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Guanine Nucleotide Effects on 8-Cyclopentyl-1,3-[^{3}H]Dipropylxanthine Binding to Membrane-Bound and Solubilized A_1 Adenosine Receptors of Rat Brain

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Abstract: The effects of guanine nucleotides on binding of 8-cyclopentyl-1,3-[^{3}H]dipropylxanthine ([^{3}H]DPCPX), a highly selective A_1 adenosine receptor antagonist, have been investigated in rat brain membranes and solubilized A_1 receptors. GTP, which induces uncoupling of receptors from guanine nucleotide binding proteins, increased binding of [^{3}H]DPCPX in a concentration-dependent manner. The rank order of potency for different guanine nucleotides for increasing [^{3}H]DPCPX binding was the same as for guanine nucleotide-induced inhibition of agonist binding. Therefore, a role for a guanine nucleotide binding protein, e.g., G_i, in the regulation of antagonist binding is suggested. This was confirmed by inactivation of G_i by *N*-ethylmaleimide (NEM) treatment of membranes, which resulted in an increase in [^{3}H]DPCPX binding similar to that seen with addition of GTP. Kinetic and equilibrium binding studies showed that the GTP- or NEM-induced increase in antagonist binding was not caused by an affinity change of A_1 receptors for [^{3}H]DPCPX but by an increased B_{max} value. Guanine nucleotides had similar effects on membrane-bound and solubilized receptors, with the effects in the solubilized system being more pronounced. In the absence of GTP, when most receptors are in a high-affinity state for agonists, only a few receptors are labeled by [^{3}H]DPCPX. It is suggested that [^{3}H]DPCPX binding is inhibited when receptors are coupled to G_i. Therefore, uncoupling of A_1 receptors from G_i by guanine nucleotides or by inactivation of G_i with NEM results in an increased antagonist binding. **Key Words:** Adenosine receptors—8-Cyclopentyl-1,3-[^{3}H]dipropylxanthine—Antagonist binding—Guanine nucleotide effects. **Klotz K.-N. et al.** Guanine nucleotide effects on 8-cyclopentyl-1,3-[^{3}H]-dipropylxanthine binding to membrane-bound and solubilized A_1 adenosine receptors of rat brain. *J. Neurochem.* **54,** 1988–1994 (1990).

A_1 adenosine receptors are members of the family of receptors that couple to an effector system via a guanine nucleotide binding protein (G protein). In the past, A_1 receptors have been characterized by means of biochemical and pharmacological methods (for review, see Lohse et al., 1988). It is generally accepted that G protein-coupled receptors can occur in two different affinity states for agonists. These receptors are in a high-affinity state when they are coupled to a G protein. GTP binding to the G protein induces dissociation of the receptor–G protein complex and shifts receptors to a low-affinity form (Gilman, 1987). GTP regulation of agonist binding has been shown for membrane-bound and solubilized A_1 adenosine receptors (Gavish et al., 1982; Goodman et al., 1982; Lohse et al., 1984*a*; Klotz et al., 1986).

Conflicting results have been obtained for GTP effects on antagonist binding to adenosine receptors. In bovine and rat brain, no GTP modulation was detected (Goodman et al., 1982; Lohse et al., 1984*a*; Klotz et al., 1986), whereas other studies showed slightly increased affinity in rat adipocytes (Ramkumar and Stiles, 1988) or increased B_{max} values in rat hippocampus and bovine brain (Yeung and Green, 1983; Stiles, 1988) on GTP addition. In the past, the examination of GTP effects was hampered by the fact that only the weak antagonist radioligand 1,3-[^{3}H]diethyl-8-phenylxanthine ([^{3}H]DPX) was available. Recently,

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Abbreviations used: ADA, adenosine deaminase; CHAPS, 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate; [^{3}H]-DPCPX, 8-cyclopentyl-1,3-[^{3}H]dipropylxanthine; [^{3}H]DPX, 1,3-[^{3}H]diethyl-8-phenylxanthine; G protein, guanine nucleotide binding protein; G_i and G_s, inhibitory and stimulatory guanine nucleotide binding protein, respectively; NEM, *N*-ethylmaleimide; [^{3}H]PIA, *R*-*N*^{6}-[^{3}H]phenylisopropyladenosine; [^{3}H]XAC, [^{3}H]xanthine amine congener.

[^{3}H]xanthine amine congener ([^{3}H]XAC) (Jacobson et al., 1986) with nanomolar affinity, but high nonspecific binding, became available and was used by the group of Stiles (Ramkumar and Stiles, 1988; Stiles, 1988). The introduction of 8-cyclopentyl-1,3-[^{3}H]dipropylxanthine ([^{3}H]DPCPX) (Bruns et al., 1987; Lohse et al., 1987) with high selectivity and subnanomolar affinity for the A_1 receptor enabled a closer look at GTP modulation of antagonist binding. We consistently observed an increase in binding of [^{3}H]DPCPX on GTP addition at both membrane-bound and solubilized receptors. Therefore, we studied GTP modulation of antagonist binding in more detail and report now conditions that show more pronounced GTP effects on antagonist binding.

MATERIALS AND METHODS

Materials

R-N^6-[^{3}H]Phenylisopropyladenosine ([^{3}H]PIA) was purchased from Du Pont-New England Nuclear (Dreieich, F.R.G.), and [^{3}H]DPCPX was from Amersham Buchler (Braunschweig, F.R.G.). All nucleotides were obtained from Boehringer Mannheim (Mannheim, F.R.G.). 3-[3-(Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and polyethylenimine were from Sigma (Deisenhofen, F.R.G.). All other chemicals were of the highest purity available.

Preparation of membranes

Membranes from whole rat brains were prepared as described by Lohse et al. (1984*b*). Pretreatment of membranes with *N*-ethylmaleimide (NEM) before radioligand binding was done as described recently (Klotz et al., 1988).

Solubilization of rat brain membranes

The solubilization of rat brain membranes was performed as described previously (Klotz et al., 1986) with some modifications. The initial washing step with EDTA-containing buffer was omitted, because results were not fundamentally different with EDTA-treated membranes (see Discussion). Membranes were suspended in ice-cold water containing 1% CHAPS at a protein concentration of 5 mg/ml and incubated for 15 min on ice. The extract was diluted with the respective incubation buffer to a CHAPS concentration of 0.4% and then centrifuged for 30 min at 100,000 *g*. The supernatant was used as the solubilized receptor preparation.

Radioligand binding assay

Radioligand binding was performed as reported previously (Lohse et al., 1987) with the following modifications. All binding experiments were done in 50 m*M* histidine buffer at pH 6 (free histidine base adjusted with HCl), because the GTP effect on antagonist binding was more pronounced at pH values of <7. Experiments at pH 7.4 were done in 50 m*M* Tris-HCl. [^{3}H]DPCPX (or [^{3}H]PIA) was incubated at a final concentration of 0.2 n*M* (or 1 n*M*) with 50 μg (or 100 μg) of membrane protein (or solubilized receptors from the same amount of protein) in a total volume of 250 or 500 μl in saturation experiments. Membranes were incubated with either radioligand for 2 h at 25°C and then filtered over Whatman GF/B glass fiber filters. Solubilized receptors were incubated with [^{3}H]DPCPX at 12°C, and the binding reaction was stopped after 2 h by filtration over polyethylenimine-treated filters (Bruns et al., 1983). [^{3}H]PIA was equilibrated for 20 h, because membranes were not treated with EDTA. The endogenous Mg^{2+} levels are sufficient to slow the association rate, as has been shown earlier (Klotz et al., 1986). Adenosine deaminase (ADA) was present at a concentration of 0.2 U/ml. This concentration is supposed to be high enough to remove endogenous adenosine, because [^{3}H]DPCPX binding was maximally enhanced at a concentration of ADA as low as 0.003 U/ml. Nonspecific binding was determined in the presence of 10 μ*M* *R*-PIA or 1 m*M* theophylline for [^{3}H]DPCPX and [^{3}H]PIA binding, respectively. Radioligand bound refers to specific binding or as indicated.

Data analysis

Saturation data were analyzed with the nonlinear curve-fitting program SCTFIT (De Lean et al., 1982*a*). Kinetic data were fitted to monoexponential equations (Lohse et al., 1984*a*), and concentration–response curves were analyzed by nonlinear regression as described by Lohse et al. (1986).

RESULTS

GTP consistently induced a small increase in binding of the high-affinity antagonist [^{3}H]DPCPX in rat brain membranes. This effect was dependent on the pH of the incubation buffer and was more pronounced at pH values of <7. At pH 6, a 40–80% increase in [^{3}H]DPCPX binding was observed. Figure 1 shows the concentration dependence of the effect for different guanine nucleotides. The EC_{50} of 3 μ*M* for the GTP-induced stimulation of [^{3}H]DPCPX binding resembled the IC_{50} for inhibition of binding of the agonist [^{3}H]PIA, which is ~9 μ*M* at pH 6 (data not shown).

NEM pretreatment of membranes, which inactivates the inhibitory G protein (G_i), induced a concentration-dependent increase in [^{3}H]DPCPX binding (Fig. 2). The NEM-induced increase in antagonist binding followed the same concentration dependence as the NEM-induced decrease in binding of the agonist [^{3}H]PIA.

The association time courses in Fig. 3 show that both

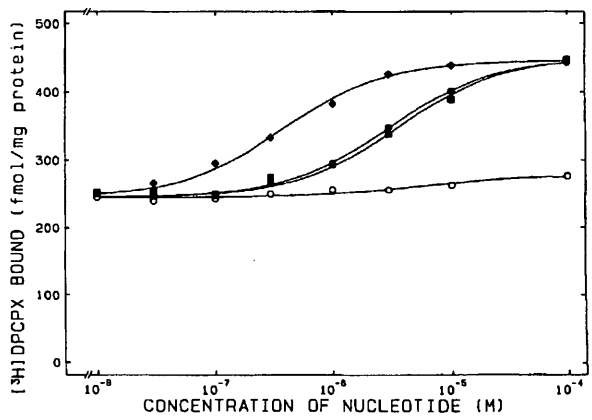

FIG. 1. Concentration dependence of the increase in [^{3}H]DPCPX binding for different guanine nucleotides. Specific binding of [^{3}H]DPCPX to membranes is shown in the presence of increasing concentrations of guanosine-5'-*O*-(3-thio)triphosphate (♦), GTP (●), GDP (■), and GMP (○).

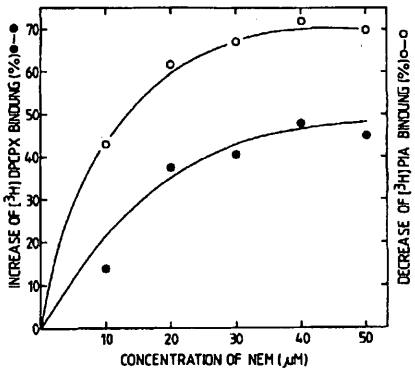

FIG. 2. Concentration dependence of NEM pretreatment of rat brain membranes on [^{3}H]DPCPX and [^{3}H]PIA binding. Membranes were treated with increasing concentrations of NEM; then, radioligand binding was measured as described in Materials and Methods. Shown are the percent increase in [^{3}H]DPCPX binding (●) and the percent decrease in [^{3}H]PIA binding (○).

GTP and NEM pretreatment increased equilibrium binding of [^{3}H]DPCPX, which is reached at all conditions after ~1 h. GTP addition to control membranes after binding equilibrium was attained enhanced [^{3}H]DPCPX binding to the level of the GTP curve. GTP had no further effect in NEM-pretreated membranes.

To clarify whether the increased antagonist binding is caused by a change in kinetic parameters and a resulting affinity change or not, rate constants for association and dissociation were determined. The experiments shown in Fig. 4 suggest that no change in kinetic parameters occurred. The presence of GTP or pretreatment with NEM had only minor effects on rate constants; thus, the K_D values calculated from these data are in close agreement (Table 1). This indicates that GTP affected [^{3}H]DPCPX binding with no change of the affinity of A_1 receptors for this antagonist.

To confirm further the lack of an effect of GTP on affinity of [^{3}H]DPCPX for A_1 receptors, we performed equilibrium binding studies. The saturation experiments shown in Fig. 5 clearly demonstrate that GTP increased [^{3}H]DPCPX binding by increasing the B_{max} value, with almost no change in the K_D value. In addition, it is shown that inactivation of G_i by NEM pretreatment resulted in a similar increase of the B_{max} value, again with only a minor effect on the K_D. Kinetic and equilibrium binding data are summarized in Table 1. In contrast to this observation in saturation experiments in the absence of ADA, a change in the K_D value was observed on addition of GTP or ADA (data not shown).

The reversibility of the GTP effect is shown in Table 2. Membranes were first incubated with GTP, which increased binding of [^{3}H]DPCPX. Washing the membranes with buffer reduced binding to the level of control membranes. Addition of GTP to washed membranes enhanced binding to similar levels as before the washing procedure. The presence or absence of ADA during the pretreatment had no effect on the results.

It has been shown that solubilized A_1 adenosine receptors remain coupled to a G protein, because agonist binding is GTP modulated, as is binding to membrane-bound receptors (Gavish et al., 1982; Klotz et al., 1986). Similar experiments were, therefore, performed with solubilized receptors. Again, binding in the presence of GTP is highest at pH 6, but in contrast to membrane-bound receptors, a marked GTP effect was also observed in the physiological pH range.

Saturation of solubilized A_1 receptors with [^{3}H]DPCPX demonstrated that GTP increased the B_{max} value by almost fivefold, whereas the K_D value was not substantially affected (Fig. 6). In accordance with the results for membrane-bound receptors, the GTP effect is not caused by a change in the affinity for the antagonist. The reversibility of the GTP effect was also observed when control and GTP-treated membranes were washed and then solubilized (Table 2).

The magnitude of the GTP-induced increase of [^{3}H]DPCPX binding to solubilized receptors seemed to be highly dependent on the solubilization procedure. Centrifugation of the membrane extract at a CHAPS concentration of 1% (see Materials and Methods) resulted in a twofold increase, whereas about a fourfold increase was measured after centrifugation at 0.2–0.6% CHAPS (data not shown). The effect of the protein concentration during solubilization was even more pronounced. Control binding of [^{3}H]DPCPX in the absence of GTP decreased with increasing protein concentration (Fig. 7). In the presence of GTP, binding was almost constant at protein concentrations of 1–5 mg/ml. This resulted in a relative GTP-induced increase by a factor of up to about eightfold. The decrease in binding at 10 mg/ml might be caused by a reduced solubilization yield. The presence of adenosine or ADA

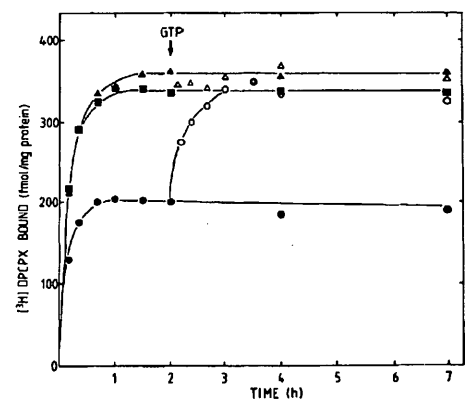

FIG. 3. Effect of GTP and NEM pretreatment of membranes on [^{3}H]DPCPX binding. Membranes were incubated in the absence (●) or presence of 100 μM GTP (■) or after NEM pretreatment of membranes (▲). The arrow indicates GTP addition to control (○) or NEM-pretreated (△) membranes after binding equilibrium was attained.

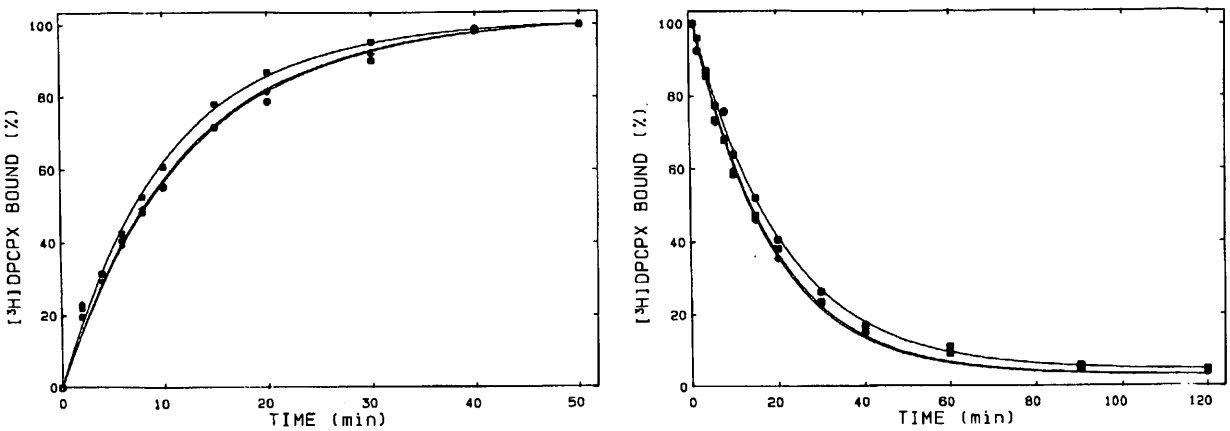

FIG. 4. Association (**left**) and dissociation (**right**) time courses for [^{3}H]DPCPX binding. Specific binding is shown from a representative experiment as a percentage of the respective equilibrium binding. Rate constants for association (k_1) are 0.212, 0.188, and 0.154 nmol^{-1} min^{-1} in the absence (●) or presence of 100 μM GTP (■) or after NEM pretreatment of membranes (♦), respectively. Dissociation was induced by theophylline addition to a final concentration of 1 mM. The rate constants for dissociation (k_{-1}) are 0.0502, 0.0559, and 0.0573 min^{-1} in the absence or presence of GTP or after NEM pretreatment, respectively.

during solubilization did not influence the GTP effect determined in the solubilized preparation. Moreover, the same [^{3}H]DPCPX binding was measured whether GTP was present already during solubilization or only during binding.

DISCUSSION

The effects of guanine nucleotides on antagonist binding to A_1 adenosine receptors are still contested. Some guanine nucleotide effects might be overlooked by the use of the weak antagonist [^{3}H]DPX. [^{3}H]DPCPX is a radiolabeled antagonist with high affinity and selectivity for the A_1 receptor and a high specific radioactivity and therefore proved to be a useful tool for investigating guanine nucleotide effects on antagonist binding. In addition, we took advantage of incubation conditions that dramatically increased the guanine nucleotide effect on binding of [^{3}H]DPCPX, in particular at solubilized receptors. Incubation at pH 6 in histidine buffer was determined to be a very useful condition for this purpose.

TABLE 1. *Comparison of kinetic and equilibrium data for [^{3}H]DPCPX binding to A_1 adenosine receptors from rat brain membranes*

Membrane	Kinetic K_D (nM)	Equilibrium K_D (nM)	Equilibrium B_{max} (fmol/mg)
Control	0.24	0.45	330
GTP	0.30	0.31	620
NEM-treated	0.37	0.27	580

Kinetic K_D values were calculated from the rate constants shown in Fig. 4, and equilibrium data are from Fig. 5.

The guanine nucleotide-induced increase in [^{3}H]DPCPX binding and the inhibition of binding of the agonist [^{3}H]PIA had a similar rank order of potency, an observation suggesting a role of G_i in both processes. This suggestion was further supported by inactivation of G_i by NEM pretreatment of membranes, which induced an identical increase in binding as the presence of GTP with the same concentration dependence as the NEM-induced inactivation of agonist binding. Binding data from kinetic and equilibrium experiments showed that enhanced binding of [^{3}H]DPCPX cannot be attributed to a change in affinity of A_1 receptors for this antagonist. Saturation analysis showed an increased B_{max} value on GTP addition.

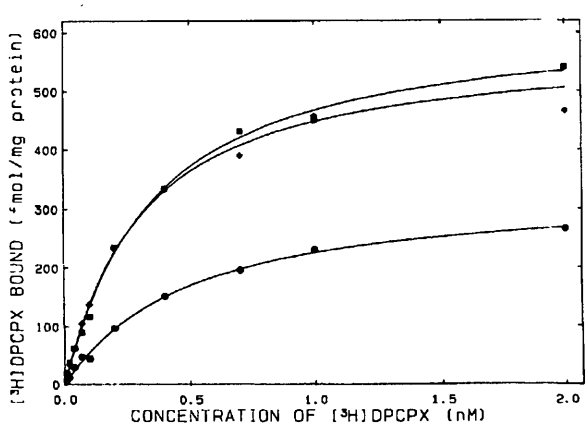

FIG. 5. Saturation of [^{3}H]DPCPX binding to rat brain membranes. Membranes were incubated in the absence (●) or presence of 100 μM GTP (■) or after NEM pretreatment of membranes (♦) with increasing concentrations of [^{3}H]DPCPX. Specific binding is shown from a representative experiment. Nonlinear curve fitting gave K_D and B_{max} values of 0.45 and 330, 0.31 and 620, and 0.27 nM and 580 fmol/mg, respectively.

TABLE 2. *Reversibility of the GTP-induced increase of [³H]DPCPX binding*

	First incubation	Washed	Washed + GTP
Membranes, control	291 ± 29	281 ± 31	364 ± 51[a]
Membranes, 100 μM GTP	405 ± 49[a]	295 ± 35	377 ± 55[a]
Solubilized, control	—	121	513
Solubilized, 100 μM GTP	—	176	489

Membranes were first incubated in the absence or presence of 100 μM GTP and then washed three times with buffer. After the washing step, a portion of the membranes was solubilized. [³H]DPCPX binding was measured with and without GTP after the first incubation and after the washing procedure. ADA was present throughout the entire procedure. For membranes, data (in fmol/mg) are mean ± SEM values from five independent experiments; for solubilized receptors, data are shown from a representative experiment.

[a] $p < 0.05$ versus the respective control.

The lack of an effect on the apparent affinity ruled out the possibility that the GTP-induced increase was a consequence of the presence of endogenous adenosine, supposing a competitive interaction between ligand and receptor. Dissociation of endogenous adenosine caused by a GTP-induced shift of high-affinity binding sites to the low-affinity state would not change the B_{max} value but would increase the apparent affinity of the receptors for [³H]DPCPX. An affinity change was observed in saturation experiments in the absence of ADA on addition of GTP or ADA. This demonstrates that A_1 receptors in rat brain membranes show the attribute of a competitive system. The possibility that removal of endogenous adenosine may be the reason for the GTP-induced increase of antagonist binding in this study could further be ruled out by showing reversibility of the GTP effect.

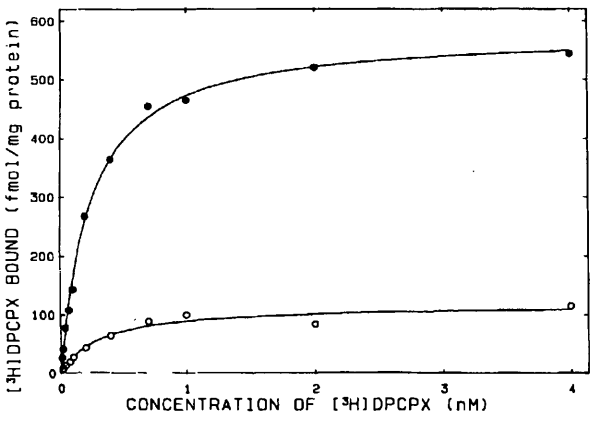

FIG. 6. Saturation of [³H]DPCPX binding to solubilized receptors. Solubilized receptors were incubated in the absence (○) or presence of 100 μM GTP (●) with increasing concentrations of [³H]DPCPX. Specific binding is shown from a representative experiment. Nonlinear curve fitting gave K_D and B_{max} values of 0.29 and 120, and 0.21 nM and 580 fmol/mg, respectively.

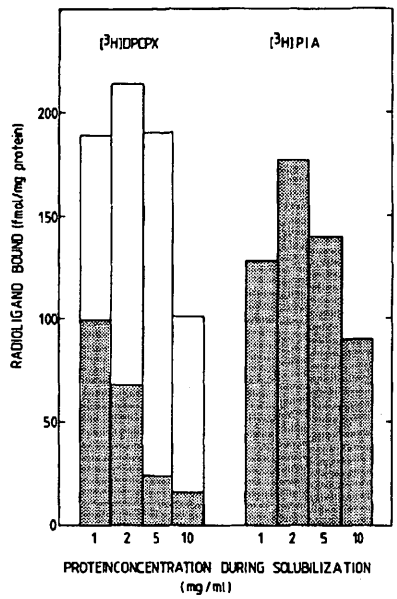

FIG. 7. Dependence of [³H]DPCPX and [³H]PIA binding on protein concentration during solubilization. Radioligand binding is shown in the absence (stippled columns) and presence of 100 μM GTP (open columns).

EDTA pretreatment of membranes was omitted in this study, because it did not qualitatively change results. It slightly diminished the GTP effect, because it induced a small increase of [³H]DPCPX binding by itself. Divalent cations like Mg^{2+} or Ca^{2+} have the opposite effects on receptor–G protein coupling compared with GTP. It is conclusive, therefore, that EDTA pretreatment and GTP have similar effects on G protein-dependent events.

In several receptor systems, e.g., dopamine (De Lean et al., 1982*b*) and muscarinic (Burgisser et al., 1982) receptors, it has been shown that antagonists bind to two different affinity states in the absence of GTP. In these models, agonist and antagonist affinities were reciprocally regulated by GTP. In contrast, at A_1 adenosine receptors, only one affinity state for antagonists could be detected (Yeung and Green, 1983; Lohse et al., 1984*a*, 1987; Klotz et al., 1986; Ramkumar and Stiles, 1988). This seems to be a fundamental difference to the above receptor systems.

Several studies with A_1 receptors done in brain reported no effect of guanine nucleotides on antagonist binding (Gavish et al., 1982; Goodman et al., 1982; Lohse et al., 1984*a*; Klotz et al., 1986) or an enhanced B_{max} value (Yeung and Green, 1983), whereas Ramkumar and Stiles (1988) reported a small effect of guanine nucleotides on affinity of the antagonist [³H]XAC in adipocyte membranes with no change in the B_{max} value. The increased affinity is attributed to an enhanced association rate, whereas the dissociation rate is not affected. The proposal that bound radioligand stabilizes the receptor in a guanine nucleotide-insensitive state

and thus the dissociation rate is not modulated by guanine nucleotides could not be confirmed in our model. At both membrane-bound and solubilized receptors, GTP addition after binding equilibrium was attained in the absence of GTP increased [^{3}H]DPCPX binding to the level of the GTP curve (Fig. 3). It is not clear whether these represent true tissue differences.

A GTP-induced increase in antagonist binding was also seen at solubilized receptors and exhibited characteristics similar to those at membrane-bound receptors. Because G proteins seem to be involved in the modulation of antagonist binding, this result was expected, because solubilized A_1 receptors still interact with a G protein (Gavish et al., 1982; Klotz et al., 1986). It is assumed that GTP binding to G proteins induces dissociation of receptor–G protein complexes and shifts receptors to a low-affinity state for agonists. Thus, [^{3}H]PIA binding to solubilized receptors is abolished in the presence of GTP (Klotz et al., 1986). [^{3}H]DPCPX binding, on the other hand, is low under conditions when receptors are preferentially in the high-affinity state for agonists. The striking similarity between [^{3}H]DPCPX binding in the presence of GTP and binding of the agonist [^{3}H]PIA in the absence of GTP lends support to the idea that [^{3}H]DPCPX labels A_1 receptors that are uncoupled from a G protein. Competition of agonists for [^{3}H]DPCPX binding, however, clearly showed that this radioligand labels both G protein-coupled and -uncoupled receptors (Lohse et al., 1987). This discrepancy may be explained by coupling of A_1 receptors to different G proteins (Munshi and Linden, 1989). Thus, antagonists may bind to, e.g., RG_i but not RG_o (or vice versa), whereas agonists bind to both coupled complexes.

The high variability of the GTP effect in the solubilized system could be attributed to slightly different solubilization protocols. In particular, the protein concentration during solubilization greatly influences the extent of the GTP-induced increase of [^{3}H]DPCPX binding. The decreased binding with increasing protein concentration can be explained by increasing amounts of receptors coupled to G_i. Uncoupling these complexes by GTP addition or inactivation of G_i by NEM pretreatment of membranes thus induces an increase in antagonist binding. This idea corresponds to findings from reconstitution experiments with β-adrenergic receptors and stimulatory G protein (G_s) into phospholipid vesicles (Cerione et al., 1984). These authors found increasing GTPase activity of G_s, even in the absence of an agonist, when reconstitution was performed with increasing concentrations of G_s and β-adrenergic receptors. This was attributed to an increasing portion of receptor–G_s complexes.

Our present data suggest a role for a G protein in the modulation of antagonist binding at A_1 receptors. The finding that the extent of guanine nucleotide-induced increase in binding of the antagonist [^{3}H]DPCPX is dependent on incubation conditions at both membrane-bound and solubilized receptors probably reflects different amounts of A_1 receptors coupled to G proteins. In particular, with solubilized receptors we were able to increase dramatically the extent of the GTP-induced increase of [^{3}H]DPCPX binding by changing solubilization conditions, which might influence protein interactions. GTP effects on antagonist binding reported previously were relatively small; thus, clear-cut conclusions were hard to draw. With the use of the high-affinity antagonist [^{3}H]-DPCPX and conditions demonstrating about an eightfold increase in [^{3}H]DPCPX binding by GTP in our present study, we overcame these problems and clearly showed that no affinity change for this antagonist is induced by guanine nucleotides. It is not clear, however, whether the mechanism of GTP modulation of binding is the same for agonists and antagonists.

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