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Determinants of α_2-Adrenergic Receptor Activation of G Proteins: Evidence for a Precoupled Receptor/G Protein State

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SUMMARY

The ability of agonist-occupied α_{2D}-adrenergic receptors to activate G proteins was measured in membranes from PC-12 cells stably expressing the cloned receptor, using guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding as an endpoint. Epinephrine (EPI) stimulated [^{35}S]GTPγS binding in a Mg^{2+}-dependent manner, showing both micromolar and millimolar cation affinities. Prior treatment of cells with pertussis toxin completely eliminated the EPI stimulation. The presence of GDP decreased basal [^{35}S]GTPγS binding and increased the proportion of EPI-stimulated binding. Increasing concentrations of Na^+ also reduced basal [^{35}S]GTPγS binding but had less effect on EPI-stimulated binding, such that the agonist response was proportionately greater at higher Na^+ levels. In saturation binding studies with [^{35}S]GTPγS only low affinity binding was observed in the presence of 100 mM Na^+, whereas in the absence of Na^+ a high affinity component was also present, indicating a Na^+-regulated receptor/G protein interaction. EPI induced high affinity [^{35}S]GTPγS binding in the presence of Na^+ and increased the affinity of the high affinity component under Na^+-free conditions. The selective α_2-adrenergic antagonist rauwolscine produced rightward shifts of EPI dose-response curves and decreased the basal level of [^{35}S]GTPγS binding across the same range of concentrations. The extent of decrease was dependent upon the α_2-adrenergic receptor expression level, indicating that α_2-adrenergic receptors contribute to basal G protein activation in the absence of agonist. The ability of rauwolscine to decrease basal [^{35}S]GTPγS binding was diminished as the level of Na^+ was increased, suggesting that both agents act to reduce receptor/G protein interaction, by distinctive mechanisms. α_2-Adrenergic receptor antagonists reduced basal G protein activation with a rank order for maximal effectiveness that was different from their receptor binding affinities. These results support the existence of precoupling between α_{2D}-adrenergic receptors and G proteins; coupling can be diminished by both Na^+ and antagonists, whereas agonists increase the efficiency of receptor/G protein coupling.

α_2R regulate a variety of physiological activities via their ability to activate G proteins. In almost all cases receptor responses are blocked by pertussis toxin, indicating the involvement of G_i or G_o (1). Examples of α_2R-mediated responses include the contraction of vascular smooth muscle (2), inhibition of the release of insulin (3), inhibition of lipolysis (4), and inhibition of neurotransmitter release (5). Although in many cases the effector systems mediating these receptor responses remain obscure (6, 7), G protein activation is clearly the proximal event elicited by α_2R agonists.

As members of the large group of G_i/G_o-coupled receptors, α_2R share certain commonalities in addition to pertussis toxin sensitivity, including Na^+-dependent agonist and antagonist binding and general receptor structural features. Thus, in the case of α_2R (8), dopamine D_2 receptors (9), and δ-opiate receptors (10) Na^+ causes a rightward shift of agonist displacement curves, reflecting reduced receptor affinity. Conversely, Na^+ causes an increase in the affinity of antagonists for these receptors (11–13). Initially this dual effect of Na^+ was interpreted as indicating its promotion of the antagonist-preferring conformation of these receptors (14), whereas more recent studies have proposed that Na^+ effects reflect its modulation of receptor affinity for G protein (11, 13).

The postulated third cytoplasmic loop is characteristically larger for G_i/G_o-coupled receptors, compared with receptors coupling to G_s or other pertussis toxin-insensitive G proteins (15, 16). At the same time, the carboxyl-tail segment attached to the seventh transmembrane domain is shorter in G_i/G_o-coupled receptors. These shared structural features suggest that this group of receptors may also share some binding or functional features.

Previous studies of α_2R binding behavior suggest precoupling of receptor to G protein in the absence of agonist. Neubig *et al.* (17) carried out a kinetic analysis of [^{3}H]yohimbine binding to

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ABBREVIATIONS: α_2R, α_2-adrenergic receptor(s); EPI, epinephrine; RAU, rauwolscine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate.

platelet α_2R and concluded that approximately 30% of receptors existed in a precoupled state. Jagadeesh *et al.* (11) examined the influence of Na^+ and GTP on [^{3}H]RAU binding in arterial membranes and its displacement by EPI and proposed that their modulatory effects resulted from distinct actions that reduced receptor/G protein affinity and precoupling. The existence of precoupling is readily accommodated by the ternary model of receptor/G protein coupling as initially proposed by DeLean *et al.* (18) and subsequently applied to describe the binding of antagonists and agonists to D_2 receptors in pituitary cell membranes (19). Recently Costa *et al.* (20) have expanded the predictions of the ternary model with specific inclusion of the modulatory influence of Na^+, using opiate receptor binding to verify its applicability.

In the current studies we have examined the ability of α_{2D}R (RG20) to activate G proteins in PC-12 cell membranes both in the absence and in the presence of agonist.[2] Based upon the inhibitory effects of Na^+ and antagonists on basal rates of [^{35}S]GTPγS binding, our results indicate the presence of functional precoupling of α_{2D}R to G proteins.

Experimental Procedures

Materials. [^{35}S]GTPγS (1255 Ci/mmol) and [^{3}H]RAU (78 Ci/nmol) were purchased from DuPont-New England Nuclear (Boston, MA). Tissue culture supplies were purchased from JRH Bioscience (Lenexa, KS). RAU was obtained from Atomergic Chemicals (Farmingdale, NY), phentolamine from Ciba-Geigy, and WB 4101 from Research Biochemicals Inc. (Natick, MA); other drugs and reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell culture. PC-12 cells stably expressing a cloned rat α_{2D}R (21) and RIN5AH cells (kindly provided by Dr. Ake Lernmark, University of Washington, Seattle) were grown as monolayers at 37° (95% O_2/5% CO_2) in Dulbecco's modified Eagle's medium with high glucose (4.5 g/liter), supplemented with 10% fetal calf serum, 5% horse serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone. Cells were routinely assayed for maximal [^{3}H]RAU binding. PC-12 cells transfected with α_{2D}R expressed a range of receptor densities. Individual clonal cell lines expressing 0.37–3.6 pmol/mg of protein were used in this study.

Membrane preparations. Cells were washed twice with phosphate-buffered saline (137 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), harvested with a rubber policeman, and pelleted at 4° at 200 × *g*. The pellet was resuspended in 5 ml/dish of lysis buffer (5 mM Tris·HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride) at 4° and was homogenized with a Dounce homogenizer. The cell lysate was then centrifuged at 34,000 × *g* for 15 min and the pellet was resuspended in membrane buffer (50 mM Tris·HCl, pH 7.5, 0.6 mM EDTA, 5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride) on ice. In some studies Mg^{2+} was deleted, to assess its role. Aliquots were rapidly frozen in liquid nitrogen and stored at −80° until used.

[^{35}S]GTPγS binding . [^{35}S]GTPγS binding was measured with a modification of the assay used by Hilf and Jakobs (22). Cell membranes were thawed and diluted with 10 mM Tris·HCl buffer. Membrane protein (4–8 μg) was mixed with reaction mixture (50 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, unless otherwise specified, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 2 μM GDP, 1 μM propanolol, and 2–3 nM [^{35}S]GTPγS, unless otherwise specified), in a total volume of 100 μl. The incubation was started by addition of reaction mixture to the membrane suspension and was carried out in at least triplicate for 10 min at 25°. The reaction was terminated by rapid filtration through GF/C filters under vacuum. The filters were washed four times (with 4 ml of 50 mM Tris·HCl, pH 7.5, containing 5 mM $MgCl_2$ and 100 mM NaCl) and then counted in scintillation cocktail. Nonspecific binding was determined in the presence of 10 μM GTPγS and subtracted from total bound radioactivity. The concentration of free Mg^{2+} in the reaction mixture was calculated using a K_d of EDTA for Mg^{2+} at pH 7.5 of 1 μM (23).

Results

Binding of [^{35}S]GTPγS to PC-12 cell membranes containing α_{2D}R was stimulated by EPI in a concentration-dependent manner (Fig. 1), whereas EPI was without effect in control PC-12 cells that were not transfected with α_{2D}R (see Fig. 8). RAU, a selective α_2R antagonist, caused a rightward shift of the EPI dose-response curve with a K_B value of 39.3 ± 9.6 nM, confirming its α_2R origin. In addition, RAU also lowered the basal level of [^{35}S]GTPγS binding (Fig. 1). The actions of both EPI and RAU were modulated by monovalent and divalent cation levels, as well as by the concentrations of both GDP and GTPγS. Experiments were carried out to characterize the potential role of each component in regulating signal transduction under physiological conditions.

Increasing the concentration of Mg^{2+} from 0.1 μM to 10 μM caused a 5-fold increase in basal levels of [^{35}S]GTPγS binding, whereas binding in the presence of EPI was increased 11-fold over the same range (Fig. 2, *left*). Moreover, supramicromolar levels of Mg^{2+} were an absolute requirement for demonstrating EPI stimulation of binding. Further increase of Mg^{2+} up to 30 mM had little or no influence on basal [^{35}S]GTPγS binding but markedly increased EPI-stimulated binding, reaching a peak between 3 and 10 mM, at which point agonist-stimulated binding was >4 times the level of basal binding. Based upon this result, 5 mM Mg^{2+} was used in subsequent studies unless otherwise indicated. A comparison with other divalent cations at a concentration of 5 mM indicated that, whereas each increased basal binding, only Mg^{2+} supported the agonist-dependent binding of [^{35}S]GTPγS (Fig. 2, *right*).

Previous studies have led to the conclusion that agonists activate G proteins by modulating their affinity for GDP and/

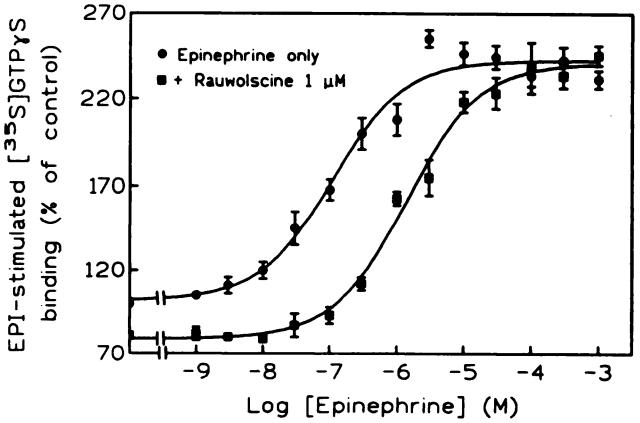

Fig. 1. RAU inhibition of both basal and EPI-stimulated binding of [^{35}S]GTPγS. Binding of [^{35}S]GTPγS to PC-12/α_{2D} membranes was determined in the absence and presence of 1 μM RAU, with increasing concentrations of EPI, for 10 min at 25°. Data were calculated as means ± standard errors from at least three independent experiments.

[2] The human C-10 and rat RG20 genes encode receptors that are 89% homologous at the amino acid level but differ in their ligand recognition properties, primarily in their affinity for the receptor antagonists RAU and yohimbine. To indicate the differences in ligand recognition and to encompass the identification of such a site, the RG20 gene product is referred to as the α_{2D}R. However, the C-10 and RG20 gene products likely represent species homologues and appear to be functionally identical.

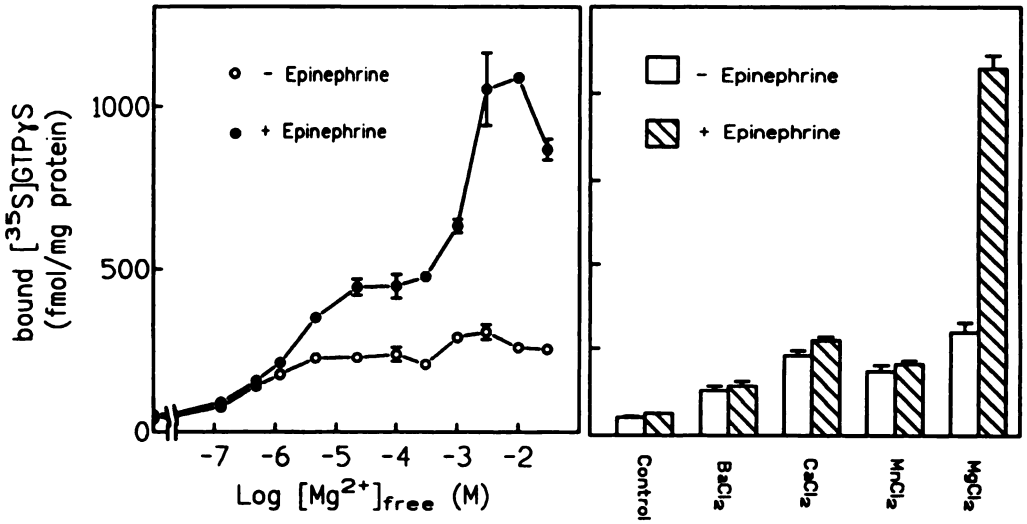

Fig. 2. Mg^{2+} enhancement of both basal and EPI-stimulated [^{35}S]GTPγS binding to PC-12/α_{2D} membranes. *Left*, binding was determined in the absence or presence of 100 μM EPI, with increasing concentrations of free Mg^{2+}. In the absence of Mg^{2+}, 1 mM EDTA was added. The concentration of free Mg^{2+} was calculated as described (23). *Right*, the specificity of Mg^{2+} in enhancing EPI-stimulated [^{35}S]GTPγS binding was determined with 5 mM concentrations of the indicated chloride salts of four divalent cations. The data shown are from triplicate determinations in a representative experiment. The experiment was repeated three times with similar results. The values are means ± standard errors of triplicate determinations.

or GTP, such that GDP binding is disfavored and GTP binding is favored (24). When the concentration of GDP was increased over the range of 0.03 μM to 30 mM, basal binding of [^{35}S] GTPγS was suppressed, with little influence on the absolute ability of EPI to increase binding (Fig. 3). This resulted in an increase in the percent stimulation caused by EPI, from 31% in the absence of GDP to 183% with 30 μM GDP. The concentration of GDP was set at 2 μM for standard assay conditions unless otherwise noted.

To assess the involvement of G_i or G_o in the action of EPI, groups of cells were treated for 18 hr with pertussis toxin (100 ng/ml) before membrane preparation. The time course of [^{35}S] GTPγS binding was then compared with that for membranes from untreated cells, both in the presence and in the absence of EPI. In normal membranes EPI produced an increase in the initial rate of [^{35}S]GTPγS binding, whereas in pertussis toxin-treated membranes this action of EPI was completely blocked (data not shown). Thus, the EPI-induced [^{35}S]GTPγS binding is due to, most likely, α_{2D}R activation of G_i or G_o or, less likely, some other pertussis toxin-sensitive G protein.

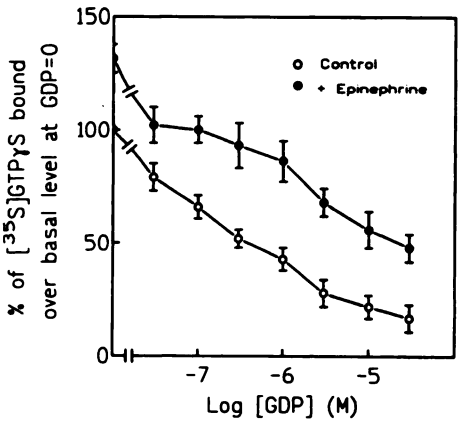

Fig. 3. Effect of GDP on [^{35}S]GTPγS binding. Binding of [^{35}S]GTPγS to PC-12/α_{2D} membranes was determined in the absence or presence of 100 μM EPI, with increasing concentrations of GDP. The data shown are means ± standard errors of six experiments, each performed in triplicate.

Na^+ levels are an important modulator of both α_2R binding (8, 11) and functional response (25, 26), and these effects may relate to the influence of Na^+ on R/G interaction (11). In α_{2D}R transfectants, Na^+ (20–200 mM) progressively decreased basal [^{35}S]GTPγS binding (Fig. 4, *left*). The major decrease occurred at concentrations up to 60 mM, with a lower sensitivity being evident at higher levels. [^{35}S]GTPγS binding in the presence of EPI was less affected by the level of Na^+, especially at lower concentrations (i.e., up to 60 mM), such that the percent increase caused by EPI increased from 62% at 0 mM to 176% at 60 mM Na^+. The level of Na^+ was set at 100 mM in our standard assay conditions unless otherwise noted. When tested at 100 mM, a number of other monovalent cations were also able to both suppress basal [^{35}S]GTPγS binding and allow higher EPI stimulation (Fig. 4, *middle*). However, at lower concentrations Na^+ was more effective than either K^+ or *N*-methylglucamine in suppressing basal binding (Fig. 4, *right*).

To characterize the influence of Na^+ on EPI potency and efficacy, dose-response curves were obtained at 0, 20, and 100 mM concentrations of Na^+. As shown in Fig. 5, Na^+ progressively decreased the basal level of [^{35}S]GTPγS binding, whereas the absolute amount of increase caused by EPI was largely unaffected. In effect, this increased the relative importance of the agonist-induced increase above the lower basal level. The potency of EPI was decreased by approximately 4.5-fold in the presence of 100 mM Na^+, suggesting a relationship between its effects on ligand affinity and its modulation of G protein activation.

To further characterize the basis for the influence of EPI and Na^+ on [^{35}S]GTPγS binding, saturation studies were carried out in which membranes were incubated with various concentrations of the nucleotide for 10 min in the presence or absence of 100 mM Na^+, 100 μM EPI, or their combination. As shown in the Scatchard plots of Fig. 6 and in Table 1, [^{35}S] GTPγS binding in the absence of Na^+ exhibited nonlinear behavior, indicating the presence of high and low affinity binding components (K_d values of 1.8 and 84 nM) or some other form of complex binding. In the presence of Na^+ only a single,

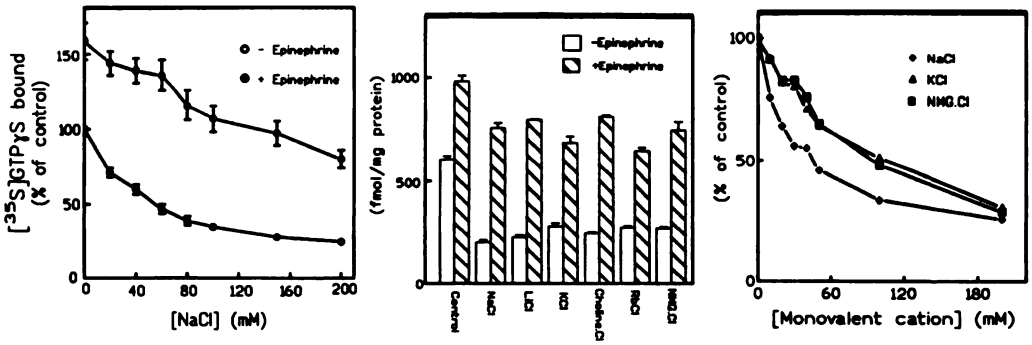

Fig. 4. Effect of monovalent cations on [^{35}S]GTPγS binding. PC-12/α_{2D} membranes were incubated for 10 min at 25° in the presence of NaCl at the indicated concentrations, in the absence or presence of 100 μM EPI (*left*) or in the presence of 100 mM concentrations of the indicated chloride salts (*middle*). *Right*, differential suppression of basal [^{35}S]GTPγS binding by NaCl, KCl, or *N*-methylglucamine (*NMG*) chloride. The data shown are means ± standard errors of three or four experiments, each performed in triplicate.

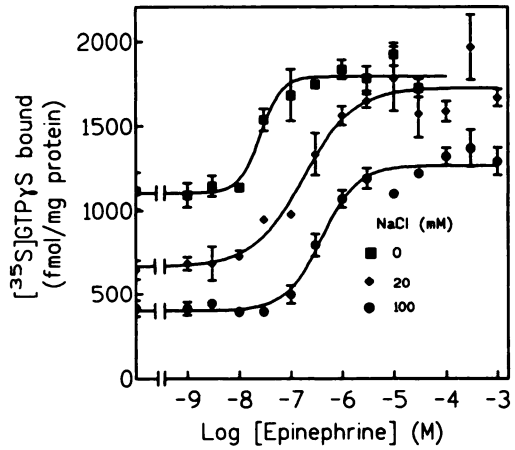

Fig. 5. Effect of Na^+ on EPI-stimulated [^{35}S]GTPγS binding. PC-12/α_{2D} membranes were incubated with 0, 20, or 100 mM NaCl and increasing concentrations of EPI. The data shown are triplicate determinations from a representative experiment. The experiment was repeated three times with similar results.

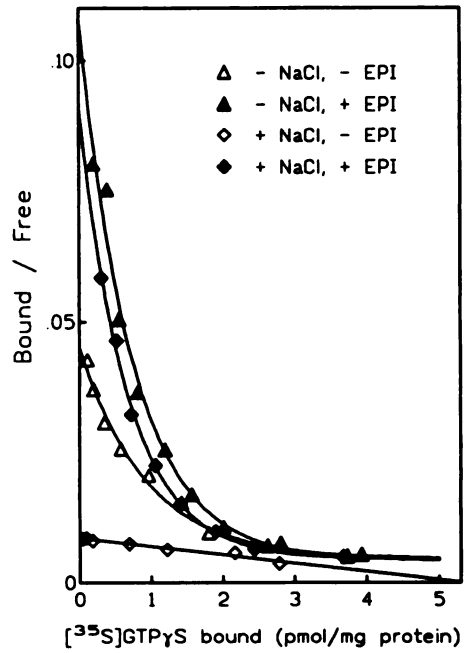

Fig. 6. Scatchard plots of saturation [^{35}S]GTPγS binding in PC-12/α_{2D} membranes. Binding of [^{35}S]GTPγS to PC-12 cell membranes was determined in the absence or presence of 100 μM EPI or 100 mM NaCl, with increasing concentrations of [^{35}S]GTPγS from 0.2 to 50 nM amd 2 μM GDP, at 25° for 10 min. The data shown are from triplicate determinations from a representative of three separate experiments.

lower affinity, component was evident (K_d = 40 nM), consistent with the reduction of basal [^{35}S]GTPγS binding noted above. In the presence of EPI nonlinear binding behavior was observed both in the absence and in the presence of Na^+, consistent with the ability of EPI to increase [^{35}S]GTPγS binding at any level of Na^+, as shown in Fig. 4. The binding curves in the presence of EPI were biphasic and shifted upward and to the right, compared with unstimulated binding in the absence of Na^+. The presence of agonist increased the apparent [^{35}S]GTPγS affinity of the high affinity component by 2.7-fold, compared with the Na^+-free condition. The lower affinity binding component was not significantly affected by EPI in the absence of Na^+, whereas in the presence of Na^+ EPI caused an almost 3-fold decrease in the affinity of this component. It should be noted that calculation of the fitted values for the lower affinity component involves a higher degree of extrapolation and therefore the values may be less reliable. The major influence of receptor interaction with G protein therefore appears to involve the induction of apparent high affinity [^{35}S]GTPγS binding. This action occurs in the absence of Na^+ for the unliganded receptor, whereas the agonist EPI can further increase the apparent [^{35}S]GTPγS affinity.

As shown in Fig. 1, RAU not only displaced the EPI dose-response curve to the right but also reduced its starting point. To further characterize the effect of RAU on basal [^{35}S]GTPγS binding, an antagonist dose-response curve was generated (Fig. 7); the curve showed that the IC_{50} value for RAU inhibition (48 nM) was similar to its K_B for EPI antagonism (39 nM) and its K_d in α_{2D}R [^{3}H]RAU binding studies (27), indicating that a single binding site (i.e., the ligand recognition site of the α_{2D}R) produces both effects. This leads to the conclusion that the α_{2D}R is spontaneously active in promoting [^{35}S]GTPγS binding and that occupation of the ligand recognition site by the antagonist RAU reduces this spontaneous activity.

To investigate the possibility that the high density of α_{2D}R (3.6 pmol/mg of protein) in our receptor expression system might be responsible for their spontaneous coupling, we examined the effect of RAU in membranes from clones expressing lower receptor density. As shown in Fig. 7, the percentage of maximal inhibition of basal [^{35}S]GTPγS binding was progressively lower as receptor density decreased. However, at 370 fmol/mg of protein, a density 3-fold less than the native expres-

TABLE 1

Effect of Na^+ on basal and EPI-stimulated [^{35}S]GTPγS binding

PC-12/α_{2D} membranes were incubated with reaction mixture with increasing concentrations of [^{35}S]GTPγS from 0.2 to 50 nM, in the absence and/or presence of 100 mM NaCl and 100 mM EPI, at 25° for 10 min. Nonspecific binding was determined in the presence of 10 μM GTPγS and was subtracted from total bound radioactivity. Saturation curves were analyzed by computerized interactive nonlinear least-squares regression. K_{d_H} and K_{d_L} are the apparent dissociation constants reflecting high and low apparent affinity states of G protein for GTP as the consequence of GDP/GTP exchange. The extrapolated maximal [^{35}S]GTPγS binding density was 9.5 ± 0.49 pmol/mg when measured in the presence of 2 μM GDP at 25° in a 10-min incubation and 17.1 ± 0.9 pmol/mg when measured at 50 min. The data shown are from triplicate determinations in a representative experiment that was repeated three times with similar results.

Group	Apparent affinity	
	K_{d_H}	K_{d_L}
	nM	
$-Na^+$, −EPI	1.80 (13%)	83.6 (87%)
$-Na^+$, +EPI	0.67 (11%)	69.9 (89%)
$+Na^+$, −EPI		40.4 (100%)
$+Na^+$, +EPI	0.94 (11%)	113.0 (89%)

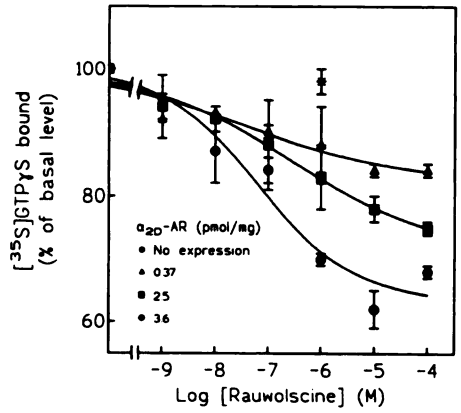

Fig. 7. Concentration-dependent reduction by RAU of basal [^{35}S]GTPγS binding. Binding was determined with increasing concentrations of RAU in the absence of NaCl in PC-12/α_{2D} membranes with receptor densities of 0, 0.37, 2.5, or 3.6 pmol/mg of protein. The data shown are mean ± standard error of triplicate determinations from two experiments.

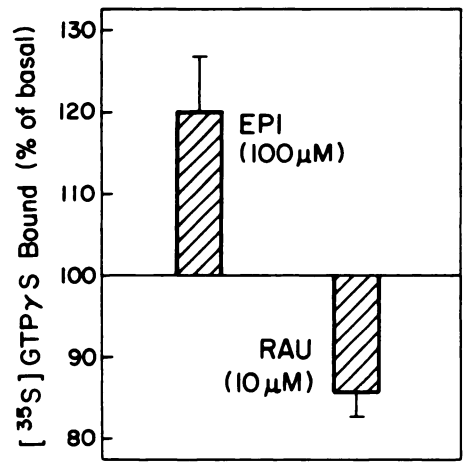

Fig. 8. EPI stimulation and RAU inhibition of [^{35}S]GTPγS binding to RIN5AH cell membranes. Binding was determined in the absence or presence of either 100 μM EPI (with 100 mM NaCl) or 10 μM RAU (without NaCl) and is expressed as a percentage of the untreated level. The data shown are means ± standard errors of four experiments, each run in triplicate.

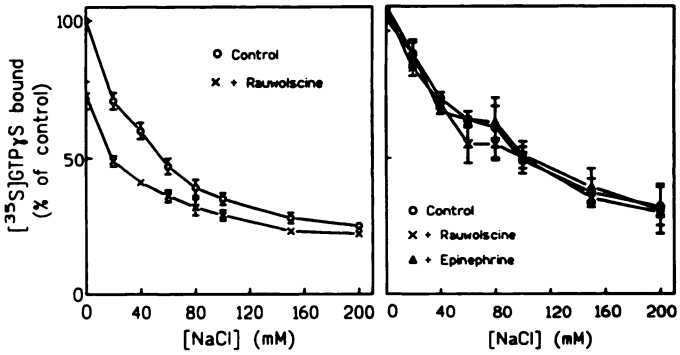

Fig. 9. Na^+ effect on basal and ligand-regulated [^{35}S]GTPγS binding in PC-12/α_{2D} membranes. *Left*, binding of [^{35}S]GTPγS to PC-12 cell membranes expressing 3.6 pmol/mg of protein α_{2D}R was determined in the absence or presence of 1 μM RAU, with increasing concentrations of NaCl. *Right*, binding in untransfected PC-12 cell membranes was unaffected by RAU or EPI (100 μM) at any level of NaCl. The data shown are means ± standard errors of at least three separate experiments.

sion level in human platelets (17), substantial inhibition could still be observed. In nontransfected cells RAU failed to inhibit basal [^{35}S]GTPγS binding, confirming the role of α_{2D}R in its action. As expected, maximal stimulation of [^{35}S]GTPγS binding by EPI (100 μM) was also reduced at lower receptor density, reaching 27% and 130% at 0.37 and 2.50 pmol/mg of protein versus 180% for the standard clone with 3.6 pmol/mg of protein.

To determine whether spontaneous α_{2D}R coupling occurred in cells normally expressing this receptor, we examined the effect of 10 μM RAU on basal [^{35}S]GTPγS binding to rat insulinoma-derived RIN5AH cell membranes. Binding was significantly ($p < 0.05$) reduced by RAU, to the extent of 14%, whereas EPI (100 μM, in the presence of 100 mM NaCl) increased binding by 20% (Fig. 8). α_{2D}R density was 50 fmol/mg of protein in these membranes.

The inhibitory effect of RAU was modulated by Na^+, as would be expected if both agents reduced basal R/G interaction. Thus, as the concentration of Na^+ was increased and basal [^{35}S]GTPγS binding decreased, the ability of RAU to further decrease binding was diminished (Fig. 9, *left*). In membranes from nontransfected PC-12 cells, neither EPI or RAU affected [^{35}S]GTPγS binding, although Na^+ caused a similar decrease in basal binding (Fig. 9, *right*).

A number of other antagonists were compared for their ability to decrease [^{35}S]GTPγS binding at concentrations at least 10-fold higher than their reported K_d values for the α_2R. As illustrated in Fig. 10, *left*, these antagonists decreased binding in a Na^+-dependent manner, although significant differences in their negative efficacy were apparent, especially in the absence of Na^+. Such differences are not consistent with displacement of endogenous agonist. Yohimbine, an isomer of RAU, was the most effective at reducing basal [^{35}S]GTPγS binding, producing a 41% decrease, which may reflect the net contribution of α_2R to basal G protein activation in this system. By comparison, Costa *et al.* (13) found that opiate antagonists caused a decrease of 17% in NG108 cells, similar to the 14% observed in RIN5AH cells expressing a native density of α_{2D}R. The rank order of negative efficacy we observed for basal binding (yohimbine = phentolamine > idazoxan = RAU > WB-4101 (Fig. 10, *right*) does not correspond to the order for ligand binding to α_{2D}R, as determined by [^{3}H]RAU competition studies (idazoxan = phentolamine > yohimbine = RAU) (27). This is consistent with the interpretation that differences in negative

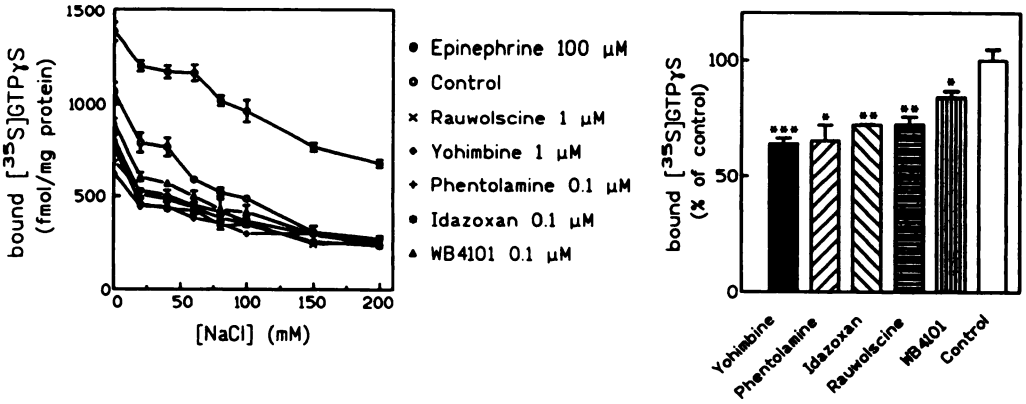

Fig. 10. Decrease of basal [^{35}S]GTPγS binding in PC-12/α_{2D} cell membranes by competitive α_2R antagonists. *Left*, [^{35}S]GTPγS binding was determined at the indicated concentration of antagonists in the presence of increasing concentrations of Na^+. *Right*, comparison of antagonist inhibition of [^{35}S]GTPγS binding at 0 mM Na^+. The data shown are from triplicate determinations from a representative experiment. The experiment was repeated twice with similar results. Significant differences from control at $p < 0.05$ (*), 0.01 (**) or 0.001 (***) by Student's *t*-test are indicated.

efficacy are caused by differences among antagonists in their relative affinity for free α_2R versus the α_2R/G complex, as opposed to differences in their affinity for the free receptor.

Discussion

α_2R agonists exert their effects via the activation of G proteins that have generally been found to be sensitive to pertussis toxin, indicating the involvement of G_i or G_o. In the present study we have demonstrated the ability of EPI to increase the binding of [^{35}S]GTPγS in membranes from PC-12 cells expressing a cloned α_{2D}R and have characterized the modulatory influences of GDP, divalent cations, and monovalent cations on agonist-stimulated binding. Although EPI stimulated [^{35}S]GTPγS binding via α_2R, these receptors also provided a significant degree of G protein activation in the absence of agonist, an activity that could be diminished by antagonists. Our results illustrate the intrinsic ability of α_2R to interact with and activate G proteins and the ability of ligands to allosterically regulate this interaction in both positive and negative directions. This behavior is entirely consistent with the predictions of a ternary complex model of receptor function.

EPI-induced [^{35}S]GTPγS binding exhibited an absolute requirement for Mg^{2+} and displayed a biphasic concentration dependence (Fig. 2), as has been previously described for other receptors (28). The micromolar affinity site has been attributed to the requirement for the Mg^{2+}-bound form of GTP at the G protein nucleotide binding site and is shared by basal and agonist-stimulated binding (22). The millimolar site is less well defined but appears to be more critical for the action of agonists. This suggests an influence on the agonist-dependent conformational changes of the receptor itself or on receptor/G protein interactions.

As expected, GDP caused a decrease in [^{35}S]GTPγS binding, reflecting its competition for the nucleotide binding site on the G protein. The ability of agonists to increase GTP binding has been attributed by some investigators to the lower affinity of the agonist/receptor/G protein complex for GDP, leading to increased GDP dissociation (24, 29). Other investigators have concluded that the action of the agonist-occupied receptor is mediated by an increase in GTP affinity, and this is an area of controversy. We found that EPI stimulated [^{35}S]GTPγS binding to approximately the same extent at all GDP levels, including its absence (Fig. 3). This indicates that GDP dissociation is not rate limiting in our system. In studies of muscarinic receptor stimulation of GTPγS binding, Hilf and Jakobs (22) found that 0.1 μM or higher levels of GDP were necessary to suppress basal binding and reveal agonist efficacy. This difference from our results may be due to the higher density of receptors (versus G protein) in our transfected cell system, differences in the receptor systems studied, or perhaps differences in the membrane environment, including G protein content or other accessory proteins.

Saturation binding studies with [^{35}S]GTPγS revealed that conditions that increase R/G complex formation (i.e., low Na^+ or EPI) caused the appearance of a high affinity component (Fig. 6). The presence of agonist augmented [^{35}S]GTPγS affinity and was able to overcome the inhibitory action of Na^+. The two components of [^{35}S]GTPγS binding appear to represent activated and nonactivated states of the G protein. Occurrence of the high affinity component in both the Na^+-free and EPI-containing conditions implies that activation of the G protein could be a consequence of either agonist stimulation or spontaneous R/G association. Na^+ and other monovalent cations diminished basal binding by up to 80% while exerting little or no effect on EPI-induced binding (Fig. 4). Na^+ was more effective at lower concentrations, whereas selectivity was not observed at higher levels, which may indicate two separate modes of influence or a preference for Na^+ at a single locus (13). This action of Na^+ was associated with the loss of the high affinity component of [^{35}S]GTPγS binding in saturation studies (Fig. 6). In previous studies with α_2R in vascular membranes, we found that Na^+ was essential to bring about complete dissociation of the R/G protein complex and that its site of action was distinct from that of guanylyl nucleotide (11). Limbird and co-workers (30) have extensively characterized the influence of Na^+ on α_{2A}R, including performing site-directed mutagenesis studies in which aspartate-79 in the second transmembrane domain of the receptor was identified as the likely site of allosteric Na^+ binding. Based upon these earlier findings, it is reasonable to propose that Na^+ binding to this location favors dissociation of the R/G complex, resulting in the reduction of basal [^{35}S]GTPγS binding that is observed.

The inhibitory action of Na^+ on basal [^{35}S]GTPγS binding indicates that α_{2D}R receptors and G proteins can interact in a functionally fruitful manner in the absence of added agonist ligands, provided that the level of Na^+ is sufficiently low. Taken

together with the previously reported effects of Na^+ on agonist and antagonist binding to α_2R, Na^+ can thus be viewed as a negative modulator of the α_2R/G protein precoupling equilibrium in a ternary model of receptor/G protein binding behavior (20). In such a model the affinities of receptor and G protein for each other are modified by the binding of agonist and antagonist ligands to the receptor. In the most generally applied version of this scheme, based largely upon β-adrenergic receptor function, formation of an R/G complex is highly dependent upon the presence of an agonist ligand, whose binding induces a receptor conformation that is favorable for G protein binding (i.e., the R + G $\rightarrow$ RG equilibrium lies to the left without agonist). However, some receptors such as α_2R may possess a higher native affinity for G protein, resulting in a significant level of precoupling of R and G (i.e., the R + G $\rightarrow$ RG equilibrium lies to the right). Recognizing that receptors may exist in two conformations, with either low (R) or high (R*) affinity for G protein, and that agonists allosterically favor the R* conformation, our results imply that the energy barrier for spontaneous conversion of R to R* is relatively low for α_{2D}R in the absence of Na^+.

Despite its ability to lower basal [^{35}S]GTPγS binding by up to 80%, Na^+ does not interfere with the agonist-induced increase, suggesting that agonist and Na^+ levels may function in concert to determine the extent of G protein activation by α_2R. Thus, in the data of Fig. 6, precoupling in the absence of Na^+ is associated with higher affinity [^{35}S]GTPγS binding, in much the same way as binding in the presence of EPI with or without Na^+. R/G association, whether spontaneous or promoted by agonist, is able to produce increases of apparent GTP affinity.

The additional [^{35}S]GTPγS binding produced by EPI in the presence of Na^+ can be attributed to the ability of EPI to increase receptor affinity for G protein, overcoming the negative allosteric influence of Na^+. This action is analogous to agonist actions proposed for nonprecoupled receptors such as β-adrenergic receptors. In the absence of Na^+, however, when an R/G complex already exists, EPI may exert an additional action, distinct from increased R/G association, to account for the higher level of GTP binding. Specifically, agonists can promote a conformation of the receptor that is more efficacious in promoting GDP/GTP interchange than is the unoccupied receptor. This property of the agonist-occupied receptor would be potentially more important for precoupled receptors. The above distinction recognizes that R/G and A/R/G complexes are not equal in their ability to bind GTP and the efficacy of agonists includes their ability both to favor R/G association and to specifically catalyze increased nucleotide exchange.

Costa *et al.* (13) have found a similar pattern of antagonist-sensitive and Na^+-dependent basal G protein activation for opiate receptors in NG-108 cell membranes, raising the likelihood that multiple receptor systems that predominantly couple to pertussis toxin-sensitive G proteins do so in a Na^+-modulated, precoupled manner. Although the phenomenon of Na^+-modulated precoupling is most readily observed in isolated membranes, the earlier work of Motulsky and Insel (25) and Connolly and Limbird (26) on α_{2A}R in intact platelets demonstrated that EPI efficacy and potency were regulated by intracellular Na^+ levels. In our studies the IC_{50} value for Na^+ was close to 20 mM (Fig. 4), which is near the prevailing cytoplasmic concentration (10–20 mM) reported by several techniques (31, 32), suggesting the potential for an important influence of intracellular Na^+ on basal and agonist-induced receptor coupling efficiency.

Although it is possible that receptor-dependent basal [^{35}S]GTPγS binding in PC-12 cell membranes could result from contaminating levels of agonist, there are several observations that argue against this possibility. 1) RAU produced a reduction of basal [^{35}S]GTPγS binding in RIN5AH cells, which do not produce catecholamines. 2) Three additional washes of PC-12 cell membranes failed to alter the ability of RAU to reduce binding. 3) High performance liquid chromatographic assay of membrane fractions for catecholamines failed to reveal measurable contamination (detection limit, 10 nM). 4) Differential negative efficacy at saturating concentrations of antagonists (Fig. 10) would not be expected if they were merely displacing agonist.

Within the predictions of the ternary model, antagonist ligands can reduce G protein activation in two ways. 1) They can competitively interfere with agonist binding. 2) They can reduce the level of precoupling. As proposed by Costa *et al.* (20), antagonists that bind with equal affinity to R and R/G states are "null" antagonists, acting only by the first mechanism, whereas antagonists binding preferentially to R are "negative" antagonists and reduce basal G protein activation. In our studies RAU and a number of α_2R antagonists exhibited negative antagonism. As predicted by the ternary model, the potential for negative antagonism was dependent upon Na^+ levels (Figs. 9 and 10). Thus, when higher Na^+ levels shifted the R/G equilibrium to the predominantly free R state, RAU lost effectiveness. Accordingly, one can postulate that both Na^+ and antagonists can induce unfavorable conformations of the α_2R for G protein interaction, although it is unclear whether they share a common conformational influence. The differences in negative antagonism we observed among α_2R antagonists at concentrations that should provide close to 100% occupancy (Fig. 10) can potentially be attributed to differences in the preferences of the antagonists for the free R state, giving rise to graded levels of partial negative antagonism. It remains to be determined whether such differences are of importance in determining the individual pharmacological profiles of α_2R antagonists.

In summary, we provide evidence for the precoupling of α_{2D}R to G proteins in membranes isolated from cells expressing the cloned receptor. Precoupling can be modulated by the level of Na^+ (or other monovalent cations) and is reduced with differential negative efficacy by a number of α_2R antagonists. Precoupling behavior can be described by the ternary model of receptor/G protein interaction and may be a general feature of those receptors that couple to G_i/G_o. Receptor subtypes and receptor/G protein pairs can be expected to differ in their extent of precoupling activity, which may also have significance for agonist efficacy at these receptors. It also seems likely that factors other than Na^+ may play a role in modulating precoupling, thus determining the relative importance of Na^+ in regulating the basal source of G protein signaling.

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