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Agonist-Specific Effects of Guanine Nucleotides on Alpha-Adrenergic Receptors in Human Platelets

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SUMMARY

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The effect of GTP on the binding affinity of alpha-adrenergic receptors for alpha-adrenergic agents was studied in human platelet lysates using direct ligand binding methods with [^{3}H]dihydroergocryptine. GTP at a concentration of 0.1 mM markedly decreased the binding affinity of the agonist (−)epinephrine for the receptors (more than 10-fold) but had no effect on the binding of antagonists. The half maximal effect of GTP on epinephrine binding occurred at a concentration of 4 μM. Gpp(NH)p was as effective as GTP at the same concentration, whereas GDP was only 80% as effective. Other nucleotides such as ATP and ITP were less effective. The extent of the GTP-induced reduction in the affinity of alpha-adrenergic agents for the receptors was directly related to the intrinsic activity of these agents for inhibition of PGE_1-stimulated adenylate cyclase. The effect of GTP appears to depend on the concurrent presence of Mg^{++}. In the absence of Mg^{++}, GTP caused only a slight decrease in the agonist binding affinity. When Mg^{++} was present without GTP, the binding affinity of the agonist (−)epinephrine was increased by 5-fold. GTP in the presence of Mg^{++} induces a state of diminished affinity of the receptor for the agonist which is lower than that induced by the nucleotide in the absence of $MgCl_2$. The relationship between GTP and $MgCl_2$ in the regulation of platelet alpha-adrenergic receptors which are inhibitory to adenylate cyclase activity appears to be analogous to their role in regulating beta-adrenergic receptors which are stimulatory for the enzyme in other tissues.

INTRODUCTION

The guanine nucleotide GTP has multiple regulatory effects on the hormone-sensitive adenylate cyclase system. GTP has been shown to be required for hormonal stimulation of adenylate cyclase in many tissues. This effect of GTP has been well documented for the stimulation of adenylate cyclase by glucagon in liver (1), by PGE_1 in S49 lymphoma cells (2), and by beta-adrenergic catecholamines in frog erythrocytes (3), among many others. In addition guanine nucleotides have been shown to regulate the functions of adenylate cyclase coupled receptors by decreasing their binding affinity for hormones or agonist drugs. This effect was first observed in liver for the glucagon receptor (4, 5), and was later demonstrated in cultured glioma cells (6) and frog erythrocytes (7) for the beta-ad-

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renergic receptors. In these latter two systems it was demonstrated, using direct ligand binding techniques, that nucleotide-induced changes in drug or hormone binding occurred only for agonists, but not antagonists.

Quite recently it was demonstrated that GTP reduced the affinity of agonist ([^{3}H]-epinephrine) binding to alpha-receptor sites in brain membranes while having no effect on binding of antagonists such as [^{3}H]WB4101 and [^{3}H]DHE[1] (8). The ability of agonists to displace the antagonists, [^{3}H]WB4101 and [^{3}H]DHE, was also unaffected by the nucleotide.

The alpha-adrenergic receptors of human platelets, which can be studied by direct ligand binding with [^{3}H]DHE (9–11), have an inhibitory effect on PGE_1 stimulated and basal adenylate cyclase (9, 11–13). This inhibitory effect is opposite in direction to the effects of most hormone receptors coupled to the enzyme which serve to increase adenylate cyclase activity. Accordingly the present studies were undertaken in an attempt to determine whether regulation of this "inhibitory" adenylate cyclase-coupled receptor by guanine nucleotide occurred, and to what extent the properties of this regulation might be analogous to those observed with stimulatory receptors, such as the beta-adrenergic receptors.

MATERIALS AND METHODS

Materials. [^{3}H]DHE with a specific activity of 39 Ci/mmole was obtained from New England Nuclear. The sources of other materials used in this study have been described previously (7).

Preparation of human platelet lysates. Platelet lysates were prepared as described previously (9, 14) with some modifications. After the platelets were washed twice with buffer I solution (Tris HCl, 50 mM, NaCl, 0.15 M, EDTA, 0.02 M, pH 7.5), they were resuspended in "lysing buffer" (Tris HCl, 5 mM, EDTA, 5 mM, pH 7.5) and frozen under liquid nitrogen. The frozen platelets were then thawed at room temperature and homogenized for 30 strokes with a motor driven teflon-tipped pestle. For the binding assay, the lysates were diluted to 10 ml with "lysing buffer" and centrifuged at 39,000 × *g* for 10 min at 4°. The resulting pellet was further washed five times by resuspension in "lysing buffer" and centrifugation. The final pellet was resuspended in either buffer containing Tris-HCl, 40 mM, $MgCl_2$, 20 mM (in excess of 1 mM EDTA) or "lysing buffer" as indicated. For the adenylate cyclase assay, the lysates obtained after thawing and homogenizing were diluted to 2 ml with "lysing buffer" and centrifuged at 39,000 × *g* for 10 min at 4°. The resulting pellet was resuspended in Tris-HCl, 75 mM, $MgCl_2$, 25 mM, and DTT, 5 mM.

Receptor binding assay. Alpha-adrenergic receptor binding was assayed in 200 μl of incubation buffer containing either 40 mM Tris-HCl, 10 mM $MgCl_2$ (in excess of 0.5 mM EDTA), pH 7.5, or 40 mM Tris-HCl, 8.5 mM $MgCl_2$ (in excess of 2.5 mM EDTA), pH 7.5. The concentration of [^{3}H]DHE in the assay was 5.0 nM. The binding reaction was initiated by the addition of lysates to the incubation medium and continued for 20 min at 25°. In order to determine the nonspecific binding, phentolamine (10 μM) was included in the incubation medium. The specific binding was defined as the difference between total and nonspecific and was generally ~70% of total binding. The incubation was terminated by adding 2 ml of Tris-HCl buffer (40 mM, pH 7.5). Separation of bound and free radioligands was accomplished by filtration as previously described (9, 14). The filter paper was further washed with 20 ml of Tris-HCl buffer. The characteristics of [^{3}H]DHE binding to human platelet alpha-adrenergic receptors have been previously documented (9–11).

Adenylate cyclase assay. Adenylate cyclase activity was assayed as previously described (14) except for the concentration of ATP being 0.1 mM and the incubation being carried out at 25° for 20 min. Protein was determined by the method of Lowry *et al.* (15).

RESULTS

Effects of GTP on the binding of alpha-adrenergic agents to the receptors. Previous studies from this laboratory and oth-

[1] The abbreviation used is: DHE, dihydroergocryptine.

ers (9–11) have shown that [^{3}H]DHE, a potent alpha-adrenergic antagonist, binds to human platelet binding sites with the characteristics expected of the physiological alpha-adrenergic receptors which induce platelet aggregation and inhibit platelet adenylate cyclase. The effect of the guanine nucleotide GTP on the binding of [^{3}H]DHE to alpha-adrenergic receptors was studied in thoroughly washed platelet lysates. After five washings to reduce endogenous GTP, the addition of exogenous GTP at 0.1 mM altered neither the total binding nor the non-specific binding of [^{3}H]DHE. To determine whether GTP might affect the binding affinity of alpha-adrenergic agents for the receptors, the displacement curves of various alpha-adrenergic agents for [^{3}H]DHE binding were determined in the presence and absence of GTP (0.1 mM). As shown in Fig. 1A GTP markedly decreased the ability of (−)epinephrine to inhibit [^{3}H]DHE binding. The displacement curve for (−)epinephrine, a full agonist, was shifted 12-fold to the right (a "1-fold" shift means no shift occurred). GTP was less effective in changing the binding affinity of clonidine which is a partial agonist in this system, producing a 4-fold shift (Fig. 1B). The displacement curve for DHE, an antagonist, was not altered at all (Fig. 1C).

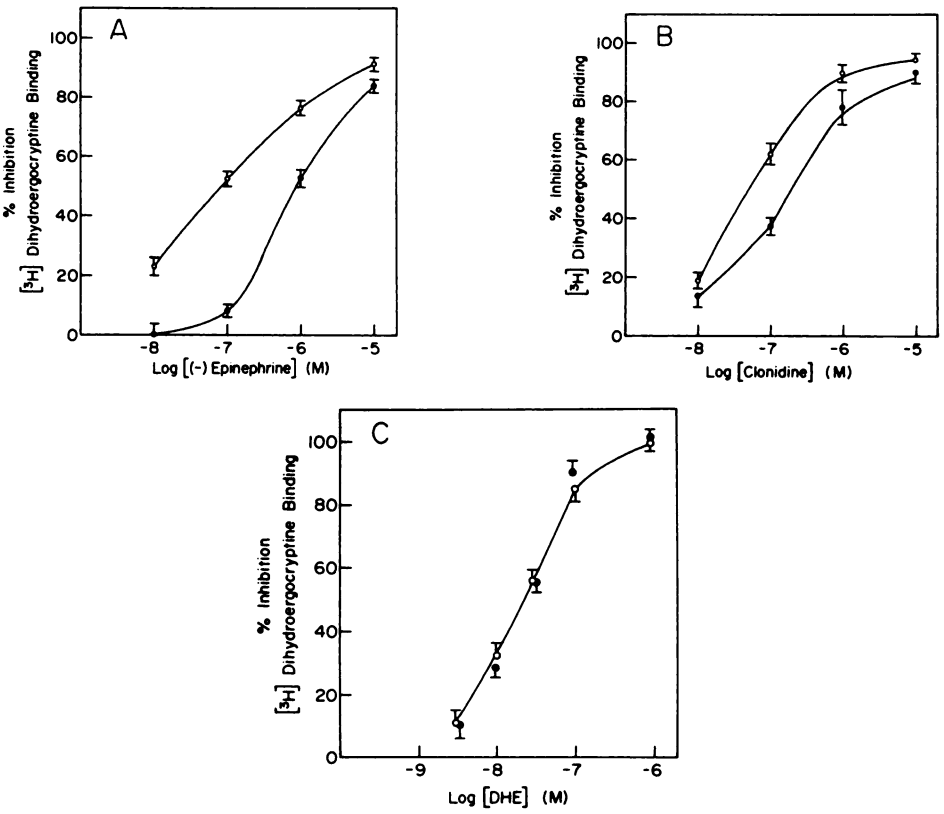

FIG. 1. *Effect of GTP on competition of (A) (−)epinephrine, (B) clonidine and (C) DHE for [^{3}H]DHE binding sites in human platelet lysates*

Lysates were prepared as described in METHODS and incubated in the presence (●——●) and absence (○——○) of GTP (0.1 mM). Binding assays were performed in buffer containing 10 mM $MgCl_2$ (in excess of 0.5 mM EDTA) and 40 mM Tris-HCl, pH 7.5 as described in METHODS. Shifts in the binding displacement curves were determined by comparing the 50% displacement concentrations of (−)epinephrine (EC_{50}) in the absence and presence of GTP. The shift was then determined as the ratio of EC_{50} in the presence of GTP to EC_{50} in the absence of GTP. A "1-fold" shift means that no shift occurred. The values shown are means and standard errors of three to twelve experiments.

The effects of GTP on the (−)epinephrine displacement curve for [^{3}H]DHE binding were twofold. Not only was the curve shifted to the right but its slope became steeper (Fig. 1A). It can be noted that in the absence of added nucleotide the displacement curve for the full agonist (−)epinephrine is considerably more shallow than that for the antagonist DHE (Fig. 1C), and the shape of the displacement curve for the partial agonist indicates an intermediate slope. GTP appears not to change the slope of the clonidine displacement curve. In the presence of maximally effective concentrations of GTP, the slopes of all of the curves are now parallel. The effect of GTP on the binding affinity of (−)epinephrine was apparent at a concentration of 1 μM (producing a 3-fold shift); the half maximal and maximal effects occurred at approximately 4 μM, and 10 μM, respectively (Fig. 2).

Other nucleotides were tested to see whether they produced effects similar to GTP (Fig. 3). At equal concentrations (0.1 mM), Gpp(NH)p, an analogue of GTP which is resistant to nucleotide phosphohydrolase action, was as effective as GTP. GDP was 80% as effective as GTP. ITP and ATP produced 6.5 and 2.5-fold shifts, respectively, in the displacement curves for (−)epinephrine. Since these nucleotide preparations may contain trace amounts of GTP the possibility exists that their effects may be due to contamination by GTP.

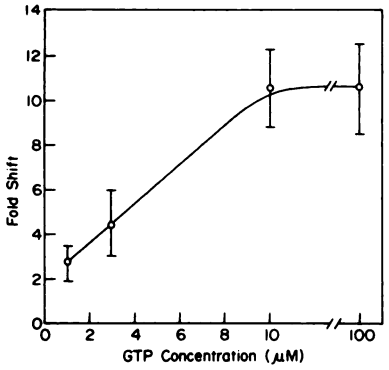

FIG. 2. *Effect of GTP concentration on "fold" shift of the (−)epinephrine curve for displacement of [^{3}H]DHE binding*

The shift in the (−)epinephrine displacement curve of [^{3}H]DHE binding was determined as described in Fig. 1. Results shown are means and standard errors of three experiments.

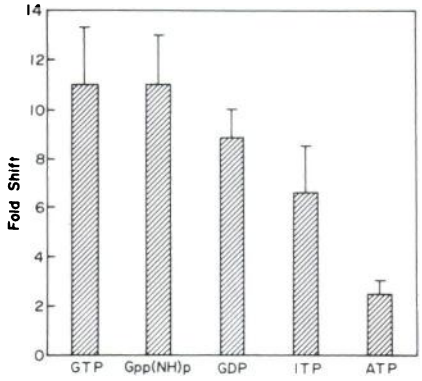

FIG. 3. *Effects of various nucleotides on (−)epinephrine receptor binding displacement curves*

The shift in the (−)epinephrine displacement curve of [^{3}H]DHE binding was determined as described in Fig. 1. The concentration of nucleotides was 0.1 mM. The values shown are means and standard errors of three to five experiments.

The GTP induced change in binding affinity of alpha-adrenergic agents was related to their abilities to inhibit PGE_1-stimulated adenylate cyclase via the alpha-adrenergic receptors. Previous studies have shown that the alpha-adrenergic receptors in human platelets are coupled to adenylate cyclase (9, 11–13). Activation of alpha-adrenergic receptors in platelets is associated with a reduction in PGE_1-stimulated adenylate cyclase. The fact that (−)epinephrine, clonidine, and DHE are a full agonist, partial agonist, and antagonist, respectively, in inhibiting PGE_1-stimulated adenylate cyclase (9, 12, 14) and that GTP displays different effects on the binding affinity of these adrenergic agents suggests that the extent of the binding affinity shift of alpha-adrenergic agents induced by GTP may be in some way related to their abilities to inhibit PGE_1-stimulated adenylate cyclase. The intrinsic activities of various alpha-adrenergic agents for inhibition of PGE_1-stimulated adenylate cyclase were determined and are shown in Table 1. As shown in Table 1, the binding affinities of (−)norepinephrine and (±)cobefrin were markedly decreased by GTP, whereas the displacement curves for oxymetazoline, phenylephrine, and methoxamine were

TABLE 1

Intrinsic activities of alpha-adrenergic agents for inhibition of PGE_1-stimulated adenylate cyclase

GTP (0.1 mM)-induced shifts in (^{3}H)DHE displacement curves, and intrinsic activities of various alpha-adrenergic agents as inhibitors of PGE_1-stimulated adenylate cyclase activity in human platelet lysates. The basal adenylate cyclase activity was 77 ± 5 pmol/mg/min (n = 4). PGE_1 at 10 μM produced a 4–5 fold stimulation. (−)Epinephrine at 1 mM inhibited PGE_1-stimulated adenylate cyclase by 40–50%. The values shown are means and standard errors of the number of experiments indicated in parentheses. The intrinsic activity of alpha-adrenergic agents was determined at a concentration of 1 mM. Dihydroergocryptine and phentolamine showed no intrinsic activity at concentrations up to 1 μM and 50 μM, respectively. A "1-fold" shift indicates that no shift occurred.

Agent	Shift in (^{3}H)DHE displacement curve	Intrinsic activity
(−)Epinephrine	14 ± 2.0 (12)	1.0 (6)
(−)Norepinephrine	12.5 ± 2.5 (2)	0.94 ± 0.02 (6)
(±)Cobefrin	13.5 ± 6.5 (2)	0.84 ± 0.04 (5)
Clonidine	4 ± 0.3 (3)	0.52 ± 0.03 (6)
Oxymetazoline	2.38 ± 0.48 (3)	0.14 ± 0.02 (7)
Phenylephrine	2.16 ± 0.6 (3)	0.37 ± 0.04 (6)
Methoxamine	1.46 ± 0.29 (3)	0.09 ± 0.04 (8)
Phentolamine	1.0 (3)	0 (6)
DHE	1.0 (3)	0 (6)

shifted only slightly (1.4–2-fold), similar to that of clonidine. The shifts in binding affinity of alpha-adrenergic agents caused by GTP correlated very well with their intrinsic activities as inhibitors of PGE_1-stimulated enzyme activity ($r = 0.96$).

Mg^{++} requirement for the GTP effect. The beta-adrenergic receptors in frog erythrocytes are also regulated by guanine nucleotides (7, 16). GTP selectively decreases the binding affinity of agonists without altering antagonist binding. This effect of GTP on the beta-adrenergic receptors in frog erythrocytes is dependent on the presence of Mg^{++} (16). We observed the above effects of GTP on human platelet lysates when Mg^{++} was present in the medium (10 mM in excess of 0.5 mM EDTA). To determine whether Mg^{++} was required, we investigated the effect of GTP on (−)epinephrine binding affinity for the receptors in the absence of exogenous Mg^{++}. Tris-HCl buffer containing 2.5 mM EDTA was used as the incubation buffer to ensure that most of the endogenous free Mg^{++} was chelated. As shown in Fig. 4 in the absence of exogenous Mg^{++}, GTP produced only a slight effect on the (−)epinephrine displacement curve for [^{3}H]DHE (2-fold shift). We found that whether GTP was present or absent, Mg^{++} altered neither the binding affinity nor the specific binding of [^{3}H]DHE at 5

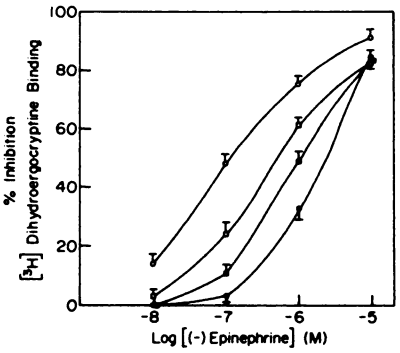

FIG. 4. *Effect of GTP on (−)epinephrine receptor binding displacement curves in the presence and absence of $MgCl_2$*

When the competition of (−)epinephrine for [^{3}H]-DHE binding sites was determined in the absence of $MgCl_2$, the incubation buffer was Tris-HCl, 40 mM, EDTA, 2.5 mM, pH 7.5. Displacement curves for (−)epinephrine in the absence (□——□) and presence (■——■) of GTP (0.1 mM) are shown. When the competition of (−)epinephrine for [^{3}H]DHE binding sites was determined in the presence of $MgCl_2$, the incubation buffer was Tris-HCl, 40 mM, EDTA, 2.5 mM, $MgCl_2$, 8.5 mM (in excess of 2.5 mM EDTA), pH 7.5. The displacement curves for (−)epinephrine in the absence (○——○) and presence (●——●) of GTP (0.1 mM) are shown. The values plotted are means and standard errors of three to seven experiments. It can be noted that in these experiments in the presence of $MgCl_2$, GTP produces a somewhat greater shift in the (−)epinephrine displacement than that shown in Fig. 1. The platelet preparations used in these experiments are distinct from those in Fig. 1.

nM, which is below the saturation concentration. This suggests that the total number of [^{3}H]DHE binding sites was also not changed by Mg^{++}. When $MgCl_2$ (8.5 mM in excess of 2.5 mM EDTA) was added without GTP, the binding affinity of (−)epinephrine was increased 5-fold. GTP (0.1 mM) in the presence of Mg^{++} greatly reduced the binding affinity of (−)epinephrine and produced a state of a lower affinity of the receptors for (−)epinephrine than that induced by GTP in the absence of Mg^{++}. When Mg^{++} was present at a concentration of 1.25 mM (in excess of 2.5 mM EDTA), the maximal effect of GTP on agonist binding affinity occurred. The half-maximal effect of GTP was seen at 0.3 mM free $MgCl_2$ (Fig. 5) which is identical to the concentration of Mg^{++} which half maximally augments the binding of the agonist [^{3}H]hydroxybenzylisoproterenol to the beta-adrenergic receptors and [^{3}H]PGE_1 to the prostaglandin receptors in frog erythrocyte membranes (16).

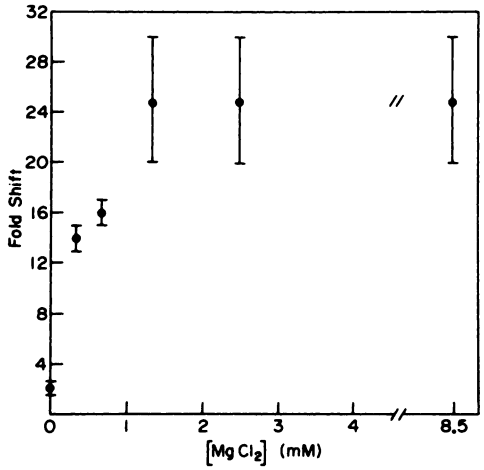

FIG. 5. *Effect of $MgCl_2$ concentrations on the GTP-induced shift in the (−)epinephrine displacement curve of [^{3}H]DHE binding*

Shifts in binding displacement curves were determined in the presence of various concentrations of $MgCl_2$ (in excess of 2.5 mM EDTA) by comparing the 50% displacement concentrations of (−)epinephrine in the presence and absence of 0.1 mM GTP as described in Fig. 2. The values shown are means and standard errors of two to three experiments. These experiments were performed with different platelet lysates than those described in Fig. 4.

DISCUSSION

The data presented here clearly suggest that guanine nucleotides are able to profoundly influence the function of the adenylate cyclase-coupled alpha-adrenergic receptors of human platelets. The effect is very selective in that the GTP-induced shift in binding affinity is greatest for full agonists such as (−)epinephrine, and intermediate for partial agonists such as clonidine, while no shifts were observed for pure antagonists such as phentolamine or DHE. This selective GTP effect in human platelets is similar to the agonist-selective sodium effect on alpha-adrenergic receptors observed previously in rabbit platelets (14).

A recent report by U'Prichard and Snyder described the effect of GTP on alpha-adrenergic receptor binding in calf and rat brain (8). Unlike the findings in human platelets, it was found that GTP had no effect on the binding affinity of agonists at [^{3}H]DHE binding sites. However, it was reported that GTP reduced the binding of the agonists [^{3}H]epinephrine and [^{3}H]norepinephrine by decreasing the binding affinity to alpha-adrenergic receptors without affecting the binding of antagonists. It was suggested that, in the brain, [^{3}H]agonist and [^{3}H]antagonist bind to distinct sites of alpha-adrenergic receptors. Our own data obtained with human platelet lysates are thus not entirely consistent with those reported for brain membranes. In the platelet system, agonist, antagonist and partial agonist ligands all appear to compete for the same sites, but the effects of the guanine nucleotides are agonist specific.

The mechanism by which GTP reduces the binding affinity of agonists for alpha-adrenergic receptors in human platelet is unknown. U'Prichard and Snyder reported that GTP decreased the binding affinity of [^{3}H]agonists for brain alpha-adrenergic receptors mainly by enhancing the dissociation of [^{3}H]agonist from the receptors (8). Similar findings have also been found for beta-adrenergic receptors in frog erythrocytes (17) and glucagon receptors (18) in rat liver. Whether this is also true for platelet alpha-adrenergic receptors remains to be determined. Attempts to perform agonist binding with [^{3}H]catecholamines in

platelet lysates have thus far been frustrated by very low levels of specific binding.

Our studies appear to suggest that the GTP effect on receptor binding is apparent only when the alpha-adrenergic receptors are coupled to some component(s) of adenylate cyclase as a result of interaction with an alpha-adrenergic agonist. The same finding has also been reported and well characterized for beta-adrenergic receptors in frog erythrocytes (7) and S49 lymphoma cell membranes (2, 19).

Jakob *et al.* reported that the inhibition of PGE_1-stimulated adenylate cyclase induced by the stimulation of alpha-adrenergic receptors in human platelets required the presence of GTP (20). In the absence of GTP, the effect of alpha-adrenergic receptors disappeared. It is not known whether the GTP binding site which mediates the reduction in alpha-agonist binding affinity is a distinct site or corresponds to the sites involved in mediating the GTP effect on alpha-adrenergic inhibition of platelet adenylate cyclase.

In brain tissue, GTP can reduce the binding affinity of [^{3}H]agonists for alpha-adrenergic receptors in the absence of Mg^{++} (8). However, in human platelets, much of the GTP effect requires the presence of Mg^{++}. In the absence of Mg^{++}, GTP produces only a slight effect on agonist binding. These findings suggest that the alpha-adrenergic receptors of human platelets which are inhibitory to adenylate cyclase are regulated by guanine nucleotides by mechanisms which are similar to those for stimulatory receptors such as the beta-adrenergic receptors. We have previously proposed a "dynamic receptor affinity" model (3, 16, 21) for the beta-adrenergic receptor system which may thus also be applicable to inhibitory receptors such as the platelet alpha-adrenergic receptors. This model postulates the initial formation of a "low affinity" agonist receptor complex, which is followed by the formation of a "high affinity" coupled state of the receptor which is formed uniquely by agonist in the presence of Mg^{++}. This high affinity intermediate is postulated to represent some form of complex between agonist, receptor, and some component(s) of the adenylate cyclase system. This intermediate is envisaged as being uniquely sensitive to the actions of regulatory guanine nucleotides which in a concerted mechanism: 1) break up the high affinity complex, thus reducing agonist affinity and 2) thereby activate (or in this case inhibit) adenylate cyclase. Binding of antagonist, which does not promote formation of the high affinity intermediate, is thus not sensitive to guanine nucleotides or divalent cations.

The model described here for alpha-adrenergic receptors in human platelets is of course speculative. However, it is consistent with all of the data obtained. The picture of nucleotide and divalent cation regulation of these inhibitory adenylate cyclase-coupled alpha-adrenergic receptors is strikingly analogous to that observed with stimulatory receptors such as the beta-adrenergic receptors. These findings suggest that comparable coupling mechanisms, at least insofar as guanine nucleotides and divalent cations are concerned, may be involved for both stimulatory and inhibitory adenylate cyclase coupled receptors.

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