Modulation of [^{3}H]DPCPX binding to membrane bound and solubilized A_1 adenosine receptors by guanine nucleotides

KARL-NORBERT KLOTZ, ROGER KEIL,
FRANZ-JOSEF ZIMMER AND ULRICH SCHWABE

Pharmakologisches Institut
der Universität Heidelberg
Im Neuenheimer Feld 366
D-6900 Heidelberg, FRG

INTRODUCTION

A_1 adenosine receptors, which are coupled to adenylate cyclase via the inhibitory guanine nucleotide binding protein G_i, can occur in two different affinity states for agonists both in membranes (Lohse et al., 1984a) and in the solubilized form (Klotz et al., 1986). It has been shown that they are in a high affinity form in the G-protein coupled state, similar to other G-protein coupled receptors. GTP induces dissociation of the receptor-G-protein complex and shifts the receptors to a low affinity state for agonists. It is assumed that antagonists do not discriminate between these affinity states and GTP does therefore not influence antagonist binding. Nevertheless, several reports describe GTP-effects on antagonist binding to e.g. A_1 adenosine receptors (Yeung and Green, 1983; Ramkumar and Stiles, 1988), D_2 dopamine receptors (De Lean et al., 1982) or ß-adrenergic receptors (Lang and Lemmer, 1985). We consistently observed a small, but reproducible increase in the binding of the A_1 receptor selective antagonist [^{3}H]DPCPX (8-cyclopentyl-1,3-[^{3}H]dipropylxanthine) upon GTP addition. Therefore we decided to study the GTP-effect on antagonist binding to membrane-bound and solubilized receptors in detail.

METHODS

Rat brain membranes were prepared according to Lohse et al. (1984b) and receptors were solubilized as described previously (Klotz et al., 1986) with some modifications. Membranes were dissolved in 1% CHAPS in H_2O instead of buffer and were then diluted before centrifugation with the respective buffer used in the experiment. Radioligand bind-

ing was performed in 50 mM histidine buffer at pH 6 or as indicated. Membranes were incubated at room temperature for 2 h. Solubilized receptors were incubated with [^{3}H]DPCPX for 2 h and with [^{3}H]PIA for 20 h at 12°C. Details are described by Lohse et al. (1987) and Klotz et al. (1986). GTP was used at a final concentration of 100 M if not indicated otherwise. Pretreatment of membranes with NEM (1 mM or as stated) was performed as described elsewhere (Klotz et al., 1988).

RESULTS

The GTP-dependent increase in binding of [^{3}H]DPCPX to membranes was measured at different pH-values. All subsequent binding studies were performed at pH 6, because the GTP-effect was larger at this pH compared to pH 7.5 (Fig 1, left panel). The concentration dependence of the increase in [^{3}H]DPCPX binding was tested for different guanine nucleotides. GTPγS increased binding with an EC_{50}-value of 0.4 M while GTP and GDP were about equally effective with EC_{50}-values of 3.0 and 3.6 M respectively. GMP was ineffective in concentrations up to 100 M. Inactivation of G_i by NEM-pretreatment of membranes induced also an increase in antagonist binding with a concentration dependence similar to the NEM-induced inactivation of agonist binding (not shown).

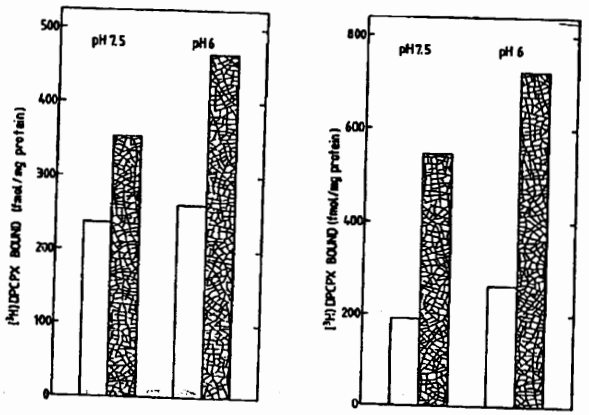

Figure 1

[^{3}H]DPCPX binding to membrane-bound (left panel) and solubilized receptors (right panel) in the presence (patterned columns) or absence of GTP (open columns).

The kinetic experiment shown in Fig 2 demonstrates that [^{3}H]DPCPX binding to membranes was increased by both GTP addition and NEM-pretreatment of membranes. The association time course was not changed compared to control membranes. Dissociation was induced after 90 min by addition of theophylline and also no change in dissociation time course was observed. Rate constants measured in kinetic experiments are summarized in Table 1. K_D-values calculated from rate constants are almost identical, suggesting that GTP or NEM-pretreatment do not influence the affinity of receptors for antagonists. Equilibrium binding data derived from saturation experiments also show that GTP does not affect K_D-values but rather increased the B_{max}-value. Again, NEM-pretreatment caused the same changes as the presence of GTP (Table 1).

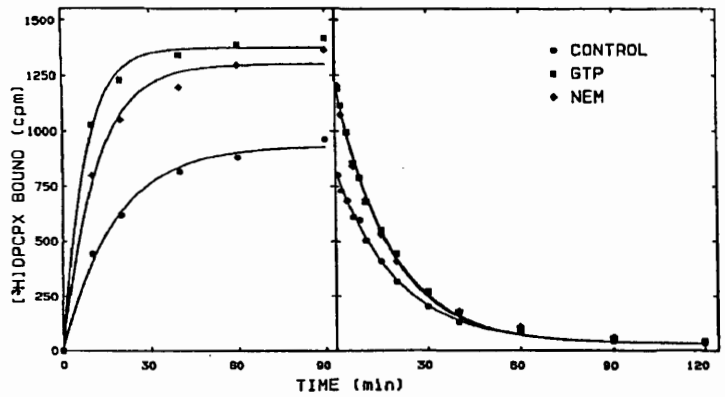

Figure 2

Association and dissociation kinetics of [^{3}H]DPCPX at membranes in the presence or absence of GTP or after NEM-pretreatment.

The GTP-induced increase in [^{3}H]DPCPX binding at solubilized receptors turned out to be much more pronounced than at membrane-bound receptors (Fig 1, right panel). The GTP-effect is somewhat larger at pH 6 compared to physiological pH-values. Different guanine nucleotides increased [^{3}H]DPCPX binding with EC_{50}-values (Fig 3) very similar to the IC_{50}-values for inhibition of agonist binding at solubilized A_1 receptors (Klotz et al., 1986).

Table 1. Kinetic and equilibrium data for [^{3}H]DPCPX binding to rat brain membranes.

Kinetic	Control	GTP	NEM-treated
k_1 ($min^{-1} \times nM^{-1}$)	0.212	0.188	0.154
k_{-1} (min^{-1})	0.0502	0.0559	0.0573
K_D (nM)	0.24	0.30	0.37
Equilibrium			
K_D (nM)	0.45	0.31	0.27
B_{max} (fmol/mg)	330	620	580

In agreement with the data for membrane-bound receptors association time courses for solubilized receptors are similar in the presence and absence of GTP (Fig 4). NEM-pretreatment caused the same increase in antagonist binding as the presence of GTP. GTP-addition after attaining binding equilibrium in the absence of GTP increased binding to about the level of the GTP-curve while no additional effect is observed with NEM-pretreated membranes.

Saturation experiments with [^{3}H]DPCPX show that GTP does also not induce a change in the K_D of solubilized receptors, but considerably increases B_{max}-values (Table 2).

Table 2. Binding data from a saturation experiment with [^{3}H]DPCPX at solubilized receptors.

	Control	GTP
K_D (nM)	0.29	0.21
B_{max} (fmol/mg)	120	580

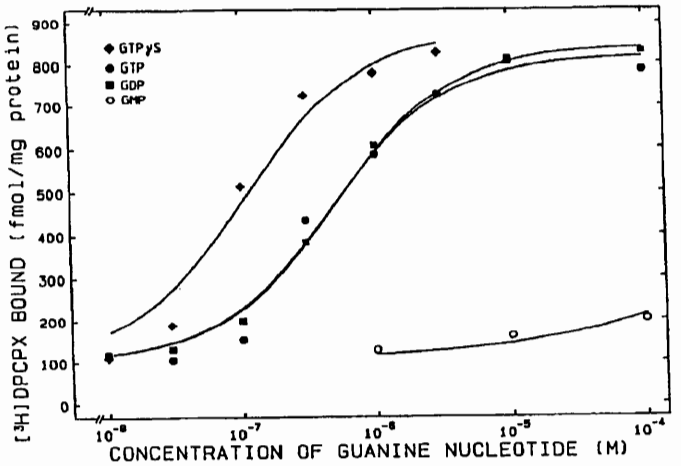

Figure 3
Concentration dependence of guanine nucleotide-induced increase in [^{3}H]DPCPX binding at solubilized receptors.

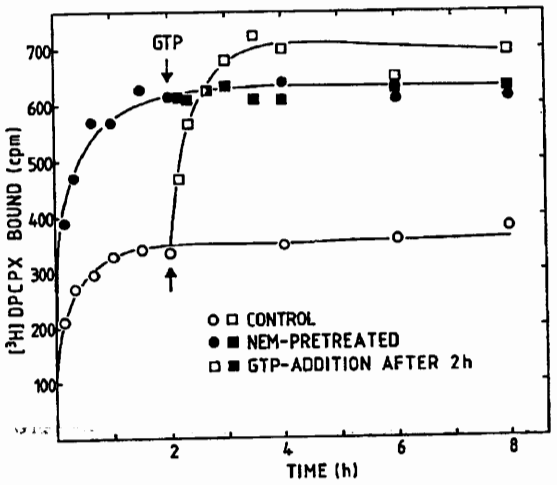

Figure 4
Association time course of [^{3}H]DPCPX binding at solubilized receptors in the presence or absence of GTP or after NEM-pretreatment.

The extent of the GTP-induced increase in [^{3}H]DPCPX binding at solubilized receptors was different in different experiments and seemed to be dependent on the solubilization protocol. Fig 5 shows that [^{3}H]DPCPX binding was doubled in the presence of GTP when membrane extracts were centrifuged at 1% CHAPS while centrifugation at 0.2 to 0.6% CHAPS allowed an about fourfold increase (see "Methods"). The GTP-effect was even more dependent on the protein concentration during solubilization. Control binding in the absence of GTP was reduced with increasing protein concentrations. In the presence of GTP [^{3}H]DPCPX binding was only slightly affected up to a protein concentration of 5 mg/ml. Therefore an increasing net GTP-effect resulted of up to eightfold (Fig 6). The decrease in binding at higher protein concentrations was probably a result of reduced solubilization yield. Binding of the agonist [^{3}H]PIA exhibited a similar protein dependence as [^{3}H]DPCPX in the presence of GTP.

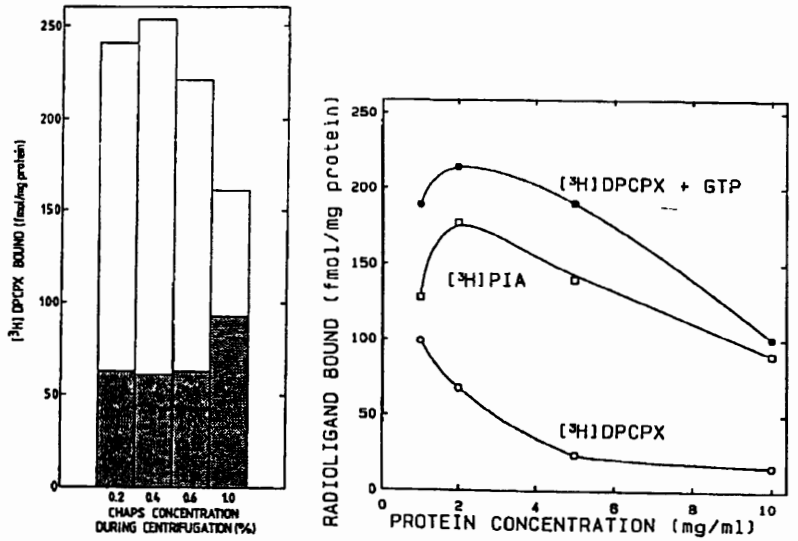

Figure 5

Effect of CHAPS concentration during centrifugation on GTP-induced increase (open columns) in [^{3}H]DPCPX binding. Control binding is shown by dark columns.

Figure 6

Effect of protein concentration during solubilization on [^{3}H]DPCPX and [^{3}H]PIA binding.

DISCUSSION

The GTP-induced increase in [^{3}H]DPCPX binding observed in radioligand binding experiments at membrane-bound A_1 adenosine receptors could be enhanced to almost twofold by incubation at pH 6 in histidine buffer. Under this condition different guanine nucleotides exhibited a rank order of potency similar to their inhibitory activity in agonist binding, suggesting a role of G_i in the regulation of both agonist and antagonist binding. This was further confirmed by inactivation of G_i. NEM-pretreatment of membranes resulted in a similar increase in [^{3}H]DPCPX binding as the presence of GTP. The rate constants for association and dissociation of [^{3}H]DPCPX were not markedly affected, thus K_D-values calculated on the basis of kinetic parameters were also very similar. GTP-induced increase of [^{3}H]DPCPX binding seemed therefore not to involve affinity changes for antagonists at A_1 receptors. Saturation experiments showed also only minor effects on affinity of [^{3}H]DPCPX but B_{max}-values were doubled. Similar results were obtained with solubilized receptors, which still bind agonists in a GTP-modulated manner (Gavish et al., 1982; Klotz et al., 1986). The lack of an effect on the K_D-value for [^{3}H]DPCPX argues against the possibility that the GTP-effect might be caused by dissociation of endogenous adenosine from the receptors by shifting them to the low affinity state for agonists.

The GTP-effect on antagonist binding at solubilized receptors was much more pronounced as at membrane-bound receptors and was highly dependent on the solubilization protocol. In particular, changing the protein concentration during solubilization dramatically influenced the magnitude of the increase in [^{3}H]DPCPX binding. The decrease in [^{3}H]DPCPX binding with increasing protein concentrations during solubilization observed in the absence of GTP might serve as a clue to a mechanistic explanation for the GTP-effect. Assuming that receptor-G-protein-complexes (R-G) are only partially detected by [^{3}H]DPCPX could explain that GTP, by dissociating R-G, increases [^{3}H]DPCPX binding. The striking similarity between agonist binding ([^{3}H]PIA) in the absence of GTP and antagonist binding ([^{3}H]DPCPX) in the presence of GTP further supports this assumption. The decrease in [^{3}H]DPCPX binding in the absence of GTP also fits to data from Cerione et al. (1984). They found increasing GTPase activity of G_o upon reconstitution with ß-receptors at increasing concentrations of both proteins in phospholipid vesicles. This increasing GTPase activity, which occured in the absence of an agonist, reflects enhanced coupling between G_o and ß-receptors. An increasing

coupling of G_i and A_1 receptors during solubilization at increasing protein concentrations could also account for the GTP-effect in the above model.

In summary, we have shown that binding of the antagonist [^{3}H]DPCPX can be modulated by GTP. Inactivation of G_i by NEM-treatment of membranes induces a similar increase of [^{3}H]DPCPX binding as the presence of GTP, supporting the idea that G_i is also involved in modulating antagonist binding. The enhanced binding at both solubilized and membrane-bound receptors is caused by an increase of the B_{max}-value with no change in receptor affinity for the antagonist. It is suggested that G_i, which is coupled to A_1 receptors also in the solubilized state, inhibits antagonist binding. A functional dissociation of receptors from G-proteins by GTP or by inactivation of the G-protein with NEM results therefore in an increased antagonist binding.

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