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RECIPROCAL MODULATION OF AGONIST AND ANTAGONIST BINDING TO INHIBITORY ADENOSINE RECEPTORS BY 5′-GUANYLYLIMIDODIPHOSPHATE AND MONOVALENT CATIONS[1]

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Abstract

Previous work from this laboratory showed that rat hippocampal membranes contain adenosine receptors that mediate GTP-dependent inhibition of adenylate cyclase activity (Yeung, S. -M. H., and R. D. Green (1983) J. Biol. Chem. *258:* 2334–2339). Furthermore, we reported that guanine nucleotides decrease agonist and increase antagonist binding to these adenosine receptors. The present study examines the effects of monovalent cations and guanine nucleotides, alone and in combination, on the binding of agonist ([^{3}H]N^6(L-phenylisopropyl)adenosine) and antagonist ([^{3}H]diethylphenylxanthine) radioligands to adenosine receptors in rat hippocampal membranes. Low concentrations of monovalent cations (≤100 mM) did not affect agonist binding. 5′-Guanylylimidodiphosphate (Gpp(NH)p) alone increased the K_D of the agonist without affecting the maximal number of sites labeled by the agonist (B_{max}); in the presence of monovalent cations, Gpp(NH)p both increased the K_D and decreased the number of sites labeled by the agonist. In contradistinction, Gpp(NH)p increased the maximal number of sites to which the antagonist bound without affecting its K_D, while monovalent cations decreased the K_D of the antagonist both in the absence and the presence of Gpp(NH)p. It is proposed that both agonist and antagonist-receptor complexes exist in three distinct affinity states and that the transitions between these states are modulated by guanine nucleotides and monovalent cations. The results are consistent with a model in which the agonist radioligand measures binding to high and middle agonist affinity states of the receptor but does not measure binding to a low agonist affinity state, while the antagonist radioligand measures binding to the middle and low agonist affinity states but not binding to the high agonist affinity state. According to this model the receptor exists in high agonist-low antagonist and high antagonist-low agonist affinity states and a third state with intermediate affinities for both agonists and antagonists.

Binding of radioligands to receptors coupled to adenylate cyclase has been widely studied. It is now clear that guanine nucleotides decrease agonist binding to receptors that mediate the inhibition (negatively coupled receptors) as well as those that mediate the stimulation (positively coupled receptors) of adenylate cyclase activity (Limbird, 1981; Cooper, 1982). Negatively coupled receptors seem to differ from positively coupled receptors in that antagonist binding to positively coupled receptors is, in general, unaffected by guanine nucleotides, while antagonist binding to negatively coupled receptors is increased by guanine nucleotides. This latter effect has been demonstrated for muscarinic cholinergic (Burgisser et al., 1982; Hosey, 1982), D-2-dopaminergic (DeLean et al., 1982), α_2-adrenergic (U'Prichard et al., 1982; Woodcock and Murley, 1982), and R_i adenosine receptors (Yeung and Green, 1983). In addition, monovalent cations have marked effects on ligand binding to negatively coupled receptors that are not apparent with positively coupled receptors (Limbird, 1981; Cooper, 1982). We recently reported that the R_i adenosine receptor agonist [^{3}H]-N^6-cyclohexyladenosine ([^{3}H]CHA) binds to R_i adenosine receptors in rat hippocampal membranes with three distinct affinities depending on the assay conditions (Yeung and Green, 1983). High agonist affinity binding occurs in the absence of exogenous guanine nucleotide; the addition of 100 μM 5′-guanylylimidodiphosphate (Gpp(NH)p) increases the K_D and decreases the B_{max} of [^{3}H]CHA. Data were presented which suggested that, in the presence of Gpp(NH)p, [^{3}H]CHA binds to two different states of the R_i adenosine receptor, a middle affinity state which is measured directly with [^{3}H]CHA and a low agonist affinity state which can be measured from CHA/[^{3}H]diethylphenylxanthine ([^{3}H]DPX, an antagonist) competition curves. In addition, we reported that Gpp(NH)p increases [^{3}H]DPX binding to R_i receptors. Goodman et al. (1982) reported somewhat similar studies on membranes from guinea pig brain and bovine forebrain. In contrast to our results, these workers reported that guanine nucleotides increase the K_D of [^{3}H]CHA without affecting the B_{max} and that guanine nucleotides are without effect on the binding of [^{3}H]DPX. A potentially important difference between our studies and those of Goodman et al. (1982) is that our incubations contained approximately 50 mM NaCl (added in the glycylglycine buffer used), while, unless specifically added, the incubations of Goodman et al. (1982) were devoid of monovalent cations. The

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studies presented in this communication characterize the interactions between monovalent cations and Gpp(NH)p on [^{3}H] N^6(L-phenylisopropyl)adenosine ([^{3}H]-L-PIA, an agonist) and [^{3}H]DPX binding to R_i receptors in hippocampal membranes. These experiments were performed with [^{3}H]-L-PIA rather than [^{3}H]CHA because of the higher specific activity of the [^{3}H]-L-PIA that is available. Limited experiments with [^{3}H] CHA (results not given) gave results identical to those reported. The results of these studies apparently resolve some of the differences between results we previously reported (Yeung and Green, 1983) and data reported by Goodman et al. (1982) and, more importantly, give further insight into the modulation of ligand binding to R_i adenosine receptors by guanine nucleotides and monovalent cations.

Materials and Methods

Materials. [^{3}H]-L-PIA (49.9 Ci/mmol) and [^{3}H]DPX (13.4 Ci/mmol) were purchased from New England Nuclear. Sources of other drugs and chemicals were the same as previously detailed (Yeung and Green, 1983).

Preparations of membranes. Sprague-Dawley rats (150 to 350 gm, either sex) were killed by decapitation, and the hippocampi were rapidly dissected. Hippocampi were either processed immediately or stored intact in liquid nitrogen for later use. Hippocampal membranes were prepared by the method previously employed (Yeung and Green, 1983) with the exception that the hippocampi were homogenized in 10 mM L-histidine/1 mM EDTA, pH 7.5, and the initial centrifugation was at 12,000 × *g* for 10 min. The membranes were resuspended in the histidine/EDTA buffer for study.

Ligand-binding assays. Incubation mixtures (100 μl) contained 30 μl of membrane preparation (150 to 250 μg of protein), 10 mM L-histidine (in addition to that added in the membrane preparation), 4 mM $MgCl_2$, 2.5 units/ml of adenosine deaminase, ^{3}H-ligand, and other additions as noted. Incubations were performed in duplicate or triplicate at 37°C for 15 min, at which time 3 ml of ice-cold wash buffer (1 mM glycylglycine, pH 7.5, 1 mM $MgCl_2$) were added, and the sample was rapidly poured onto a Whatman GF/A filter under reduced pressure and washed three times with 4-ml volumes of the same buffer. Samples containing 0.1 mM L-PIA were used to correct for nonspecific binding. Scatchard plots of [^{3}H]-L-PIA binding were constructed using a single concentration of [^{3}H]-L-PIA and varying concentrations of cold L-PIA. Parameters determined with this protocol gave values indistinguishable from those determined using varying concentrations of radiolabeled ligand. Scatchard plots of [^{3}H]DPX binding were constructed from experiments in which the concentrations of radioligand were varied. Parameters were determined by least squares analysis; B_{max} values are expressed in terms of milligrams of protein. Protein was determined by the method of Lowry et al. (1951).

Results

The effects of NaCl, KCl, and NH_4Cl on the binding of [^{3}H] -L-PIA to hippocampal membranes in the absence and the presence of 100 μM Gpp(NH)p were determined (Fig. 1). (Concentrations of Gpp(NH)p between 0.1 and 100 μM produce dose-dependent decreases in agonist radioligand binding to R_i adenosine receptors both in the absence (Goodman et al., 1982) and presence (S. -M. H. Yeung and R. D. Green, unpublished observations) of monovalent cations. Gpp(NH)p, 100 μM, produces a near maximal, if not maximal, effect and was used in all of the studies reported herein.) Fifty and 100 mM concentrations of the monovalent cations had negligible effects on the binding in the absence of Gpp(NH)p (Fig. 1, *solid lines*) but significantly decreased binding when Gpp(NH)p was present (Fig. 1, *dashed lines*). Four hundred millimolar concentrations of NaCl and NH_4Cl decreased the binding both in the absence and the presence of Gpp(NH)p. While 400 mM KCl marginally affected binding in the absence of Gpp(NH)p, it greatly depressed binding when Gpp(NH)p was present. NH_4Cl appeared to be more potent than NaCl and KCl both in the absence and presence of Gpp(NH)p.

Figure 2 summarizes an experiment in which the binding of

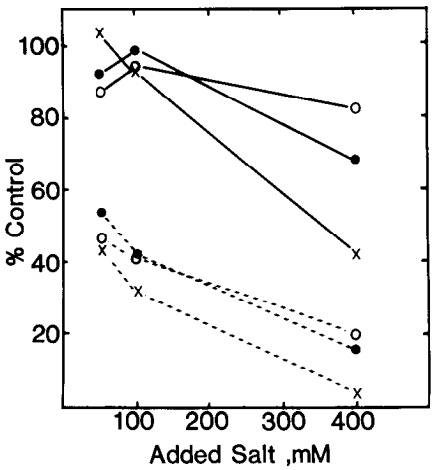

Figure 1. Effects of NaCl (●), KCl (○), and NH_4Cl (*X*) on [^{3}H]-L-PIA binding to hippocampal membranes in the absence (—) and presence (- - -) of Gpp(NH)p (100 μM). The *points* shown are the means of triplicate determinations. Data are expressed as percentages of the ±Gpp(NH)p control values. Membranes were incubated with 2 nM [^{3}H]-L-PIA (−Gpp(NH)p) or 10 nM [^{3}H]-L-PIA (+Gpp(NH)p). The control values were 340 and 181 fmol/mg of protein, respectively.

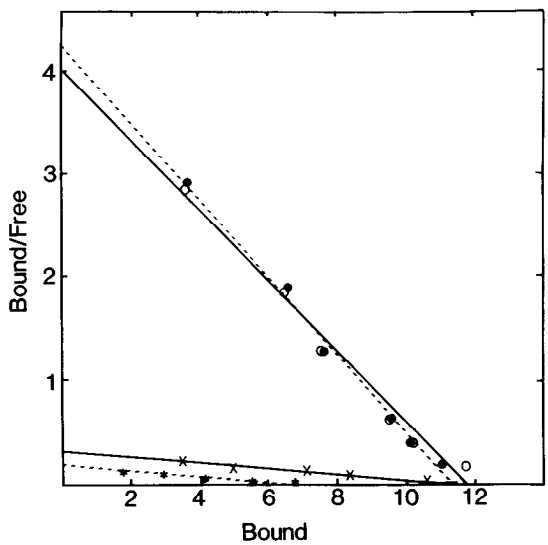

Figure 2. Scatchard plots of [^{3}H]-L-PIA binding determined simultaneously under four different conditions: (*1*) control (○—○), (*2*) 100 mM NaCl (●- - -●), (*3*) 100 μM Gpp(NH)p (X—X), and (*4*) 100 mM NaCl + 100 μM Gpp(NH)p (*- - -*). Samples were assayed in duplicate. *Ordinate, Bound/Free* (picomoles per sample·M) × 10^{-7}. *Abscissa, Bound* picomoles per sample × 10^2. The parameter estimates were as follows: K_D, nanomolar; B_{max} (femtomoles per milligram of protein): control, 4.2 (783); NaCl, 2.6 (752); Gpp(NH)p, 34.9 (752); and NaCl + Gpp(NH)p, 48.4 (519).

varying concentrations [^{3}H]-L-PIA was determined under four conditions: (*1*) control, (*2*) 100 mM NaCl, (*3*) 100 μM Gpp(NH)p, and (*4*) 100 mM NaCl + 100 μM Gpp(N)p. The results of this and two other similar experiments are summarized in Table I. It is important to note that the effect of Gpp(NH)p to increase the K_D of L-PIA was the same in the absence and presence of NaCl, while the effect to decrease the B_{max} was markedly increased, if not dependent on, the presence of NaCl. Under the conditions of these experiments NaCl (100 mM) alone did not have a significant effect on the binding of [^{3}H]-L-PIA. Scatchard analyses of [^{3}H]-L-PIA binding in the presence of higher concentrations of NaCl and NH_4Cl but in the absence of Gpp(NH)p were also performed (Fig. 3). Under

these conditions the monovalent cations (200 to 800 mM) caused dose-dependent increases in the K_D of L-PIA and decreases in the numbers of sites to which the agonist radioligand bound. NH_4Cl was more potent than NaCl (see the legend to Fig. 3 for parameter estimates).

The effects of NaCl and NH_4Cl on the binding of the antagonist [^{3}H]DPX to hippocampal membranes were determined in the absence (Fig. 4, *solid lines*) and presence (Fig. 4, *dotted lines*) of 100 μM Gpp(NH)p. Gpp(NH)p alone increased the binding of [^{3}H]DPX. NaCl, at 50 and 100 mM, did not affect the [^{3}H]DPX binding in the absence or presence of Gpp(NH)p; however, these same concentrations of NH_4Cl increased the binding of [^{3}H]DPX both in the absence and presence of Gpp(NH)p. Four hundred millimolar concentrations of both salts increased the binding of [^{3}H]DPX in both the absence and the presence of Gpp(NH)p. The effects of 400 mM NH_4Cl, 100 μM Gpp(NH)p, and the combination of NH_4Cl and Gpp(NH)p on Scatchard plots of [^{3}H]DPX binding were determined (Fig. 5*A*). The results from four similar experiments are summarized in Table II. Four hundred millimolar NH_4Cl decreased the K_D of [^{3}H]DPX both in the absence and the presence of Gpp(NH)p. NH_4Cl did not appear to affect the B_{max} determined under either of these conditions. On the other hand, Gpp(NH)p appeared to increase the B_{max} both in the absence and the presence of NH_4Cl. We had previously reported that [^{3}H]DPX binding to hippocampal membranes was increased by *N*-ethylmaleimide (NEM) pretreatment and that Gpp(NH)p was without effect on [^{3}H]DPX binding to NEM-pretreated preparations. In the present experiments we also determined the effect of NH_4Cl on [^{3}H]DPX binding to NEM-pretreated hippocampal membranes (Fig. 5*B*, Table II). The effects of NH_4Cl on [^{3}H]DPX binding were qualitatively similar in control and NEM-pretreated preparations, i.e., NH_4Cl decreased the K_D without affecting the B_{max} in both cases.

TABLE I

Effect of NaCl (100 mM) and Gpp(NH)p (100 μM) on the binding of [^{3}H]PIA to hippocampal membranes[a]

Group	K_D	B_{max}
	nM	*fmol/mg of protein*
Control	3.0 ± 0.6	787 ± 11
NaCl	2.3 ± 0.2	762 ± 18
Gpp(NH)p	33.7 ± 0.8	680 ± 45
NaCl, Gpp(NH)p	42.6 ± 5.1	432 ± 55

[a] All values are means ± SE; $N = 3$.

Discussion

This report shows that both guanine nucleotides and monovalent cations reciprocally regulate the affinities of agonists and antagonists for R_i receptors in rat hippocampal membranes. Results are presented that show that Gpp(NH)p increases the K_D of L-PIA in the absence and the presence of 100 mM NaCl. However, the effect of Gpp(NH)p to decrease the B_{max} of L-PIA is enhanced if not dependent on the presence of

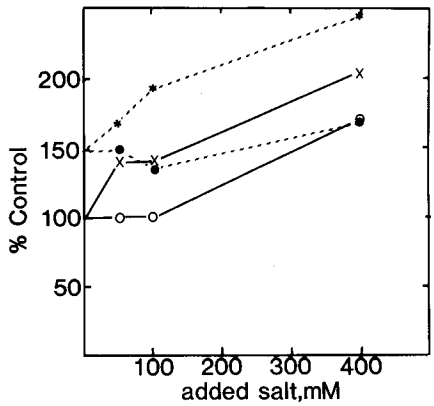

Figure 4. Effects of NaCl (○, ●) and NH_4Cl (*X*, *) on [^{3}H]DPX binding to hippocampal membranes in the absence (—) and presence (- - -) of 100 μM Gpp(NH)p. *Points* shown are averages of triplicate determinations. [^{3}H]DPX, 190 fmol/mg of protein, specifically bound when hippocampal membranes were incubated with 50 nM [^{3}H]DPX in the absence of Gpp(NH)p or added salt. All data are expressed as percentages of this value.

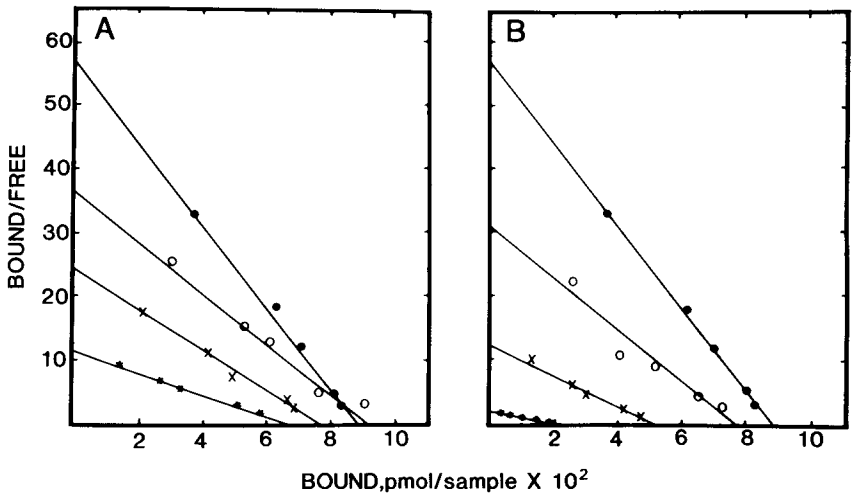

Figure 3. Scatchard plots of [^{3}H]-L-PIA binding to hippocampal membranes in the presence of different concentrations of NaCl (*A*) and NH_4Cl (*B*). The same control curve is shown in both panels as the entire experiment was performed in duplicate at the same time. *Ordinate, Bound/Free* (picomoles per sample · M) × 10^{-6}. *Abscissa, Bound*, picomoles per sample × 10^2. Control (●), 200 mM salt (○), 400 mM salt (*X*), and 800 mM salt (*). The parameter estimates were as follows, K_D, nanomolar; B_{max} (femtomoles per milligram of protein): control, 1.5 (827); 200 mM NaCl, 2.6 (871); 400 mM NaCl, 3.1 (720); 800 mM NaCl, 8.6 (644); 200 mM NH_4Cl, 2.4 (708); 400 mM NH_4Cl, 4.2 (496); and 800 mM NH_4Cl, 11.5 (229).

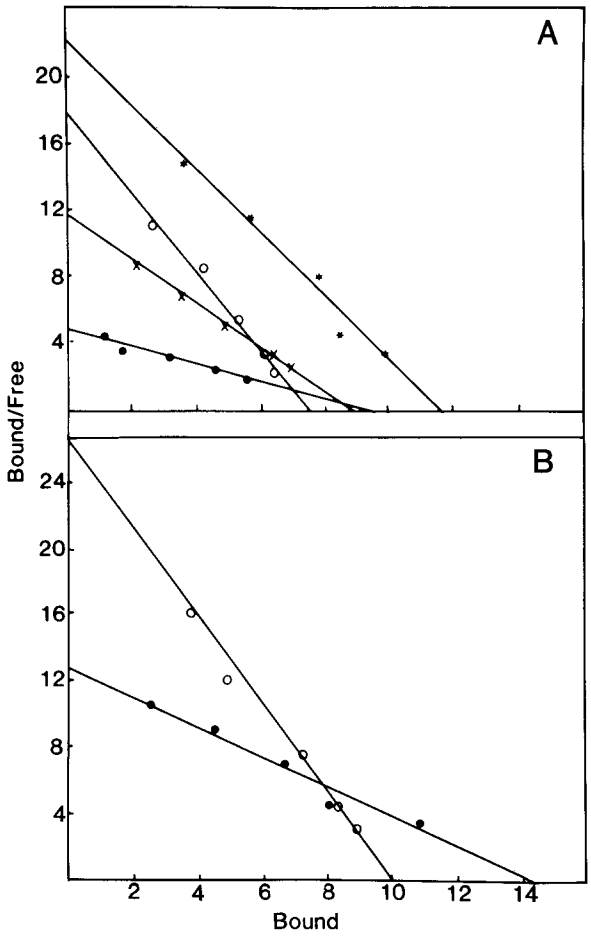

Figure 5. Scatchard plots of [^{3}H]DPX binding to control (*A*) and NEM-pretreated (*B*) hippocampal membranes. NEM-pretreated membranes were prepared as described by Yeung and Green (1983). Assays were performed in triplicate. *Ordinate, Bound/Free,* (femtomoles per sample · M) × 10^{-9}. *Abscissa, Bound,* femtomoles per milligram of protein × 10^{-2}. The parameter estimates were as follows: K_D, nanomolar; B_{max} (femtomoles per milligram of protein): control (●), 196 (923); 400 mM NH_4Cl (○), 40.5 (742); 100 μM Gpp(NH)p (*X*), 60.9 (867); NH_4Cl + Gpp(NH)p (*), 52.6 (1157); NEM pretreated (●), 110 (1405); NEM pretreated + NH_4Cl (○), 37.1 (997).

TABLE II

Effects of NH_4Cl, Gpp(NH)p, and NEM pretreatment on [^{3}H]DPX binding to hippocampal membranes

All groups were studied in each preparation with the exception of the NEM/NH_4Cl group, which was studied in three of the four experiments. Values shown are means ± SEM.

Group	K_D	B_{max}
	nM	*fmol/mg of protein*
Control	185.5 ± 23.7	696 ± 183
NH_4Cl, 0.4M	66.4 ± 23.9	680 ± 37
Gpp(NH)p, 100 μM	132.2 ± 25.3	1030 ± 143
NH_4Cl + Gpp(NH)p	68.1 ± 13.6	1012 ± 64
NEM pretreated	106.6 ± 6.5	1056 ± 139
NEM pretreated + NH_4Cl	38.0 ± 3.5	853 ± 75

monovalent cations. Thus, the difference between the results previously reported from this laboratory (Yeung and Green, 1983) and those reported by Goodman et al. (1982) appears to be due to the fact that in our previous study (Yeung and Green, 1983) incubations contained NaCl in the glycylglycine buffer employed. Therefore, we found that Gpp(NH)p both increased the K_D for agonist binding and decreased the maximal number of sites to which the agonist bound (Yeung and Green, 1983), while Goodman et al. found that guanine nucleotides increased the K_D for agonist binding without affecting the maximal number of sites to which the agonist bound. The present results suggest that [^{3}H]-L-PIA forms high affinity complexes with the R_i receptors in the absence of added guanine nucleotide and that all of these complexes are converted to an intermediate affinity state in the presence of Gpp(NH)p. In the presence of both Gpp(NH)p and 100 mM NaCl some of the binding to this intermediate affinity state remains, but some of the binding is converted to a lower affinity state that is not detected with the concentration range of [^{3}H]-L-PIA studied. Higher concentrations of the monovalent cations themselves increase the K_D and decrease the B_{max} of [^{3}H]-L-PIA. It is not possible to determine from the present experiments if the effects exerted by the lower concentrations of monovalent cations in the presence of Gpp(NH)p and those exerted by higher concentrations of the monovalent cations in the absence of added guanine nucleotide are mediated by the same monovalent cation site. In both cases NH_4Cl is more potent than NaCl. Hosey (1983) previously reported that NH_4Cl is more potent than NaCl in modulating the binding of acetylcholine to cardiac muscarinic receptors. The different potencies of NH_4Cl and NaCl in Hosey's and the present experiments are consistent with the assumption that these effects are due to the monovalent cations and not to the anions or nonspecific ionic effects.

In contrast to the effects of the monovalent cations on agonist binding, the binding of submaximal concentrations of the antagonist [^{3}H]DPX was increased in the presence of NaCl or NH_4Cl. Once more NH_4Cl appeared to be more potent than NaCl. This increased binding was attributable to a decrease in the K_D rather than an increase in the B_{max}. This effect of NH_4Cl was the same in the absence and the presence of Gpp(NH)p and in NEM-pretreated membranes, suggesting that the effect on the monovalent cation does not involve the N_i guanine nucleotide regulatory protein involved in the coupling of inhibitory receptors to adenylate cyclase (Cooper, 1982). It should also be noted that the effect of Gpp(NH)p to increase the B_{max} of [^{3}H]DPX was not affected by the presence of monovalent cation, unlike the situation with agonist binding in which the effect of Gpp(NH)p to decrease the B_{max} was enhanced by the presence of monovalent cation.

The present results are consistent with a model in which three affinity states for R_i receptor:ligand complex exist such that A ⇌ B ⇌ C. Assume that (*1*) state A has high agonist-low antagonist affinity, state B has intermediate agonist and antagonist affinity, and state C has low agonist-high antagonist affinity; (*2*) the agonist ligands [^{3}H]-L-PIA and [^{3}H]CHA measure binding to A and B directly, while the antagonist ligand measures binding to B and C directly; and (*3*) guanine nucleotides modulate the transition between A and B, while monovalent cations modulate the transition between B and C. With regard to [^{3}H]-L-PIA: (*1*) control—detect binding to A + B; (*2*) + GPP(NH)p—all A → B, overall K_D ↑, B_{max} →; (*3*) monovalent cations—no effect, and (*4*) Gpp(NH)p + monovalent cation, A → B and some B → C, K_D ↑, B_{max} ↓. The latter decrease in B_{max} occurs because this low affinity agonist form of the receptor cannot be detected with L-PIA. With regard to [^{3}H]DPX: (*1*) controls—detect binding to B; (*2*) Gpp(NH)p, A → B, K_D →, B_{max} ↑; (*3*) + monovalent cation, B → C, K_D ↓, B_{max} →; and (*4*) Gpp(NH)p + monovalent cation, A → B → C, K_D ↓, B_{max} ↑. According to this model the R_i adenosine receptor exists in agonist-preferring and antagonist-preferring states, which suggests that alkylxanthines, such as DPX, inhibit the effects of R_i receptor agonists by an allosteric rather than by a competitive mechanism. The possible existence of agonist-preferring and antagonist-preferring states of receptors is im-

plicit in the general discussion of cooperative models of drug action presented by Colquhoun (1973) and has been proposed for other specific receptor types, including opiate receptors (Pert and Snyder, 1974; Pasternak and Snyder, 1975) and dopamine receptors (Creese et al., 1965). The relationships between the three affinity states for agonist binding to R_i receptors and the inhibition of adenylate cyclase activity are not yet clear. The finding that the inhibition of adenylate cyclase activity via R_i receptors is dependent on the presence of guanine nucleotide (Londos et al., 1978; Cooper et al., 1980) and is amplified by monovalent cations (Cooper et al., 1980; Aktories et al., 1981) would suggest that state B or C must be formed to inhibit adenylate cyclase activity.

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