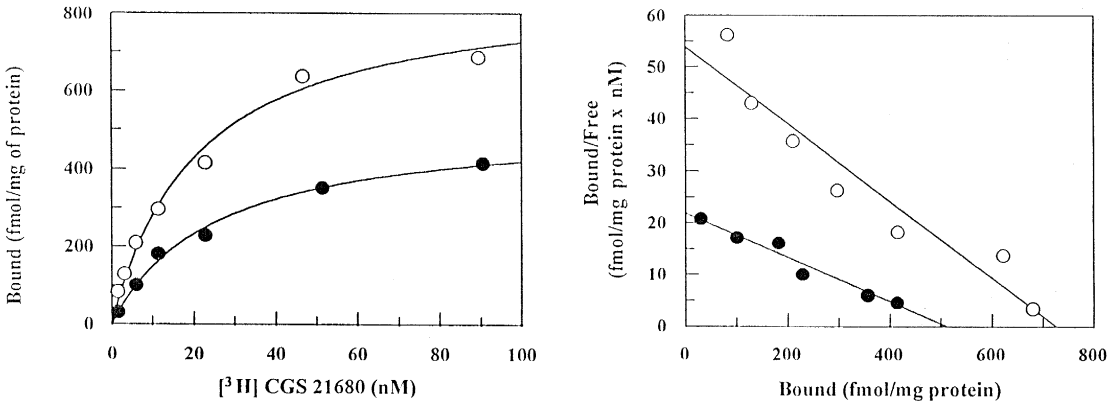

Fig. 6. Saturation curves (left panel) and derived Scatchard plots (right panel) of [^{3}H]CGS 21680 binding to A_{2a} adenosine receptors in native and NEM treated membranes. Membranes were incubated with increasing concentrations of [^{3}H]CGS 21680 ranging from 0.5 to 90 nM. Saturation binding data were transformed using the EBDA/LIGAND and GRAFIT Version 3.0 computer programs. (○) Native membranes preincubated in buffer B for 30 min at 37°C and washed; (●) membranes pretreated with 1 mM NEM for 30 min at 37°C and washed three times with buffer as described under Section 2. For native membranes, the K_d and B_{max} values were 13.5 nM and 725 fmol/mg protein, while for treated membranes the K_d and B_{max} values were 23.4 nM and 520 fmol/mg protein. Each point represents the mean of duplicate determinations. These results are representative of experiments conducted three additional times.

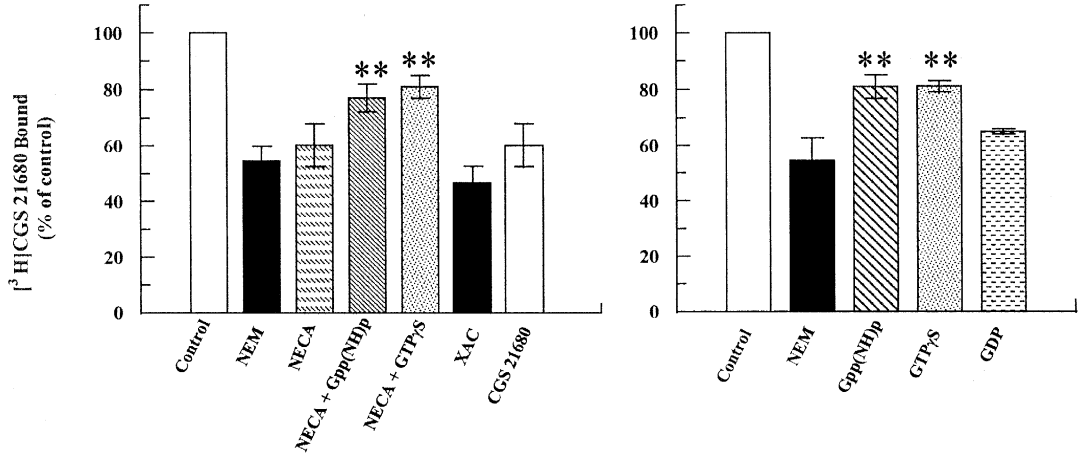

Fig. 7. Inhibition of [^{3}H]CGS 21680 binding to A_{2a} adenosine receptor and effects of ligands and guanine nucleotides. Membranes were preincubated with 1 mM NEM in buffer B (pH 7.7) for 30 min at 37°C in the presence and absence of the following compounds: 780 nM NECA; 780 nM NECA + 100 μM GTPγS; 780 nM NECA + 100 μM Gpp(NH)p; 2.9 μM XAC; 780 nM CGS 21680; 100 μM GTPγS; 100 μM Gpp(NH)p; 250 μM GDP. Controls were similarly preincubated in either buffer B or buffer B containing compounds but without NEM. Specific binding of [^{3}H]CGS 21680 (5 nM) is expressed as percent of controls. The values are the means $\pm$ S.E. of 4 to 6 separate experiments performed in duplicate. * $P < 0.05$, ** $P < 0.01$: difference between values of NEM $-$ and NEM plus compound-treated membranes is statistically significant, paired *t*-test.

tration of 250 μM, GDP showed a modest protective effect (23%) (Fig. 7, right panel), while GMP (250 μM) was not effective (data not shown).

4. Discussion

In the model proposed by Jacobson et al. [27], several disulfide bridges are present in the extracellular loops of A_{2a} adenosine receptors. Thus, reducing reagents such as DTT might inactivate the receptor by opening structurally important disulfide bridges. Additionally, agonist and antagonist ligands may produce a conformational change of the receptor so as to bury one or more disulfide bridges. Alternatively, if the disulfide bridges are located near the binding sites of the receptor it is conceivable that binding of the ligand would shield them from reduction by DTT. Molecular modeling of the A_{2a} adenosine receptor [24] does not implicate the participation of the 7 to 8 cysteine residue located in the extracellular loops in forming the ligand binding site. However, these multiple linkages may be important in maintaining normal function by the receptor, in particular, binding and activation.

The presence of disulfide bridges between conserved cysteine residues in the extracellular loops of G protein coupled receptors has been extensively investigated. Using site-directed mutagenesis, rhodopsin, β_2-adrenergic, and muscarinic acetylcholine receptors have been studied. Biochemical analysis of rhodopsin indicated that two cysteine residues are involved in an intramolecular disulfide bridge that links the second and third extracellular loops of the receptor [40]. Moreover, in experiments with mutants of rhodopsin in which one or both of the cysteines were substituted with Ala [41], findings were consistent with the presence of a disulfide bridge between the conserved cysteine residues. The disulfide bridge does not appear to be required for binding of the ligand retinal, but rather for the stability of the activated intermediate, metarhodopsin II [41]. The metarhodopsin II state of rhodopsin is equivalent to the high affinity state of hormone receptors. For the β_2-adrenergic receptor the information is less consistent. Some studies indicate that there are two extracellular bridges [29,30], while other work does not support the presence of a disulfide bridges between the two conserved cysteine residues [31]. The thiol compound DTT has been shown to have a direct effect on β-adrenergic receptors [42,43].

Pretreatment of rat striatal membranes with DTT caused a decline of the total number of [^{3}H]CGS 21680 binding sites and a decrease of their affinity

for the radioligand. Guanine nucleoside triphosphate still modulated agonist binding to these low-affinity binding sites in a dose-dependent manner. Binding activity was partially protected if rat striatal membranes were treated with DTT in the presence of agonist or antagonist ligands. These results pointed out that DTT inhibition was occurring either at the ligand binding site or at a site that was closely coupled conformationally to the ligand-binding event.

After analyzing chimeric A_1/A_3 receptors, Olah et al. [25] suggested that the second extracellular loop of adenosine receptors plays an important role in agonist and antagonist binding. A cysteine residue conserved in all adenosine receptor subtypes is in the second extracellular loop of both bovine A_1 and rat A_3 adenosine receptors. This cysteine residue has been hypothesized to be involved in disulfide bridge formation in all adenosine receptor subtypes [44]. However, when rat cortical membranes were preincubated with different concentrations of DTT and binding of a selective agonist N^6-[^{3}H]cyclohexyladenosine ([^{3}H]CHA) to A_1 adenosine receptors was measured, no significant decrease of specific binding was detectable (data not shown). The differences between A_1 and A_{2a} adenosine receptors indicate that cysteine residues in the extracellular loops of adenosine receptor subtypes may play different roles in receptor stabilization and high affinity agonist binding. Recently, the importance of the extracellular loops in the binding of ligands to the human A_{2a} adenosine receptor has been examined through site-directed mutagenesis [45]. This study indicated that glutamate residues in the second extracellular loop were required for ligand binding. The role of three cysteine residues present in this same loop has not yet been investigated. Replacement of a cysteine residue in the third extracellular loop with a glycine has no effect in agonist or antagonist binding, suggesting that any potential disulfide bridge formed with another cysteine residue would not be essential for structural integrity of the receptor. However, this observation does not exclude formation of disulfide bridges between cysteine residues in the second extracellular loop as being important in stabilizing the high affinity state of the receptor for agonist ligands.

In the VI transmembrane domain of the rat A_{2a} adenosine receptor, cysteine residues are located at positions 240 and 249. In the three-dimensional model of the rat A_{2a} adenosine receptor [24], the hydrophobic C2-substituents of selective A_{2a} agonists are accommodated in a lipophilic environment that includes Cys^{249}. The involvement of a cysteine residue in ligand binding is supported by experimental data obtained with a site-directed chemical modification approach [21]. Studies employing site-directed mutagenesis of the human β_2-adrenergic receptor [46] have suggested that a conserved cysteine residue (Cys^{285}) in the VI transmembrane domain is important for agonist mediated activation of adenylyl cyclase. In the rat A_{2a} adenosine receptor, Cys^{240} might play a similar role. In order to evaluate the importance of sulfhydryl groups we investigated the effect of the thiol-alkylating reagent NEM on agonist binding to rat striatal membranes. Preincubation of membranes with NEM caused a time-dependent decrease of specific binding. The maximal effect was a 50% inhibition indicating that a limited number of receptors was sensitive to this treatment. Moreover, NEM treatment resulted in a 2-fold decrease of receptor affinity for the radioligand and a modest reduction of the maximal number of binding sites. Thus, the presence of free sulfhydryl groups appears to be more important for high affinity agonist binding than for receptor stability. Neither agonist nor antagonist ligands were able to prevent the effects of NEM. This finding indicates that the reactive sulfhydryl groups modified by the alkylating reagent are not in close proximity to the receptor binding site or in any other location that changes conformation upon ligand binding. Therefore, a site of modification does not appear to be at Cys^{249}. Additionally, we found that the nonhydrolyzable analogs of GTP, GTPγS and Gpp(NH)p, were able to prevent the effect of NEM.

NEM has been shown to modify cysteine residues on pertussis toxin sensitive G proteins [47], and similar to pertussis toxin ADP-ribosylated $G_{i/o}$ proteins, NEM-treated $G_{i/o}$ proteins cannot be activated by receptors [48]. However, it is thought that NEM-treatment does not uncouple G_s proteins from receptors [47]. Our findings raise the possibility that the modified sulfhydryl groups are not present on the receptor, but rather are on the $G_{s\alpha}$ subunit. If the reactive sulfhydryl groups are on the A_{2a} adenosine receptor, binding of a nonhydrolyzable analog of GTP to the $G_{s\alpha}$ subunit may have an allosteric effect on receptor conformation such that the reactive cys-

teine residues are buried and no longer sensitive to alkylation. Alternatively GTP analogs may directly protect reactive sulfhydryl groups on the $G_{s\alpha}$ subunit from alkylation, while irreversible modification of these same groups by NEM would lock the subunit in a conformation similar to that induced by guanine nucleotide binding.

For β_1-adrenergic receptors in turkey erythrocyte membranes, an effect of NEM treatment on ligand binding has been reported [49,50]. However, β_1-adrenergic receptors were only inactivated when membranes were exposed to NEM in the presence of a β-adrenergic agonist. The alkylating reagent had no effect on the free or antagonist-bound forms of the receptor, while guanine nucleotides prevented the inactivation [50], and it was suggested that the reactive sulfhydryl groups might reside on G_s [32,50]. Therefore, NEM appears to have similar effects on two different types of G_s-coupled receptors. However, it should be noted that rat A_{2a} adenosine receptors are sensitive to NEM treatment even in the absence of agonist ligands. This difference may be due to the presence of the natural agonist, adenosine, in our membrane preparation.

In conclusion, our data suggest that disulfide bridges are important for the structural integrity of the rat A_{2a} adenosine receptor and for proper agonist binding. One or more of these disulfide bridges appear to be located in receptor regions that are closely coupled conformationally to the ligand binding event. At present, we cannot discriminate whether the NEM-alkylated sulfhydryl groups are on the A_{2a} adenosine receptor or on the $G_{s\alpha}$ subunit.

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