

Chemical Modification of A₁ Adenosine Receptors in Rat Brain Membranes

EVIDENCE FOR HISTIDINE IN DIFFERENT DOMAINS OF THE LIGAND BINDING SITE*

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Chemical modification of amino acid residues was used to probe the ligand recognition site of A₁ adenosine receptors from rat brain membranes. The effect of treatment with group-specific reagents on agonist and antagonist radioligand binding was investigated. The histidine-specific reagent diethylpyrocarbonate (DEP) induced a loss of binding of the agonist R-N⁶-[³H]phenylisopropyladenosine ([³H]PIA), which could be prevented in part by agonists, but not by antagonists. DEP treatment induced also a loss of binding of the antagonist [³H]8-cyclopentyl-1,3-dipropylxanthine ([³H]DPCPX). Antagonists protected A₁ receptors from this inactivation while agonists did not. This result provided evidence for the existence of at least 2 different histidine residues involved in ligand binding. Consistent with a modification of the binding site, DEP did not alter the affinity of [³H]DPCPX, but reduced receptor number. From the selective protection of [³H]PIA and [³H]DPCPX binding from inactivation, it is concluded that agonists and antagonists occupy different domains at the binding site.

Sulfhydryl modifying reagents did not influence antagonist binding, but inhibited agonist binding. This effect is explained by modification of the inhibitory guanine nucleotide binding protein. Pyridoxal 5-phosphate inactivated both [³H]PIA and [³H]DPCPX binding, but the receptors could not be protected from inactivation by ligands. Therefore, no amino group seems to be located at the ligand binding site. In addition, it was shown that no further amino acids with polar side chains are present. The absence of hydrophilic amino acids from the recognition site of the receptor apart from histidine suggests an explanation for the lack of hydrophilic ligands with high affinity for A₁ receptors.

A₁ adenosine receptors from different tissues and species have been well characterized by pharmacological and biochemical methods (for review, see Ref. 1). They are coupled to adenylate cyclase via the inhibitory guanine nucleotide binding protein G_i, while A₂ receptors are coupled in a stimulatory manner via G_s (2, 3). Adenosine modulates several physiological functions in the central nervous system and in peripheral tissues via these membrane-bound receptors (for review, see Ref. 4). The apparent molecular weight of the

binding subunit of the A₁ receptor has been determined by photoaffinity labeling to be about 35,000 (5-7). In addition, it has been demonstrated that the A₁ receptor is a glycoprotein (8). In order to obtain further information about the molecular structure of the receptor protein, we used the method of chemical modification with reagents, which are specific for amino acids or functional groups as a useful tool to probe the recognition site of receptors and other proteins. In the past, a great variety of proteins have been investigated with this method (9-14). In combination with radioligand binding techniques, it is possible to probe a receptor binding site without isolation of the receptor protein. In this study, we demonstrate that A₁ adenosine receptors have at least in part distinct agonist and antagonist binding domains. In addition, we propose a possible explanation for the lack of hydrophilic ligands with high affinity for this receptor subtype.

EXPERIMENTAL PROCEDURES

Materials

8-Cyclopentyl-1,3-[³H]dipropylxanthine ([³H]DPCPX)¹ was obtained from Amersham Buchler (Braunschweig, FRG) and [³H]PIA from Du Pont-New England Nuclear (Dreieich, FRG). FSNA and PMC were purchased from Aldrich (Steinheim, FRG). DEP, PLP, sodium borohydride, TNM, NEM, DTT, EEDQ, and phenylglyoxal were purchased from Sigma (Deisenhofen, FRG). All other chemicals were of the highest available purity.

Methods

Membrane Preparation

Rat brain membranes were prepared as described by Lohse *et al.* (15).

Chemical Treatment of Membranes

Membranes were treated with different agents at a protein concentration of about 1 mg/ml as described below. For chemical treatment of membranes in the presence of adenosine receptor agonists and antagonists, membranes were preincubated with the indicated concentrations of the ligands for 1 h at room temperature in 50 mM Tris/HCl, pH 7.4. Then, membranes were centrifuged (3 min at 13,000 × g) and resuspended in the corresponding buffer for the chemical treatment with the same ligand concentrations as during the preincubation period. At the end of the modification reaction, membranes were centrifuged, and the ligands were removed by washing the membranes three times in 50 mM Tris/HCl, pH 7.4. Then, membranes

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¹ The abbreviations used are: DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; PIA, R-N⁶-phenylisopropyladenosine; DDCHA, β-2',3'-dideoxy-N⁶-cyclohexyladenosine; DEP, diethylpyrocarbonate; DTT, dithiothreitol; EEDQ, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; FSNA, 4-fluorosulfonyl-1-hydroxy-2-naphthoic acid; NEM, N-ethylmaleimide; PLP, pyridoxal 5-phosphate; PMC, phenylmercuric chloride; PMSF, phenylmethylsulfonyl fluoride; TNM, tetranitromethane; MES, 4-morpholineethanesulfonic acid.

were resuspended in this buffer for radioligand binding. Control experiments were performed in the presence of the respective buffers, ligands, and solvents without the chemical agents. The concentration of organic solvents never exceeded 1% and had no effect on radioligand binding. Adenosine deaminase (0.2 unit/ml) was present throughout the entire incubation period.

DEP—The histidine-specific agent DEP was freshly dissolved in ethanol at a concentration of 200 mM and further diluted in water. Membranes were treated with DEP according to Maksay and Ticku (10) in 20 mM potassium phosphate buffer, pH 7, for 15 min at room temperature.

NEM and PMC—Membranes were treated with these sulfhydryl reagents in 50 mM Tris/HCl, pH 7.4, for 15 min at room temperature.

PLP—In order to modify amino groups, membranes were treated with PLP to form a Schiff base, which was subsequently reduced with sodium borohydride (10). A stock solution of 0.5 M PLP in 1 M KOH was freshly prepared and adjusted to pH 7. The reaction was performed in 20 mM potassium phosphate buffer, pH 7, for 30 min at room temperature. Then, sodium borohydride was added to a final concentration of 25 mM, and the samples were incubated for additional 20 min at room temperature.

Modification of Tyrosine—Nitration of membranes was performed according to Gurwitz and Sokolovsky (16). TNM was freshly dissolved in ethanol, and membranes were treated in 50 mM Tris/HCl, pH 8, for 30 min at room temperature. Treatment of membranes with FSNA was performed at room temperature for 30 min in 50 mM Tris/HCl, pH 8, containing 10 mM MgCl₂ (17).

EEDQ—Carboxyl groups were modified by treating membranes with EEDQ for 1 h at room temperature in 20 mM potassium phosphate buffer, pH 7 (18). EEDQ treatment in 50 mM Tris/MES, pH 7, gave similar results.

Phenylglyoxal—The arginine-specific reagent phenylglyoxal (19) was used as a freshly prepared stock solution (100 mM in dimethyl sulfoxide). Membranes were treated at a final concentration of 1 mM for 1 h at room temperature in 50 mM Tris/HCl, pH 7.4.

PMSF—Membranes were treated with PMSF (dissolved in ethanol) at final concentrations up to 1 mM in 50 mM potassium phosphate buffer, pH 7.4, for 1 h at room temperature.

Radioligand Binding

Radioligand binding was carried out as described by Lohse *et al.* (20). In brief, 50 to 100 μ g of protein were incubated in a total volume of 250 μ l of Tris/HCl, pH 7.4 (500 μ l in saturation experiments), with the agonist [³H]PIA (usually 1 nM) or the antagonist [³H]DPCPX (usually 0.2 nM) in the presence of 0.2 unit/ml of adenosine deaminase. After 2 h at 25 °C, the binding reaction was stopped by filtration through Whatman GF/B glass fiber filters. Nonspecific binding of [³H]PIA was determined in the presence of 1 mM theophylline or 1 μ M DPCPX and of [³H]DPCPX in the presence of 10 μ M R-PIA. Radioligand binding is always presented as specific binding.

Data Analysis

Saturation and competition experiments were analyzed by nonlinear curve-fitting with the program SCTFIT (21).

RESULTS

Effect of DEP Treatment on Radioligand Binding—Treatment of rat brain membranes with the histidine-specific agent DEP resulted in a concentration-dependent loss of both agonist ([³H]PIA) and antagonist ([³H]DPCPX) binding (Fig. 1). The loss of [³H]PIA binding was more pronounced than the loss of [³H]DPCPX binding. The time dependence of the inactivation of radioligand binding is shown in Fig. 2. In the presence of the agonist R-PIA, the DEP effect on [³H]PIA binding could be prevented in part, while the antagonist theophylline had no protective effect. In contrast, [³H]DPCPX binding could be protected almost totally from inactivation by the presence of theophylline, but R-PIA had only a slight protective effect. In addition, the adenosine receptor antagonist DDCHA (*K_i* value of 5 μ M) (30), which is structurally more related to the agonist R-PIA than to the antagonist theophylline, also protected mainly [³H]DPCPX binding from inactivation (Fig. 3). The agonists *N*⁶-cyclo-

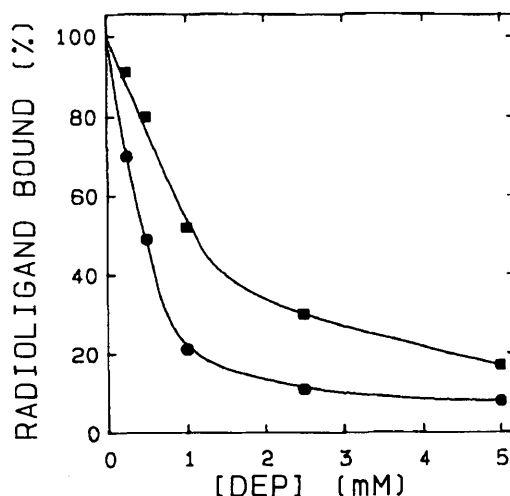


FIG. 1. Concentration-dependent reduction of radioligand binding by DEP treatment. Rat brain membranes were treated with different concentrations of DEP for 15 min at room temperature in 20 mM potassium phosphate buffer, pH 7. Membranes were then centrifuged and resuspended in 50 mM Tris/HCl, pH 7.4, for radioligand binding. Shown is the binding of the agonist [³H]PIA (1 nM) (●) and the antagonist [³H]DPCPX (0.2 nM) (■) as percent of binding to buffer-treated control membranes.

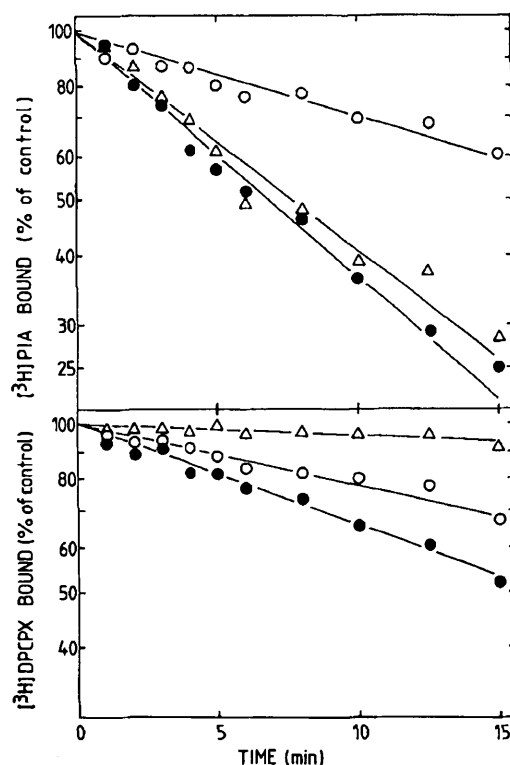


FIG. 2. Time course of reduction of radioligand binding by DEP. Rat brain membranes were preincubated in 50 mM Tris/HCl, pH 7.4, at room temperature for 1 h with 100 nM PIA (○), 1 mM theophylline (△), or buffer (●). Membranes were then centrifuged and resuspended in 20 mM potassium phosphate buffer, pH 7, with the respective ligand concentrations and treated for different times with 1 mM DEP at room temperature. At the indicated incubation times, the modification reaction was stopped with 4 volumes of ice-cold 50 mM imidazole/HCl, pH 7. Then, membranes were washed three times and resuspended in Tris buffer for [³H]PIA (upper panel) and [³H]DPCPX binding (lower panel).

hexyl- and *N*⁶-cyclopentyladenosine had protective effects similar to R-PIA. Saturation curves of [³H]DPCPX binding to A₁ adenosine receptors show that the *B_{max}* value from DEP-

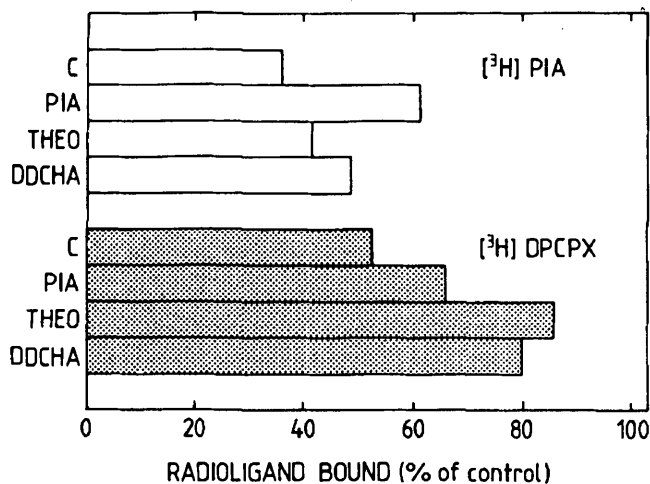


FIG. 3. Protection from inactivation by DEP. Membranes were preincubated with buffer alone as a control, with 100 nM PIA, 1 mM theophylline, or 100 μM DDCHA. Radioligand binding was measured after DEP treatment of the membranes in the presence of the ligands as described under "Experimental Procedures." Shown are the means of five experiments expressed as percent of untreated control membranes.

treated membranes was reduced, but affinity remained unchanged (Fig. 4).

Modification of Sulfhydryl Groups—It is well established that treatment with NEM, a sulfhydryl reagent, shifts A_1 adenosine receptors from a high affinity state to a low affinity state for agonists (22–24). This effect is ascribed to the alkylation of a sulfhydryl group of the inhibitory guanine nucleotide binding protein G_i . We have compared the NEM-induced inactivation of agonist binding with the effect of PMC, another sulfhydryl reagent. In contrast to NEM, PMC forms a covalent mercaptide, which can be reversed by treatment with DTT (11). Fig. 5 shows that $[^3\text{H}]$ PIA binding is inhibited by both NEM and PMC, while $[^3\text{H}]$ DPCPX binding is not affected. The NEM effect on agonist binding is not altered by the presence of 2 mM DTT. On the other hand, the PMC-induced inactivation of $[^3\text{H}]$ PIA binding is largely reversed by DTT up to 3 μM PMC.

Reaction of Amino Groups with PLP—Modification of amino groups with PLP and subsequent reduction of the Schiff base with sodium tetraborohydride induced a dose-dependent loss of $[^3\text{H}]$ PIA and $[^3\text{H}]$ DPCPX binding (Fig. 6). However, 20 mM PLP reduced $[^3\text{H}]$ PIA binding by about 85%, while $[^3\text{H}]$ DPCPX binding was reduced by only about 25%. Therefore, PLP seems to affect mainly the coupling of A_1 receptors to G_i and not the binding site of A_1 receptor. To support this hypothesis, the antagonist $[^3\text{H}]$ DPCPX was displaced with the agonist R -PIA from PLP-treated membranes. Fig. 7 shows that this treatment shifts A_1 receptors from a high affinity to a low affinity state for agonists. In control membranes, about 60% of the receptors are in a high affinity state, while, after treatment with PLP, only 30% of the binding sites exhibit high affinity for R -PIA. The slight alteration of the K_D values may represent a modification of the A_1 receptor itself. This is also suggested by the slight loss of antagonist binding upon PLP treatment. This effect could not be prevented by the presence of adenosine receptor ligands suggesting that the modification did not occur at the binding site.

Modification of Other Amino Acid Residues—A series of further amino acids were modified with specific reagents. Nitration of tyrosine residues with 100 μM TNM resulted in a loss of more than 80% of $[^3\text{H}]$ PIA binding and about 15%

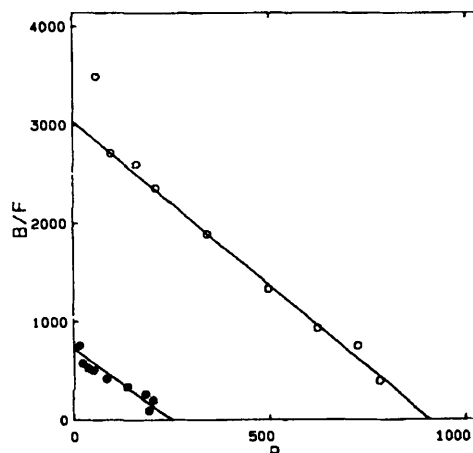
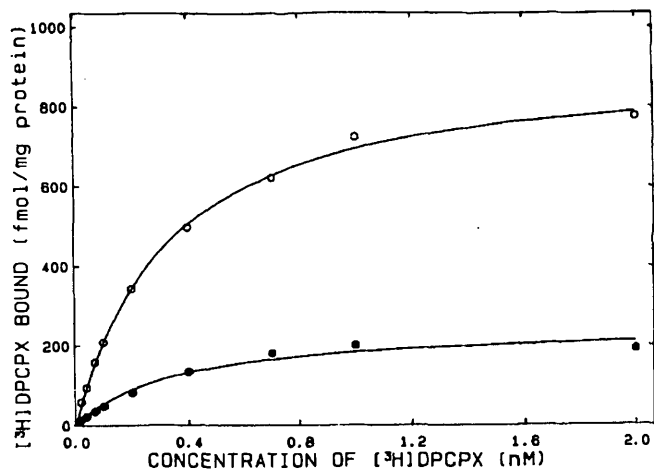


FIG. 4. Saturation of $[^3\text{H}]$ DPCPX binding to control and to DEP-treated membranes. Membranes were DEP-treated as described under "Experimental Procedures." DEP-treated (\bullet) and control (\circ) membranes were then incubated with different concentrations of $[^3\text{H}]$ DPCPX for 2 h at room temperature. The upper panel shows the saturation isotherms, and the lower panel the transformation of the data according to Scatchard. Nonlinear curve-fitting gave for control and DEP-treated membranes K_D values of 0.30 and 0.34 nM and B_{max} values of 910 and 250 fmol/mg of protein, respectively.

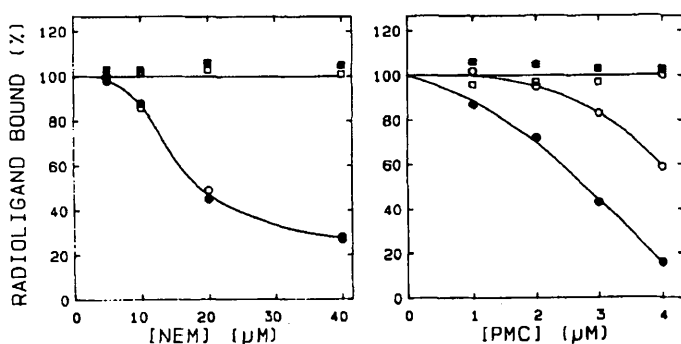


FIG. 5. Concentration dependence of NEM and PMC effects on radioligand binding. Membranes were incubated in 50 mM Tris/HCl, pH 7.4, with increasing concentrations of NEM (left) or PMC (right). After centrifugation and resuspension of the membranes, $[^3\text{H}]$ PIA (circles) and $[^3\text{H}]$ DPCPX binding (squares) was measured either in the presence (open symbols) or in the absence (filled symbols) of 2 mM DTT.

of $[^3\text{H}]$ DPCPX binding (Fig. 8). Higher concentrations of TNM (1 mM) resulted also in an increasing inactivation of $[^3\text{H}]$ DPCPX binding. Agonists and antagonists did not protect A_1 receptors from inactivation. This suggests that no tyrosine residues are located on the adenosine binding site of

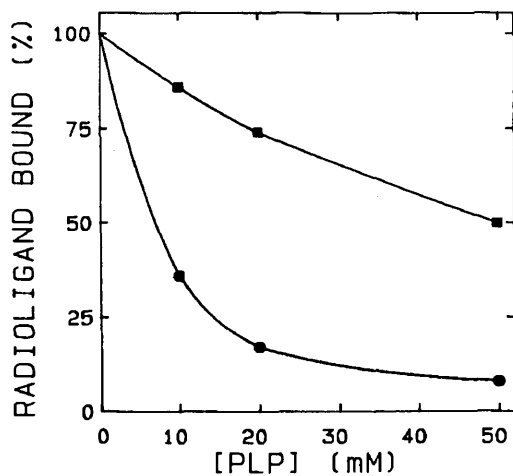


FIG. 6. Concentration dependence of PLP-induced inactivation of radioligand binding. Membranes were treated with increasing concentrations of PLP and then with 25 mM sodium tetraborohydride in 20 mM potassium phosphate buffer, pH 7, as described under "Experimental Procedures." Then, [³H]PIA (●) and [³H]DPCPX binding (■) was measured and compared with buffer-treated control membranes.

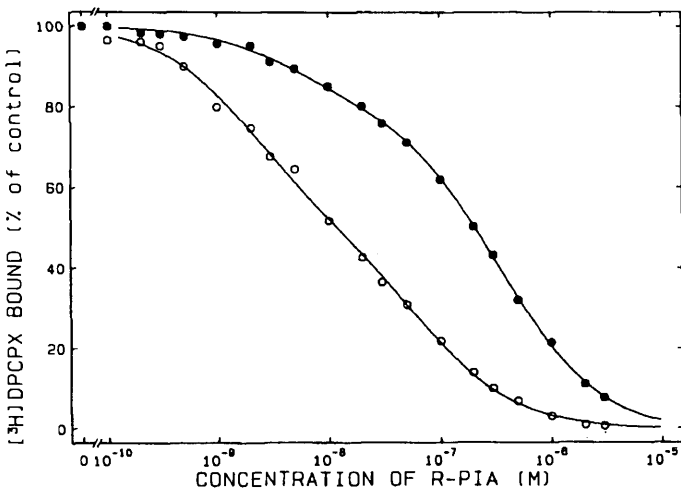


FIG. 7. PLP effect on agonist binding to A₁ receptors. Membranes were treated with 20 mM PLP and 25 mM sodium tetraborohydride, and then competition of PIA for [³H]DPCPX binding to control (○) and PLP-treated membranes (●) was measured. Nonlinear curve-fitting gave for control membranes K_D and B_{max} values for the high and low affinity states of 0.9 and 30 nM and 500 and 330 fmol/mg of protein, respectively. The values for the PLP-treated membranes are 2.9 and 136 nM and 280 and 650 fmol/mg of protein.

the receptor. In addition, FSNA, another tyrosine-specific reagent (17), had no effect on radioligand binding to A₁ receptors in concentrations up to 2 mM.

Treatment of membranes with 200 μM EEDQ caused a 40–50% inhibition of both [³H]PIA and [³H]DPCPX binding, which was prevented by the presence of theophylline, but not by the presence of agonists. In addition, radioligand binding could be protected from inactivation by the presence of nucleophilic compounds like hydroxylamine, imidazole, or 2-hydrazinoethanol. PMSF and phenylglyoxal did not influence radioligand binding. These results suggest that no aspartic acid, glutamic acid, serine, threonine, and arginine are present in the ligand binding domain of the A₁ receptor.

DISCUSSION

Chemical modification of amino acids yields information about the structure of the ligand binding site of A₁ adenosine

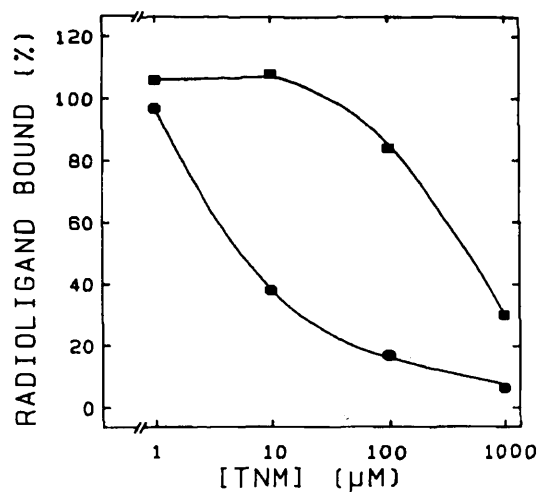


FIG. 8. Nitration of tyrosine with TMN. Membranes were treated with TNM for 30 min at room temperature in 50 mM Tris/HCl, pH 8. [³H]PIA (●) and [³H]DPCPX (■) binding was measured in 50 mM Tris/HCl, pH 7.4, and is shown as percent of binding to control membranes.

receptors. In particular, the modification of histidine residues with DEP reveals interesting features of the recognition site. DEP treatment reduced the receptor number, but it did not affect the receptor affinity for antagonists. This result is consistent with a modification of receptors at the ligand binding domain. The selective protection of [³H]PIA and [³H]DPCPX binding from inactivation by agonists and antagonists leads to the conclusion that at least 2 histidine residues should be located at or near the ligand binding site. This selective protection from inactivation leads, in addition, to the conclusion that both agonists and antagonists occupy domains of the ligand recognition site, which they do not share with each other. One can speculate that the histidine of the antagonists domain should also have some contact to the agonist domain, because R-PIA also had a small protecting effect on the inactivation of [³H]DPCPX binding. DDCHA, so far the only adenosine derivative being a pure antagonist, prevented antagonist binding from inactivation by DEP, but caused only a slight protection of agonist binding in spite of its structural similarities to adenosine receptor agonists. This result suggests also that occupation of different domains of the recognition site is necessary for agonistic or antagonistic activity of a ligand.

Our results from sulfhydryl modification with NEM confirm that no essential SH groups for ligand binding exist at the A₁ receptor protein since antagonist binding is not influenced. Agonist binding to A₁ receptors, however, was reduced by NEM treatment, as has also been shown for several other receptors which are coupled to adenylate cyclase in an inhibitory manner (25–28). Therefore, it can be assumed that the modification of a cysteine inactivates G_i, because it leads to a shift of different receptor types to a low affinity state. Similar results were obtained with PMC, which allows reversible derivatization of SH groups. The reversal of the PMC effect on agonist binding with DTT, at least at lower PMC concentrations, lends support to the idea that modification of SH groups is the mechanism for inactivation of G_i.

Modification of amino groups with PLP and subsequent reduction of the Schiff base with sodium tetraborohydride reduced both [³H]PIA and [³H]DPCPX binding to A₁ receptors with a pronounced effect on agonist binding. The shift of receptors from a high affinity state for agonists to a low affinity state suggests that modification of a lysine residue (or a N-terminal amino acid) interferes with the coupling of

the receptor and G_i similar to the effect of sulfhydryl reagents. It is not clear however if this modification takes place at the receptor protein or G_i.

Modification of tyrosine with TNM suggested that this amino acid is absent from the ligand binding site. Treatment of membranes with micromolar concentrations of TNM reduced agonist binding. At about 10-fold higher concentrations, antagonist binding was also affected. Because the inactivation of agonist and antagonist binding could not be prevented by adenosine receptor ligands, the modification probably does not occur at the binding site. The tyrosine-specific reagent FSNA had no effect on both agonist and antagonist binding. The difference to TNM may be explained by the different size of the reagents. Introduction of the small nitro group may be possible at tyrosine residues which are not accessible for the larger FSNA.

Modification of aspartic and glutamic acids with EEDQ inactivated agonist binding as well as antagonist binding, but the following points argue against a location of the modified carboxylic groups in the ligand binding site. First, in spite of inactivation of agonist radioligand binding by EEDQ, no agonist was protected from this effect. Second, apart from theophylline, several nucleophilic compounds had a protective effect on inactivation of receptors. These results suggest that modification of the carboxylic group alone is not sufficient for inactivation. The inhibitory effect of EEDQ treatment could result from cross-linking of the receptor protein with another protein located in the neighborhood (29). The presence of the amides of the acidic amino acids cannot be excluded from our experiments.

In summary, our data from chemical modification of amino acid residues present evidence that two different domains in the ligand binding site of the A₁ adenosine receptor can be distinguished. We conclude that agonists interact with a domain of the recognition site, which is different from another domain for antagonist interaction. In addition, evidence is presented that the ligand binding site lacks hydrophilic amino acids with the exception of histidine. This might be the reason for the failure in the development of hydrophilic high affinity ligands for A₁ receptors.

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