Reconstitution of Catecholamine-Stimulated Binding of Guanosine 5′-*O*-(3-Thiotriphosphate) to the Stimulatory GTP-Binding Protein of Adenylate Cyclase†

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ABSTRACT: The stimulatory GTP-binding protein (G_s) of adenylate cyclase, purified from rabbit liver, and β-adrenergic receptors, partially purified 1000–4000-fold from turkey erythrocyte plasma membranes, were coreconstituted into unilamellar phospholipid vesicles. The molar ratio of G_s to receptors in the vesicles varied from 3 to 10 in different preparations, as measured by guanosine 5′-*O*-(3-[^{35}S]thiotriphosphate) ([^{35}S]GTPγS) binding to G_s and [^{125}I]iodocyanopindolol binding to receptors. Activation of reconstituted G_s by GTPγS was stimulated up to 10-fold by the addition of the β-adrenergic agonist (−)-isoproterenol. Activation was assayed functionally by reconstitution with the catalytic unit of adenylate cyclase. Because of the relative purity of this preparation, the quasi-irreversible binding of [^{35}S]GTPγS could also be measured in the vesicles and was shown to parallel the functional activation of G_s under all conditions. Most of the assayable G_s in the vesicles could interact with the receptors and undergo agonist-stimulated activation. Agonist-stimulated activation and [^{35}S]GTPγS binding were complete in less than 3 min, even under suboptimal conditions, and could go to completion in <20 s under maximal stimulation. Agonist-stimulated binding did not require appreciable free Mg^{2+} (<0.1 mM). Activation in the absence of agonist was stimulated by free Mg^{2+}, but maximal activation took up to 10 min in the presence of 50 mM $MgCl_2$. Reconstitution increased the stability of G_s to thermal denaturation. The addition of β-adrenergic agonist further stabilized G_s, presumably by the formation of a stable agonist–receptor–G_s complex. The apparent K_d for GTPγS for agonist-stimulated binding was about 6 nM, almost 50-fold lower than that observed for Mg^{2+}-stimulated binding in the vesicles and about 100-fold lower than that observed for soluble G_s. This variation in K_d can be qualitatively accounted for by the stabilization of G_s by reconstitution and by the interaction of G_s with the agonist–receptor complex. The EC_{50} for (−)-isoproterenol for stimulation of GTPγS binding was 13 nM, far lower than its equilibrium K_d for binding to the receptor under similar conditions, 870 nM. This discrepancy, apparent spare receptors where G_s is in molar excess, reflects the ability of receptors to rapidly activate multiple G_s molecules.

The key biochemical event that underlies the activation of hormone-sensitive adenylate cyclase is the binding of GTP to a GTP-binding regulatory protein known as G_s.[1] GTP binding promotes the conversion of G_s to an "activated" state in which it can allosterically stimulate the enzymatic activity of the adenylate cyclase catalytic unit, a separate protein. It is the binding of guanine nucleotide to G_s that is stimulated by the hormone–receptor complex. If the activating nucleotide is GTP, activation is terminated relatively quickly by the hydrolysis of GTP to GDP, which does not activate. In this presumably physiological case, the extent of activation of the cyclase reflects the steady-state concentration of GTP-liganded G_s, which in turn reflects the hormone-stimulated rate of binding and the rate of hydrolysis. In the presence of a poorly hydrolyzed analogue of GTP such as GTPγS, the activation of G_s is reversed only slowly by the dissociation of nucleotide, and the activity of the cyclase is persistently elevated [for reviews see Ross & Gilman (1980) and Smigel et al. (1984)].

In order to understand this system in greater detail, we and others have attempted to purify its individual components and study them either in isolation or after their reconstitution into artificial membranes. Of the three protein components, two have been purified. β-Adrenergic receptors, which act to stimulate adenylate cyclase, have been purified from several sources using affinity chromatography (Durieu-Trautmann et al., 1980; Shorr et al., 1981). The receptor is a single, probably glycosylated, polypeptide that can range in size from about 42 to 67 kDa in different preparations. This change may indicate varying amounts of proteolysis or an intrinsic difference among β_1 and β_2 subtypes. G_s has also been purified from several sources [Northup et al., 1980; Sternweis et al., 1981; see also Smigel et al. (1984)]. It is a heterodimer that consists of a GTP-binding subunit of either 45 or 52 kDa and a 35-kDa subunit that regulates the rate of nucleotide binding (Northup et al., 1983).[2] Purified G_s can also hydrolyze GTP to GDP under appropriate conditions (Brandt et al., 1983).

We recently reported the reconstitution of partially purified β-adrenergic receptors and pure G_s into phospholipid vesicles and showed that reconstitution restored catecholamine-stimulated GTPase activity to the system (Brandt et al., 1983). Using a cruder reconstituted preparation based on that developed by Citri & Schramm (1980), we had previously shown that such reconstitution allows highly efficient interaction of the receptor with G_s (Pedersen & Ross, 1982). In this paper,

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[1] Abbreviations: DHA, dihydroalprenolol; ICYP, iodocyanopindolol; G_s, stimulatory GTP-binding protein of the adenylate cyclase system; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); kDa, kilodalton; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid, Tris, tris(hydroxymethyl)aminomethane; PEI, poly(ethylenimine); SDS, sodium dodecyl sulfate.

[2] A 8000-Da polypeptide that is stained poorly by Coomassie blue frequently cofractionates with G_s from human erythrocytes (Hildebrandt et al., 1984) and rabbit liver (A. G. Gilman, personal communication). Its function is unknown. It may be a subunit of G_s, analogous to the γ subunit of transducin (Fung et al., 1981).

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we report on the stimulatory effects of β-adrenergic receptors on G_s in reconstituted unilamellar vesicles similar to those described by Brandt et al. (1983). The purity of this preparation has allowed us to measure the activation of G_s by GTPγS according to the high-affinity binding of the nucleotide, facilitating a more complete and quantitative analysis of the coupling process. Here, we describe some functional properties of the vesicles, characterize the GTPγS binding assay, and describe the stimulation of steady-state nucleotide binding by isoproterenol. In the following (Asano & Ross, 1984), we describe kinetic studies of the receptor-mediated binding of GTPγS to reconstituted G_s that suggest a molecular mechanism of this regulatory interaction.

Experimental Procedures

Materials. G_s was purified from rabbit liver according to Sternweis et al. (1981) and stored at −80 °C in 10 mM Na-Hepes (pH 8.0)/0.1 mM dithiothreitol/1 mM EDTA/0.1% Lubrol 12A9. β-Adrenergic receptors were partially purified from turkey erythrocyte membranes by modification of the affinity chromatographic methods of Shorr et al. (1982) that have been described previously (Brandt et al., 1983). The specific activity of the receptors was 0.5–2 nmol of [^{3}H]dihydroalprenolol (DHA) bound per mg of protein. Polar lipids were prepared from turkey erythrocyte membranes as described by Brandt et al. (1983). Other phospholipids and digitonin were purchased from Sigma. Lubrol 12A9 was a gift from ICI, Ltd. [^{35}S]GTPγS (800–1500 Ci/mmol) and [^{3}H]DHA were purchased from New England Nuclear. Unlabeled GTPγS (Boehringer) was purified by elution from DEAE-Sephacel with a gradient of 0–0.5 M LiCl. The purity of both labeled and unlabeled GTPγS was at least 95%, as determined by thin-layer chromatography on PEI–cellulose plates in 0.75 M Tris–OH/0.45 M HCl/0.4 M LiCl (Bochner & Ames, 1982). [^{125}I]Iodocyanopindolol was prepared according to Engel et al. (1981). (±)-Cyanopindolol was a gift of G. Engel, Sandoz Pharmaceuticals, and alprenolol was a gift of Hässle Pharmaceuticals. BA85 nitrocellulose filters were purchased from Schleicher & Schuell. Sources of other materials are listed elesewhere (Pedersen & Ross, 1982; Fleming & Ross, 1980; Brandt et al., 1983).

Reconstitution. Reconstitution was performed according to Brandt et al. (1983). Receptors (1.0 volume in digitonin solution) and G_s (0.8–2.0 volumes in Lubrol 12A9 solution) were combined with dimyristoylphosphatidylcholine and polar lipids extracted from turkey erythrocyte plasma membranes (1:2 weight ratio; 1.0–1.8 volumes in deoxycholate/cholate solution). The mixture was reconstituted by gel filtration on Sephadex G-50 to yield unilamellar vesicles according to Brandt et al. (1983). Vesicles were collected in an eluted volume 4 times that of the original mixture. The molar ratio of G_s to receptors in the initial mixture was maintained at 3 for all experiments reported here. The final ratio in the vesicles depended on the recovery and varied from 3 to 10. Reconstituted vesicles were divided into small portions and frozen at −80 °C.

GTPγS-Binding Assay. The binding of [^{35}S]GTPγS was measured essentially as described by Northup et al. (1982). Vesicles were incubated at 30 °C in medium containing 20 mM NaHepes (pH 8), 1 mM EDTA, 100 mM NaCl, 1 mM dithiothreitol, 0.1 mM ascorbic acid, and the amounts of [^{35}S]GTPγS and $MgCl_2$ shown in the text (0.05 mL total volume). Assays were initiated by the addition of vesicles. Samples were quenched in 2 volumes of cold buffer containing 20 mM NaHepes (pH 8), 0.1 mM *dl*-propranolol, 0.1% Lubrol, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, and 0.1 mM GTP (1 mM GTP when a high concentration of GTPγS was used for binding). Binding was stable in this solution up to 3 h at 0 °C. Nucleotide bound to vesicles was separated by filtration on BA85 membrane filters (Northup et al., 1982), and the filters were washed with 14 mL of 20 mM Tris-HCl (pH 8.0)/100 mM NaCl/10 mM $MgCl_2$. Filters were dissolved for scintillation counting in 2-methoxyethanol before the addition of toluene-based scintillation cocktail. Nonspecific binding, determined in the presence of a 100-fold excess of GTPγS or a 500-fold excess of GTP, was 3–8% of total binding at steady state with 0.1 μM [^{35}S]GTPγS and was accounted for by binding of nucleotide to the filter. We report only specific binding, defined as the difference between total and nonspecific binding. The total amount of G_s present in the vesicles was estimated by the amount of [^{35}S]GTPγS bound in the presence of 50 mM Mg^{2+}, 0.1% Lubrol 12A9, and 10 μM [^{35}S]GTPγS after 30 min of incubation at 30 °C.

G_s Activation. The activation of G_s by GTPγS was carried out under conditions identical with those used to measure [^{35}S]GTPγS binding. Reactions were quenched as described above. Activated G_s was then assayed according to its ability to stimulate the activity of the catalytic unit of adenylate cyclase in plasma membranes from the cyc$^-$ variant of S49 lymphoma cells, as described by Pedersen & Ross (1982). A unit of activated G_s is defined as the amount that will increase adenylate cyclase activity by 1 nmol of cyclic AMP formed per min.

Assay data are generally expressed relative to the volume of vesicles used in each experiment rather than to the amount of protein, because protein is contributed by both the G_s and receptors and varies according to the purity of the receptor preparation and the recovery during reconstitution. The most reliable factors for normalizing specific activity are the concentration of active G_s or receptor, which can be calculated from the binding data.

Other Assays. Binding of [^{3}H]DHA to β-adrenergic receptors was assayed by the centrifugal gel filtration procedure, and the binding of [^{125}I]ICYP was assayed by the method originally described for [^{125}I]iodohydroxybenzylpindolol (Fleming & Ross, 1980). Protein was assayed by the method of Schaffner & Weissmann (1973), and lipid phosphorus was assayed after digestion in $HClO_4$ by the method of Ames (1966).

Results

Characterization of Reconstituted β-Adrenergic Receptors and G_s in Phospholipid Vesicles. When β-adrenergic receptors were substantially purified and stripped of associated membrane lipids, they retained the ability to be reconstituted into phospholipid vesicles upon the removal of detergent by gel filtration (Brandt et al., 1983). Unlike the reconstituted crude receptors described by Pedersen & Ross (1982), vesicles reconstituted using the purer receptors could not be further reconstituted by the addition of G_s without resolubilizing the vesicles with the Lubrol in the G_s solution. However, if purified receptors and G_s were mixed with phospholipids in detergent solution prior to reconstitution, then the vesicles that were obtained after gel filtration displayed appropriately coupled functions of the two proteins. This preparation was examined by negative staining and electron microscopy and found to contain unilamellar vesicles having diameters of 500–5000 Å, with most in the 1000–3000-Å range. There was some amorphous material present, but no detectable multilamellar vesicles.

The recovery of β-adrenergic ligand binding activity in the

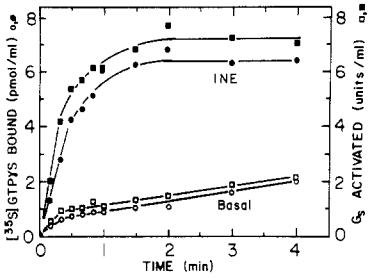

FIGURE 1: Agonist-stimulated activation of G_s and stable [^{35}S]GTPγS binding in receptor–G_s vesicles. Vesicles were incubated at 30 °C in medium containing 0.1 μM [^{35}S]GTPγS, 2 mM $MgCl_2$, 1 mM EDTA, and either 100 nM (−)-propranolol (open symbols) or 1 μM (−)-isoproterenol. At the times indicated, aliquots were withdrawn into two volumes of quenching solution and assayed for activation of G_s by reconstitution with cyc$^-$ membranes (□, ■) or for [^{35}S]GTPγS bound (○, ●) as described under Experimental Procedures. The incremental effects due to isoproterenol are shown by the solid symbols. Data are expressed according to the volume of vesicles used for each assay. The concentration of reconstituted receptors in this experiment was 1.2 nM.

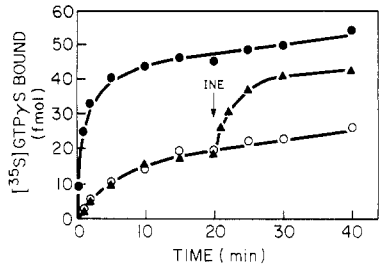

FIGURE 2: Continued responsiveness of receptor–G_s vesicles to stimulation by agonist. Vesicles were incubated at 30 °C with 100 nM [^{35}S]GTPγS and 1 mM free Mg^{2+} in the presence (●) or absence (○, ▲) of 10 μM (−)-isoproterenol. At the times shown, 25-μL aliquots were assayed for bound [^{35}S]GTPγS. At 20 min, isoproterenol (INE) (10 μM final) was added to one sample (▲). Each 25-μL aliquot contained 18 fmol of receptor.

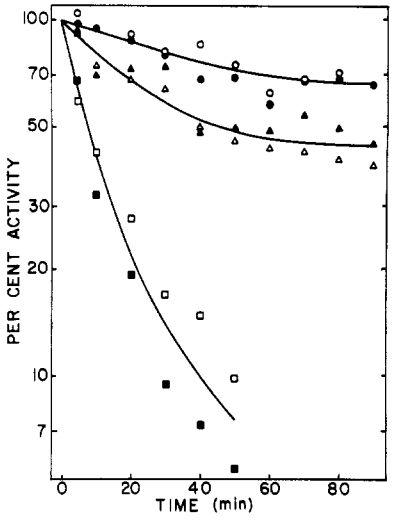

FIGURE 3: Comparison of G_s activation and [^{35}S]GTPγS binding: The stability of reconstituted G_s. Vesicles were incubated at 30 °C in 20 mM NaHepes (pH 8), 0.1 M NaCl, 1 mM dithiothreitol, 0.1 mM ascorbate, and 1 mM EDTA, plus either 2 mM $MgCl_2$ (▲, △), 2 mM $MgCl_2$ plus 1 μM (−)-isoproterenol (○, ●), or 50 mM $MgCl_2$ (■, □). At the indicated times, aliquots were withdrawn and incubated with 0.1 μM [^{35}S]GTPγS, 10 μM (−)-propranolol, and 50 mM $MgCl_2$ for 14 min at 30 °C. The reaction was terminated by addition of quenching solution, and the reaction mixture was assayed both for [^{35}S]GTPγS bound (open symbols) and for G_s activation (closed symbols). The binding of GTPγS at zero time was 2.5 (○), 2.4 (△), and 1.8 pmol/mL (□). The reconstitutive G_s activity at zero time was 2.1 (●), 2.0 (▲), and 1.4 units/mL (■).

reconstituted vesicle fraction is about 20–40%, as assayed with either [^{125}I]ICYP or [^{3}H]DHA. The incomplete recovery is caused at least in part by adsorption to the gel filtration column and the denaturing effects of Lubrol 12A9 and dithiothreitol, which were used to solubilize and stabilize G_s, respectively. The number of assayable β-adrenergic binding sites in the vesicles could not be increased by sonication or by resolubilization of the vesicles with digitonin, suggesting that no receptors were cryptic. No DHA- or ICYP-binding activity could be separated from the vesicles by rate zonal or isopycnic centrifugation in sucrose gradients. All receptors thus appear to be tightly associated with the vesicles. The K_d of reconstituted receptors for ICYP, about 50 pM (53 ± 13 pM in three experiments), was 2–3-fold higher than that observed in turkey erythrocyte membranes, the source of the receptors used here. Scatchard analysis of [^{125}I]ICYP binding (data not shown) did not suggest heterogeneity of binding sites for this ligand.

Upon reconstitution, the receptors also regained their regulatory activity, as shown by their ability to promote the activation of G_s by GTPγS (Figure 1). Activation is defined functionally as the conversion of G_s to a state in which it can stimulate the catalytic unit of adenylate cyclase (Ross & Gilman, 1980). The extent of receptor-promoted activation shown in this experiment was about 8-fold above basal at early times, and the agonist-stimulated rate of activation could be increased further by increasing the concentrations of agonist or nucleotide or both [see below; see also Asano & Ross (1984)]. The basal activation rate was unaltered by the addition of β-adrenergic antagonists.

The slow basal activation shown in Figure 1 continued for over 10 min, but basal activation has rarely been observed to approach the agonist-stimulated plateau, even after 3 h of incubation. The initial decline in the rate of basal activation does not reflect the rapid denaturation of G_s. After 20 min of incubation with GTPγS in the absence of agonist, the addition of isoproterenol caused a typical burst of rapid G_s activation that was only slightly less than that observed when agonist was added at the beginning of the experiment (Figure 2). In this experiment, activation was measured by the binding of [^{35}S]GTPγS, as discussed below.

The activation of G_s by nonhydrolyzable guanine nucleotides is associated with high-affinity binding of the nucleotide to G_s (Northup et al., 1982). Because the receptors used here were relatively pure, the specific, high-affinity binding of [^{35}S]GTPγS to G_s could also be measured in the receptor–G_s vesicles. Nonspecific binding is low, less than 10% of total binding at 100 nM GTPγS and less than 50% at 10 μM GTPγS. The data of Figure 1 both describe some properties of the activation of G_s in the vesicles and offer a comparison of the high-affinity [^{35}S]GTPγS binding with G_s activation. The time courses of the activation of G_s and the binding of [^{35}S]GTPγS were parallel, either in the presence or in the absence of isoproterenol (Figure 1). The initial rate of isoproterenol-stimulated activation was much greater than that described for vesicles reconstituted with cruder receptors, both absolutely and relative to the basal level (Pedersen & Ross, 1982). The ratio of reconstitutive activity to [^{35}S]GTPγS binding, 1.1 cyclase-activating units/pmol of GTPγS bound (Figure 1), compares favorably with the highest value obtained for pure, Lubrol-solubilized G_s, 1.4 units/pmol (Northup et al., 1982). Similar values of this ratio were observed in other experiments (e.g., 0.84, 0.83, and 0.78 unit/pmol in Figure 3). The slightly smaller values obtained by us may be due to the different assay conditions that were used, differences

in the cyc⁻ membrane preparations, or the inactivation of some of the G_s upon resolubilization of the vesicles.

The binding and reconstitutive activities of G_s are also denatured in parallel if the vesicles are incubated at 30 °C in the absence of nucleotide (Figure 3). In this experiment, vesicles were incubated without GTPγS at 30 °C either alone, in the presence of agonist, or in the presence of 50 mM $MgCl_2$. Aliquots were withdrawn at different times, and the remaining G_s was assayed both by [^{35}S]GTPγS binding and by functional activation of the adenylate cyclase catalytic unit. The stability of reconstituted G_s was much greater than that of detergent-solubilized G_s. About 50% of the reconstituted G_s remained active after 45 min at 30 °C in the presence of 1 mM Mg^{2+}, and this fraction was apparently quite stable. Under similar conditions, Lubrol-solubilized G_s displayed a $t_{1/2}$ for denaturation of only 8 min, and at least 90% of the total G_s decayed at this rate (data not shown). Reconstitution similarly stabilized G_s at higher Mg^{2+} concentrations. The $t_{1/2}$ of reconstituted G_s was about 8 min at 30 °C in 50 mM $MgCl_2$ but only about 1.5–2 min if Lubrol was added. Reconstitution enhanced the thermal stability of G_s independently of receptor. Similar stabilization was observed if G_s was simply diluted out of detergent into a sonicated dispersion of phospholipids, and the phospholipid specificity for stabilization of G_s was slight. However, G_s in the receptor–G_s vesicles was further stabilized to thermal denaturation by the addition of β-adrenergic agonists. Preliminary data indicate that the apparently biphasic denaturation of reconstituted G_s in the presence of agonist reflects in part the formation of a pool of highly stable G_s and that the size of this pool is consistently somewhat smaller than the total number of reconstituted receptors. This suggests that a stable agonist–receptor–G_s complex can form in the vesicle bilayer.

Taken together, the data of Figures 1 and 3 argue that the observed high-affinity binding of GTPγS that is promoted by β-adrenergic agonists in the reconstituted receptor vesicles represents the functional activation of the vesicle-bound G_s. The GTPγS-binding reaction is relatively easy to monitor by using the methods described here, and the experiments shown below characterize the receptor–G_s interaction by virtue of the agonist-mediated stimulation of the GTPγS-binding reaction. The activation of G_s and the binding of [^{35}S]GTPγS increase in parallel between 0.1 nM and 10 μM GTPγS (data not shown). At GTPγS concentrations above 10 μM, nonspecific binding of GTPγS compromises the binding assay but has no effect on the reconstitutive assay for G_s activation. Consequently, the reconstitutive assay for activated G_s can easily be used to monitor this reaction at nucleotide concentrations above 1 μM.

Estimation of the recovery of G_s during reconstitution and of its concentration in the vesicle preparation depends critically on the conditions and methods used for its assay in the vesicles. In previous studies, we measured the concentration of reconstituted, active G_s either according to the Mg^{2+}-stimulated binding of 10 μM [^{35}S]GTPγS (Brandt et al., 1983) or according to its cyclase-stimulating activity after Mg^{2+}-promoted activation by 10 μM GTPγS (Pedersen & Ross, 1982). In the preparation of receptor–G_s vesicles described here, we found that the amount of G_s assayable by Mg^{2+}-promoted [^{35}S]GTPγS binding was increased if the vesicles were resolubilized by the addition of Lubrol. Stimulation, which approached 50% in some preparations, occurred between 0.03 and 1 mg/mL Lubrol and was observed only at relatively high concentrations of GTPγS, above 1 μM (see Figure 4). At lower concentrations of GTPγS, Lubrol inhibited binding. It

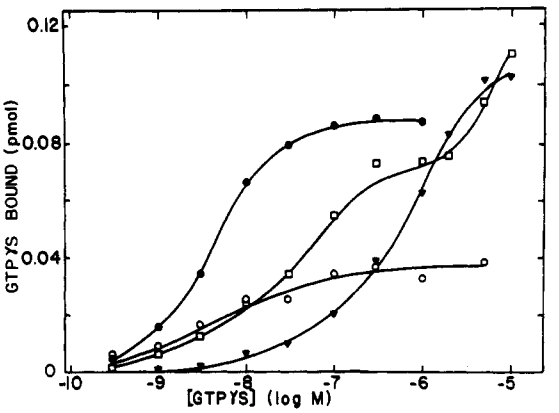

FIGURE 4: [^{35}S]GTPγS binding to receptor–G_s vesicles at increasing concentrations of nucleotide. Vesicles (8 μL) were incubated with varying concentrations of [^{35}S]GTPγS, 1 mM EDTA, and the following: 2 mM $MgCl_2$ plus 10 μM (−)-isoproterenol (●), 2 mM $MgCl_2$ (○), 50 mM $MgCl_2$ (□), or 50 mM $MgCl_2$ plus 0.1% Lubrol (▼). Incubation was carried out at 30 °C for 2 min for isoproterenol-stimulated binding and for 30 min for the other assays. Each assay contained 28 fmol of receptor.

should also be noted that 0.03 mg/mL Lubrol 12A9 uncouples receptor and G_s. For this study, we have measured the *total* amount of active, vesicle-bound G_s according to the plateau level of [^{35}S]GTPγS binding when the binding reaction was carried out in the presence of 50 mM Mg^{2+}, 10 μM GTPγS, and 0.1% Lubrol 12A9. The choice of these assay conditions for total G_s is supported in the figures below.

According to this assay, 40–70% of the G_s activity that was added to the original mixture of G_s, lipid, and receptor was recovered with the vesicles. The remainder was absorbed to the gel filtration medium or denatured. The amount of vesicle-bound G_s that was assayed by Mg^{2+}-stimulated GTPγS binding in the absence of Lubrol was usually 60–100% of this total amount. The remainder, referred to as "cryptic" G_s, could not be exposed by sonication of the vesicles either before or during the binding assay, by hypertonic or hypotonic osmotic shock, or by the addition of 10 μM alamethacin (in stoichiometric excess over lipid). About 70% (67 ± 23; n = 12 preparations) of the accessible, noncryptic G_s could also be stimulated to bind GTPγS by isoproterenol, indicating that most of the G_s that was available for Mg^{2+}-stimulated binding was also available for coupling to receptors. We do not know the cause of this small but variable amount of cryptic reconstituted G_s. Rimon et al. (1980) noted that the fraction of turkey erythrocyte adenylate cyclase that can be activated by epinephrine plus guanyl-5′-yl imidodiphosphate decreased at lower temperatures. We have preliminary evidence that increasing temperature also increases the fraction of G_s that can bind GTPγS, either after reconstitution or when G_s is diluted into dispersed phospholipids. It is still unclear if this effect reflects the same crypticity observed in the erythrocyte membrane.

Properties of Agonist- and Mg^{2+}-Promoted Stimulation of GTPγS Binding in Vesicles. The activation of G_s by GTPγS and the binding of [^{35}S]GTPγS to G_s in the vesicles were essentially irreversible at 0 °C (<10% in 1 h) and were reversed only slowly ($t_{1/2}$ > 5 min) and incompletely (25–60% remaining) at 30 °C under the assay conditions. While such slow dissociation would suggest a very high affinity of binding, the effect of the GTPγS concentration on the amount of GTPγS that bound to the vesicles suggests a more modest affinity that was strongly dependent on the conditions of assay. The data of Figure 4 show the plateau amount of GTPγS that was bound to the vesicles as a function of the nucleotide

Table I: Competitive Inhibition of Isoproterenol-Stimulated [^{35}S]GTPγS Binding to Receptor–G_s Vesicles[a]

	% binding at nucleotide concn (M) of				
	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
GTP	(94)	79	44	12	(0)
GDP	(72)	82	57	15	(0)
pp(NH)pG	91	89	80	52	26
ATP	ND	(104)	91	92	82

[a] Inhibition of the binding of 100 nM [^{35}S]GTPγS was measured after a 2-min incubation in the presence of 1 μM isoproterenol and the concentrations of other nucleotides shown. Specific binding was assayed as described under Experimental Procedures. Data are the means of two to five determinations each made in duplicate, except where shown in parentheses. ND, not determined.

concentration. Data are shown for total binding at 2 min in the presence of isoproterenol, at 30 min in the presence of 50 mM $MgCl_2$ or $MgCl_2$ plus Lubrol 12A9, or at 30 min without stimulation ("basal"; 1 mM free Mg^{2+}). The plateau level of agonist-stimulated binding was half-maximal at a GTPγS concentration of 6.3 ± 0.9 nM (n = 4), and the shape of the saturation curve was consistent with binding of nucleotide to a single class of sites. This value for the half-maximally effective concentration of nucleotide is actually a slight overestimation because the binding reaction is not complete at 2 min at very low concentrations of nucleotide [see Asano & Ross (1984)]. The concentration dependence of specifically isoproterenol-stimulated binding (i.e., total stimulated minus basal) is similar to the data in Figure 4 because the amount of basal binding at 2 min is quite low. The basal binding reaction, measured at 30 min, was also half-maximally saturated in the 5–10 nM range (6.4 ± 1.0 nM, n = 4), although the curve appears to be somewhat more shallow than that for isoproterenol-stimulated binding.

GTPγS binding stimulated by high concentrations of Mg^{2+} was of much lower apparent affinity than was agonist-stimulated binding. If the vesicles were resolubilized with Lubrol to measure binding to total G_s, the apparent affinity of Mg^{2+}-stimulated binding was half-maximal at 510 ± 80 nM. This value agrees well with that reported by Northup et al. (1982) for solubilized G_s alone. In the absence of detergent, the curve for Mg^{2+}-stimulated binding to reconstituted-G_s was quite shallow and in several experiments displayed distinct bends. Replots of the data from Figure 4 and other experiments suggest that Mg^{2+}-stimulated GTPγS binding to reconstituted G_s formally represents at least two classes of sites, one with an apparent high affinity similar to that of basal binding and another in the micromolar range, perhaps similar to that of Lubrol-solubilized G_s. In Figure 4, it is not clear that saturation was attained in the presence of 50 mM Mg^{2+} in the presence or absence of Lubrol, and binding measurements are not feasible above 10 μM GTPγS because of high nonspecific binding. However, by assaying the functional activation of G_s by GTPγS under these conditions, we have found that activation is maximal in the 3–10 μM range in the presence or absence of detergent.

The ability of other purine nucleotides to compete with [^{35}S]GTPγS for isoproterenol-stimulated binding to the vesicles was determined, and the binding displayed appropriate specificity (Table I). While the kinetics of the blockade of GTPγS binding by other nucleotides has not yet been studied in detail, the pattern of inhibition is similar to that observed in native adenylate cyclase systems. The low apparent affinity of G_s for the competing nucleotides is similar to that displayed by soluble G_s (Northup et al., 1982), implying that reconstitution does not alter the nucleotide binding site.

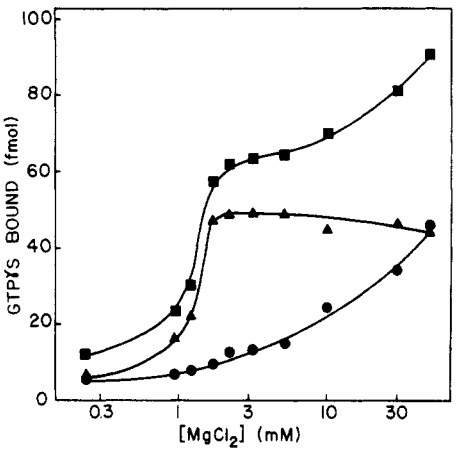

FIGURE 5: Mg^{2+} requirement for [^{35}S]GTPγS binding to receptor–G_s vesicles. Vesicles (6 μL) were incubated at 30 °C for 2 min with 0.1 μM [^{35}S]GTPγS 1.36 mM EDTA, 1 μM (−)-isoproterenol (■) or 0.1 μM (−)-propranolol (●), and various concentrations of $MgCl_2$. The incremental amount of isoproterenol-stimulated binding is also shown (▲). Each assay contained 15 fmol of receptor.

The effect of divalent cations on the GTPγS binding reaction was also dependent on whether the G_s had been reconstituted into receptor–G_s vesicles and whether agonist was present. Figure 5 describes the dependence of GTPγS binding upon Mg^{2+}, measured above a "background" of 1.36 mM EDTA. The incubation time was 2 min. The agonist-stimulated binding of GTPγS to reconstituted G_s was potentiated by low concentrations of free Mg^{2+}, with maximal stimulation by isoproterenol occurring below 0.3 mM free Mg^{2+}. The apparent half-maximally effective concentration of free Mg^{2+} was less than 0.1 mM in several experiments performed at different concentrations of EDTA. This is similar to the concentration needed for hormonal stimulation of adenylate cyclases in several biological membranes but is much lower than that required for the maximal activation of purified soluble G_s. It is therefore possible that free Mg^{2+} is not required for agonist-stimulated nucleotide binding but that Mg^{2+} serves only to form the nucleotide–Mg^{2+} complex.

At higher concentrations of Mg^{2+}, there was a further increase in total binding in the presence or absence of isoproterenol, reflecting receptor-independent stimulation by Mg^{2+} (Figure 5). When assayed at short times (2 min) in the absence of agonist, GTPγS binding to reconstituted G_s increased gradually with increasing concentrations of Mg^{2+} (up to 100 mM in other experiments). Because Mg^{2+} both makes G_s more thermolabile (Figure 3) and stimulates the GTPγS binding reaction, its effect on the extent of binding at long times is necessarily complex. If the binding reaction was allowed to approach its plateau (30 min in the absence of agonist), the effect of Mg^{2+} appeared to saturate in the 50–100 mM range (data not shown). The plateau level of binding decreased if the concentration of Mg^{2+} was increased further, probably reflecting the denaturation of G_s. The stimulatory effects of Mg^{2+} on GTPγS binding in the absence of agonist did not involve the receptor and were essentially unchanged whether or not receptor was added to the reconstitution mixture. Similar data were observed if G_s was diluted into a sonicated dispersion of either dimyristoylphosphatidylcholine or other phospholipids. The addition of 0.1% Lubrol 12A9 to the vesicles yielded a preparation that functionally resembled Lubrol-solubilized G_s in its Mg^{2+} dependence.

Regulation of the Extent of GTPγS Binding by β-Adrenergic Agonists. As noted above, the ratio of G_s molecules to receptor in the vesicles was in the range 3–10. To determine

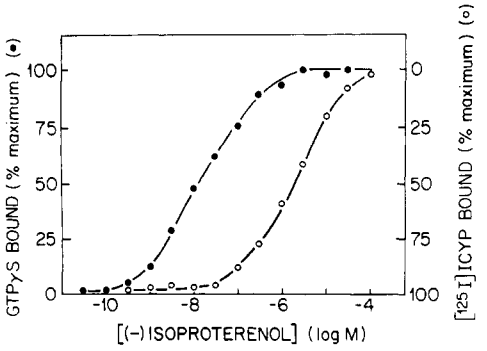

FIGURE 6: Comparison of isoproterenol binding to reconstituted receptors and consequent stimulation of GTPγS binding. [^{35}S]GTPγS binding (●) was assayed for 2 min at 30 °C in assay medium containing 0.1 μM [^{35}S]GTPγS, 1 mM EDTA, 2 mM $MgCl_2$, 0.1 mM ascorbate, and the indicated concentrations of (-)-isoproterenol. [^{35}S]GTPγS bound was 66 fmol per assay in the presence of 30 μM isoproterenol and 18 fmol per assay in its absence. Data are shown as percent of the maximum increment in the amount of GTPγS bound. Isoproterenol binding was assayed by competition with [^{125}I]ICYP binding. Vesicles were incubated in the same medium used for the [^{35}S]GTPγS-binding assay but contained unlabeled GTPγS and 60 pM [^{125}I]ICYP (1.1 times the K_d). Binding of [^{125}I]ICYP was assayed after 15 min. Specific ICYP binding was 1.1 fmol per assay in the absence of isoproterenol.

this ratio, total G_s was assayed in the presence of 50 mM $MgCl_2$, 10 μM [^{35}S]GTPγS, and 0.1% Lubrol 12A9 and receptor was assayed with [^{125}I]ICYP. Because a large fraction of the total G_s molecules could undergo receptor-stimulated activation, the ratio of G_s molecules that could be acted on by receptors to the total receptor number was also greater than unity, varying between 1.3 and 8 (average = 2.9 in 21 experiments). Thus, a single reconstituted receptor was able to sequentially promote the binding of GTPγS to multiple molecules of G_s.

The consequences of the stoichiometric relationship between G_s and β-adrenergic receptors in the vesicles were investigated further by comparing the binding of agonist to reconstituted receptors with the stimulation of GTPγS binding at different concentrations of free agonist. Isoproterenol binding to reconstituted receptor was measured by competition for binding with [^{125}I]ICYP in the presence of 100 nM GTPγS, under the conditions used for the GTPγS binding assay. The K_d of the reconstituted receptors for (-)-isoproterenol was 870 ± 160 nM (n = 4) (Figure 6) under these conditions. The presence of GTPγS, GTP, or Gpp(NH)p decreased the affinity of the receptors for agonist ligands only slightly (<3-fold), as was reported by Kelleher et al. (1983).

The potency of (-)-isoproterenol to stimulate GTPγS binding to reconstituted G_s was much greater than its affinity for the receptor (Figure 6). The concentration of (-)-isoproterenol that caused half-maximal stimulation of GTPγS binding at 2 min (EC_{50}) was 13 ± 3 nM (n = 5), less than 2% of the equilibrium K_d. This large discrepancy between K_d and EC_{50} does not reflect a stoichiometric excess of receptors because the molar ratio of coupled G_s (G_s that could be activated by agonist plus nucleotide) to receptors was always significantly greater than unity. There was no discrepancy between the affinity and functional potency of antagonists. For example, the K_d for (-)-propranolol was 1.6 nM when calculated according to its competitive inhibition of [^{125}I]ICYP binding, essentially the same as that calculated according to its ability to block the stimulation of GTPγS binding by isoproterenol, 2.3 nM.

In control studies (not shown), stimulation of GTPγS binding to receptor–G_s vesicles displayed typical β-adrenergic specificity. (-)-Isoproterenol was much more potent than the (+) isomer, and activation was blocked far more potently by (-)-propranolol than by its (+) isomer.

Discussion

The functional interactions of G_s and β-adrenergic receptors include the agonist-promoted stimulation of nucleotide binding and release by G_s, stabilization of G_s against thermal denaturation, stimulation of steady-state GTP hydrolysis by G_s, and the guanine nucleotide-sensitive enhancement of the receptor's affinity for agonists. The receptor–G_s vesicles described here provide a relatively pure preparation in which these interactions can be studied quantitatively. The vesicles contain pure G_s, but the receptors used in this study were purified only 1000–4000-fold, at best 10% pure according to [^{125}I]ICYP binding. (Theoretical purity would be 23 nmol/mg of protein for a 43 000-dalton polypeptide.) The direct molecular interaction of the two proteins is therefore not proven by these studies. However, we have recently reconstituted pure G_s with receptors that are >96% pure according to silver-stained SDS gels. In preliminary experiments, the reconstituted pure receptors have also efficiently promoted the binding of GTPγS to G_s. Thus, the single 43 000-dalton receptor polypeptide interacts directly with G_s to regulate its activity in the absence of other proteins.

The receptor–G_s vesicles described here represent a major improvement over previously reported preparations (Pedersen & Ross, 1982; Citri & Schramm 1980, 1982; Keenan et al., 1982; Gal et al., 1983; Kelleher et al., 1983). Most importantly, the purification of the receptors allowed us to increase their concentration in the vesicles such that most or all vesicles contain at least one receptor. This goal could not be attained with less pure receptors. With receptors purified to 4 nmol/mg of protein, up to 10 receptors can be incorporated into each 1000-Å diameter vesicle [see Pedersen & Ross (1982)]. In the vesicles used here, ≥70% of the reconstituted, noncryptic G_s molecules were coupled to receptors, and a single agonist-liganded receptor could promote the rapid activation of up to eight G_s molecules. These data are all roughly consistent with the calculation that random distribution of receptors and G_s among 1000-Å vesicles would yield 1–4 molecules of receptor and 6–20 molecules of G_s per vesicle by using the present reconstitution protocol. While this calculation is highly dependent on the homogeneity of vesicle diameter and on the lateral packing density of the phospholipids (Huang & Mason, 1978), its reasonable results are reassuring. The "catalytic" action of receptors, their ability to sequentially stimulate multiple G_s molecules, indicates the restoration of highly efficient coupling that is characteristic of native plasma membranes (Tolkovsky & Levitzki, 1978). This behavior supports the fidelity of the reconstitution procedure. It also forms the molecular basis of the phenomenon of apparently "spare receptors", the situation in which the EC_{50} of an agonist is much lower than its K_d (Figure 6).

The use of purified receptors has also allowed us to assay both the catecholamine-stimulated GTPase reaction (Brandt et al., 1983) and the agonist-stimulated binding of GTPγS in the relative absence of nonspecific hydrolytic or binding activities. In the cruder preparation, we used only the agonist-stimulated G_s activation by GTPγS to assess receptor–G_s coupling. We inferred the number of molecules of G_s activated from their ability to stimulate adenylate cyclase, using the reconstitutive specific activity reported for pure hepatic G_s by Northup et al. (1982). In this report, we both substantiate this functional assay and demonstrate that the stable binding of [^{35}S]GTPγS provides a simple and more directly quantitative assay either for the total number of G_s molecules present

or for those that have been activated under a given set of conditions (Figures 1 and 3). Since we can measure β-adrenergic receptors and their fractional saturation by agonists using [^{125}I]ICYP, we can now relate quantitatively the interaction of the two proteins.

The primary difficulty in reconstituting the two pure proteins was the yield obtained in the reconstitution. The recovery of both proteins from the gel filtration column was dependent on the volumes of the column and of the applied sample, on the concentration of protein, and on the choice of lipids. The use of dimyristoylphosphatidylcholine alone gave a poor yield of receptors. We believe that a major factor in the efficient production of the vesicles is preventing lipid and protein from adsorbing to the column and denaturing. Because the choice of lipids controls the yield and the stability of the receptor during reconstitution (S. E. Pedersen and E. M. Ross, unpublished results), we believe that it is difficult to assess the biological relevance of the lipid specificity displayed here or in other reconstituted systems (Kirilovsky & Schramm, 1983; Cerione et al., 1983).

Reconstitution of G_s into receptor–G_s vesicles markedly alters its responses to the concentrations of both nucleotides and Mg^{2+}. Agonist-stimulated GTPγS binding is maximal below 1 mM Mg^{2+}, whereas binding in the absence of agonist increases with increasing Mg^{2+} concentration up to 100 mM or more (Figure 5). This difference has also been noted in native membranes, where it has been interpreted as an agonist-mediated increase in the affinity of G_s for Mg^{2+} [Iyengar & Birnbaumer, 1982; see Cech et al. (1980) for review]. Alternatively, the agonist-stimulated activation of G_s may not involve Mg^{2+} except as a ligand of the nucleotide itself. Receptor-stimulated GTPγS binding requires only enough Mg^{2+} to bind nucleotide, and no other requirement for free Mg^{2+} is obvious in the presence of agonist.

The opposite effects of Mg^{2+} and agonist on the thermal stability of G_s and on its apparent affinity for GTPγS also suggest separate pathways for receptor-regulated and Mg^{2+}-regulated activation. The stability of G_s and its apparent affinity for nucleotides have already been linked mechanistically by Smigel et al. (1982) (Northup et al., 1982). These authors showed that the activated form of G_s is the GTPγS-liganded α (45 000 Da) subunit and that an initial slow step in the tight binding of GTPγS involves the dissociation of the α and β (35 000 (Da) subunits of G_s [Smigel et al., 1982, 1984; summary in Asano & Ross (1984)]. The α subunit can bind GTPγS tightly, but it is also much more labile to denaturation. By this model, the observed concentration dependence of binding primarily reflects the competition between α subunit denaturation and GTPγS binding. The apparent affinity for nucleotide is therefore inversely proportional to the denaturation rate (Smigel et al., 1982).

In this light, it is striking that reconstitution both stabilizes G_s (Figure 3) and increases its apparent affinity for GTPγS (Figure 4). Moreover, the addition of agonist further stabilizes G_s and further increases its apparent affinity, while Mg^{2+} destabilizes G_s and decreases its affinity. We therefore propose that the pathways that lead to the stable G_s–GTPγS complex are different under the influence of either agonist or Mg^{2+}. Receptor may prime G_s to bind nucleotide without promoting the initial release of its 35 000-Da β subunit. Alternatively, if the β subunit does dissociate under the influence of receptor, then the α subunit probably remains bound to a receptor–agonist complex that is quite stable. Regardless, it is likely that an agonist–receptor–G_s complex, the form of the receptor that displays highest affinity for agonist ligands, is the form that becomes primed to bind guanine nucleotide. The kinetics of the formation of this preactivation complex and its dependence on the presence of loosely bound nucleotide are discussed in the following paper (Asano & Ross, 1984).

Acknowledgments

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Catecholamine-Stimulated Guanosine 5′-*O*-(3-Thiotriphosphate) Binding to the Stimulatory GTP-Binding Protein of Adenylate Cyclase: Kinetic Analysis in Reconstituted Phospholipid Vesicles†

Tomiko Asano‡ and Elliott M. Ross*

ABSTRACT: The stimulatory GTP-binding protein of adenylate cyclase, G_s, and β-adrenergic receptors were reconstituted into unilamellar phospholipid vesicles. The kinetics of the quasi-irreversible binding of guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) to G_s, equivalent to G_s activation by nucleotide, was studied with respect to the stimulation of this process by β-adrenergic agonists and Mg^{2+}. The rate of GTPγS binding displayed apparent first-order kinetics over a wide range of nucleotide, agonist, and Mg^{2+} concentrations. In the absence of agonist, the apparent first-order rate constant, k_{app}, was 0.17–0.34 min^{-1} and did not vary significantly with the concentration of nucleotide. At 50 mM $MgCl_2$, k_{app} increased somewhat, to 0.26–0.41 min^{-1}, and remained invariant with the nucleotide concentration. In the presence of agonist, k_{app} was dependent on nucleotide concentration. At 10^{-9} M GTPγS, the addition of (−)-isoproterenol caused at most a 2-fold stimulation of k_{app}. However, k_{app} measured in the presence of isoproterenol increased as an apparently saturable function of the GTPγS concentration, such that isoproterenol caused a 17-fold increase in k_{app} at 1 μM GTPγS. The effect of isoproterenol on k_{app} also appeared to saturate at high isoproterenol concentration, yielding a $k_{app} \sim 6$ min^{-1} at high concentrations of both nucleotide and agonist. These data suggest that the receptor–agonist complex acts by increasing the rate of conversion of a lower affinity G_s–GTPγS complex to the stable activated state.

The activity of hormone-sensitive adenylate cyclase primarily reflects the extent of activation of the stimulatory GTP-binding protein, G_s.[1] Activation of G_s occurs upon the high-affinity binding of GTP, or a GTP analogue such as GTPγS, and is manifest as the ability of nucleotide-liganded G_s to bind to and stimulate the catalytic unit of adenylate cyclase. Activation is terminated either by the slow dissociation of nucleotide or, in the physiological case, by the hydrolysis of GTP to GDP. GDP does not activate G_s. Receptors for stimulatory hormones function by increasing the rate of activation of G_s by guanine nucleotides. This increase in the rate of activation increases the steady-state concentration of G_s–GTP or, in the case of nonhydrolyzed analogues, increases the rate at which the active G_s–nucleotide complex is formed [reviewed by Ross & Gilman (1980) and Smigel et al. (1984)].

The mechanism of activation of G_s by nucleotides has been addressed most thoroughly by Gilman's group using purified, detergent-solubilized G_s (Northup et al., 1982, 1983; Smigel et al., 1982). G_s is a dimer of a 45 000-Da (or 52 000-Da) GTP-binding α subunit and a 35 000-Da β subunit.[2] These authors showed that the free α subunit–nucleotide complex is the active form of G_s. GTPγS binds to G_s and activates it by a slow reaction that is tighly coupled to the dissociation of the 35 000-dalton β subunit. The binding reaction is first order in G_s, and the observed first-order rate constant for binding, k_{app}, was found to vary less than 2-fold between 10^{-8} and 10^{-4} M GTPγS (Northup et al., 1982). These findings and other data led to the following proposed mechanism for

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[1] Abbreviations: G_s, stimulatory GTP-binding protein of hormone-sensitive adenylate cyclase; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); k_{app}, apparent first-order rate constant for GTPγS binding to G_s; Da, dalton; Hepes 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

[2] A 8000-Da polypeptide also frequently copurifies with G_s (Hildebrandt et al., 1984) and probably is a γ subunit, analogous to the γ subunit of transducin (Fung, 1983). When G_s dissociates, the β and γ subunits apparently remain associated.

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