Rapid Binding of Guanosine 5′-*O*-(3-Thiotriphosphate) to an Apparent Complex of β-Adrenergic Receptor and the GTP-Binding Regulatory Protein G_s†

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ABSTRACT: When reconstituted phospholipid vesicles that contain purified β-adrenergic receptors and the GTP-binding regulatory protein G_s were preincubated with agonist before the addition of guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS), the typical receptor-stimulated GTPγS binding reaction was preceded by an even more rapid burst of GTPγS binding. This burst was studied in detail at 0 °C. The rate of the burst was second order in nucleotide and G_s [$k_{assoc} \sim 2 \times 10^7$ $(M \cdot min)^{-1}$], consistent with diffusion-controlled binding. The magnitude of the burst was always less than the number of receptors present and was roughly linear with receptor number when similarly prepared vesicles were compared. There was no obvious quantitative correlation between the burst and the amount of G_s. The species that gave rise to the burst formed with $t_{1/2} \sim 15$ min at 0 °C in the presence of agonist and decayed by ~3 min upon addition of antagonist or detergent. Formation and decay of this species was much faster at 30 °C. The data suggest that a complex of agonist, receptor, and G_s that is primed for the rapid binding of guanine nucleotide can form and be analyzed in reconstituted vesicles.

The activity of hormone-sensitive adenylate cyclase essentially reflects the extent of activation of the guanine nucleotide binding regulatory protein G_s,[1] which is caused by the binding of GTP. GTP binding is stimulated by cell surface receptors, of which the β-adrenergic receptor is prototypical. The agonist-liganded receptor functions as a noncovalent catalyst that promotes the release of GDP from the inactive G_s–GDP complex and the subsequent binding of GTP to G_s, thus generating the active G_s–GTP complex. The hydrolysis of GTP by G_s is receptor-independent and results in the inactivation of the complex [see Brandt and Ross (1986) and Smigel et al. (1984)].

The detailed mechanism of the receptor–G_s interaction during the exchange of GDP for GTP is of great interest. Cassel and Selinger (1978) first demonstrated the agonist-stimulated release of GDP from turkey erythrocyte membranes in the absence of added guanine nucleotide. They postulated the existence of significant amounts of unliganded G_s and suggested that it may exist in either an "open" or "closed" state, with receptor promoting conversion to the open state. Similar mechanisms were suggested by kinetic studies of adenylate cyclase activation in native membranes (Braun et al., 1982) and by measurement of the agonist-stimulated binding and release of guanine nucleotides by purified G_s that had been coreconstituted with purified β-adrenergic receptor (Brandt & Ross, 1986).

An alternative model holds that the agonist-liganded receptor and guanine nucleotide each bind to G_s negatively cooperatively with respect to each other, such that receptor promotes the binding of one nucleotide to G_s only by facilitating the dissociation of another nucleotide [see Ross and Gilman (1980) for review]. This model rests upon the ability of guanine nucleotides to promote the dissociation of a stable ternary agonist–receptor–G_s complex with high affinity for agonist [reviewed by Stadel et al. (1982)]. It was suggested by the observations that guanine nucleotides decrease the affinity of receptors for agonist, but not antagonist, ligands (Rodbell et al., 1971; Maguire et al., 1976) and that G_s stabilizes agonist binding only in the absence of nucleotides (Ross et al., 1978; Kelleher et al., 1983; Cerione et al., 1984). It is further supported by direct studies of the chromatographic behavior of solubilized receptor, which apparently binds G_s preferentially in the presence of agonist but not in the presence of nucleotide (Limbird & Lefkowitz, 1978; Limbird et al., 1980).

Recently Ferguson et al. (1986) showed that G proteins copurify with a stoichiometric amount of GDP. Using the homologous regulatory proteins G_i and G_o, they developed a method to remove the bound GDP. In detergent solution, the GDP-free proteins bound GTPγS in a second-order, apparently diffusion limited process. These rates were far faster than the rate of GTPγS binding to the GDP-liganded protein and were comparable to the maximum receptor-mediated rates of nucleotide binding observed previously (Asano & Ross, 1984). The facilitation of GDP dissociation from G proteins would therefore be at least kinetically adequate to account for the observed agonist-stimulated rates of binding of GTP or GTPγS without any direct facilitation of binding.

In order to study the initial steps in the receptor-stimulated binding of guanine nucleotides to G proteins in greater detail, we have examined the effect of preequilibrating receptor–G_s vesicles with agonist before the addition of nucleotide. These data suggest that the agonist-liganded receptor promotes the

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[1] Abbreviations: GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); buffer A, 50 mM NaHepes (pH 8.0)–3 mM $MgCl_2$–1 mM EDTA–0.1 M NaCl–0.5 mM sodium ascorbate; DHA, dihydroalprenolol; ICYP, iodocyanopindolol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; G_s, GTP-binding protein that stimulates adenylate cyclase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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release of GDP from G_s and that the subsequent binding of GTPγS to this form of G_s is a bimolecular process.

Materials and Methods

Materials. β-Adrenergic receptor was purified from turkey erythrocytes by the procedure of Brandt and Ross (1986). G_s was purified from rabbit liver by the method of Sternweis et al. (1981). Lipids were purchased from Avanti Polar Lipids. [^{3}H]Dihydroalprenolol and [^{35}S]GTPγS were purchased from New England Nuclear. GTPγS (Boehringer) was purified by elution from DEAE-cellulose with a 0.0–1.0 M LiCl gradient (Asano et al., 1984). Alprenolol, (−)-propranolol, Lubrol 12A9, and (−)-isoproterenol were gifts from Hassle Pharmaceuticals, Ayerst Laboratories, ICI, Ltd., and Sterling-Winthrop Research Institute. [^{125}I]ICYP was prepared according to Engel et al. (1981). (±)-Cyanopindolol was a gift of G. Engel, Sandoz Pharmaceuticals. The sources of other materials have been reported previously (Brandt & Ross, 1985, 1986; Asano et al., 1984).

Reconstitution of β-Adrenergic Receptor–G_s Vesicles. β-Adrenergic receptor, G_s, and lipids (phosphatidylethanolamine/phosphatidylserine, 3:2, dispersed in CHAPS) were combined and reconstituted by gel filtration on Sephadex G-25 as previously described (May et al., 1985; Brandt & Ross, 1986). The amount of receptor and G_s used in each reconstitution varied according to the desired receptor:G_s ratio. Vesicles containing only G_s were reconstituted by substituting the appropriate buffer for the receptor.

Assays. β-Adrenergic receptor was measured according to the specific binding of [^{3}H]dihydroalprenolol or [^{125}I]ICYP after incubation for 1.5–3.0 h at 0 °C, as previously described (Fleming & Ross, 1980). Total G_s was measured according to the binding of [^{35}S]GTPγS in the presence of 50 mM $MgCl_2$ and 0.1% Lubrol 12A9, as described by Asano et al. [1984; see also Northup et al. (1982)]. Isoproterenol-stimulated GTPγS binding was assayed essentially as described by Brandt et al. (1986). The concentrations of isoproterenol and [^{35}S]GTPγS (70–150 cpm/fmol) are noted in the text and figure legends. GTPγS binding reactions were stopped by quenching in buffer containing 20 mM NaHepes (pH 8.0), 50 mM $MgCl_2$, 1 mM EDTA, 0.1 mM GTP, 50 μM *l*-alprenolol, and 0.1% Lubrol 12A9. The temperature of the isoproterenol-stimulated nucleotide binding reactions varied as noted in the text. For all [^{35}S]GTPγS binding experiments, a "no-protein" blank value has been subtracted from the data shown. Such a blank is equivalent to the estimate of nonspecific binding described by Asano et al. (1984) because all apparent nonspecific binding reflects binding of nucleotide to the nitrocellulose filter.

Burst Measurements. To measure the rapid binding of [^{35}S]GTPγS to vesicles that had been previously equilibrated with agonist, vesicles were first incubated in 50 mM NaHepes (pH 8.0)–3 mM $MgCl_2$–1 mM EDTA–100 mM NaCl–0.5 mM sodium ascorbate (buffer A) containing the indicated concentration of (−)-isoproterenol. Unless otherwise indicated, initial exposure to agonist was for 2 h at 0 °C. In some experiments, vesicles were used to initiate GTPγS binding reactions directly following this preincubation. In other experiments, either concentrated alprenolol or detergent plus alprenolol was added to the vesicles prior to the initiation of the nucleotide binding reaction. Final concentrations were 50 μM alprenolol and 1% detergent, added as 3–100-fold concentrated solutions. The time that elapsed between this addition and initiation of the nucleotide binding reaction is indicated in the text. Subsequent nucleotide binding assays were carried out at 0 or 30 °C in buffer A that contained either

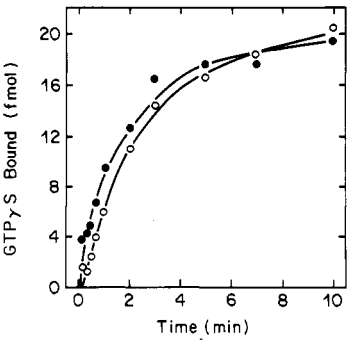

FIGURE 1: Time course of agonist-stimulated binding of [^{35}S]GTPγS to receptor–G_s vesicles with and without pretreatment with agonist. Receptor–G_s vesicles were incubated for 1 h at 0 °C in buffer A with (●) or without (○) 10 μM (−)-isoproterenol. The nucleotide binding reaction was then initiated by adding vesicles to a reaction mixture at 30 °C that contained 0.2 μM [^{35}S]GTPγS and 10 μM isoproterenol in buffer A. A separate sample (not shown) was preincubated and assayed in the absence of isoproterenol. At the indicated times, aliquots of the reaction mixture were assayed for bound [^{35}S]GTPγS as described under Materials and Methods. The data shown represent agonist-stimulated binding, i.e., the increment over the amount of binding observed in samples that were not exposed to agonist. Each sample contained 12.1 fmol of receptor and 20 fmol of receptor-sensitive ("coupled") G_s [see Asano et al. (1984)]. Data points represent the average of duplicate measurements.

10 μM isoproterenol, no adrenergic ligand, or 50 μM alprenolol. In binding assays performed on vesicles that had been quenched with detergent plus alprenolol, the assay medium also contained 0.1% detergent. The concentration of GTPγS used in each reaction is noted in the text. The standard protocol for measurement of the burst was preincubation of vesicles with 10 μM (−)-isoproterenol for 2 h at 0 °C. Vesicles were then added to a reaction mixture containing GTPγS and 50 mM alprenolol at 0 °C. The reaction was stopped after 2 min, and the amount of GTPγS bound was determined as described by Asano et al. (1984).

Results

When purified β-adrenergic receptor and G_s are reconstituted into phospholipid vesicles, β-adrenergic agonists promote an increase in the rate of GTPγS binding. The onset of receptor-stimulated binding is rapid, and no lags have been observed (Brandt & Ross, 1986; Asano & Ross, 1984). If the vesicles were first incubated with (−)-isoproterenol before the addition of GTPγS, however, a brief but rapid burst of nucleotide binding was observed (Figure 1). The burst usually amounted to 5–20% of the G_s in a routine experiment. It was complete in 5–10 s and was followed by the typical receptor-stimulated binding of GTPγS to G_s at a rate similar to that observed in vesicles that had not been preincubated with agonist [see Brandt and Ross (1986)]. The maximal amount of agonist-stimulated GTPγS binding was the same whether or not the vesicles had been preincubated with isoproterenol, and this maximum was greater than the number of receptors. The initial burst required the presence of a β-adrenergic agonist during the preincubation, and vesicles prepared without receptor or with heat-inactivated receptor did not display either the burst or any other response to agonists. Preincubation with agonist at 30 °C for as little as 2 min was adequate to cause the burst.

In order to study the rapid initial binding of GTPγS to G_s in vesicles that had been pretreated with isoproterenol, further experiments were performed at 0 °C to slow the rates of binding (Figure 2). At 0 °C, the binding of GTPγS to vesicles that had not been preincubated with agonist was quite slow

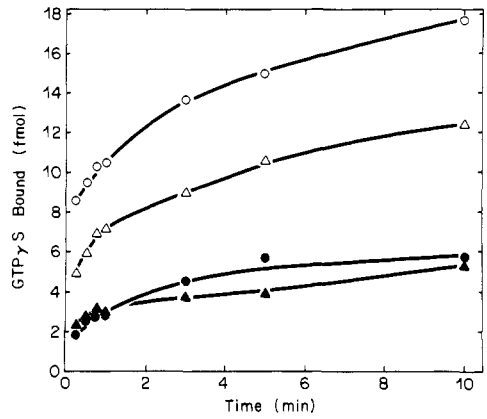

FIGURE 2: Time course of [^{35}S]GTPγS binding to receptor–G_s vesicles at 0 °C. Receptor–G_s vesicles were incubated for 1 h at 0 °C in buffer A (●, ▲) or in buffer A that contained 1 μM isoproterenol (○, △). Vesicles were then diluted with 1.14 volumes of buffer A (○, ●) or buffer A that contained 86 μM alprenolol (△, ▲) and held at 0 °C for 1 h. [^{35}S]GTPγS binding assays were then performed at 0 °C in a reaction mixture that contained 0.2 μM GTPγS and either 1 μM isoproterenol (○, ●) or 50 μM alprenolol (△, ▲). Each assay point represents 11 fmol of receptor and 51 fmol of receptor-sensitive G_s. Data shown are the average of duplicate experiments.

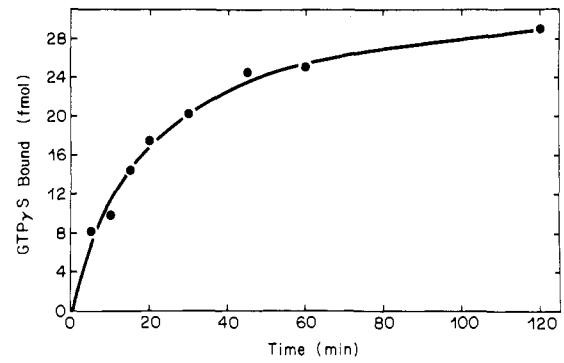

FIGURE 3: Time required for the formation of a rapidly binding pool of G_s at 0 °C. Vesicles were incubated at 0 °C in buffer A either with or without 10 μM isoproterenol. At the indicated times, aliquots were removed and used to initiate GTPγS binding reactions, also at 0 °C. The reaction mixtures all contained 0.2 μM [^{35}S]GTPγS and both 50 μM *l*-alprenolol and 2 μM isoproterenol (final concentrations). Binding reactions were stopped after 2 min, and the amount of [^{35}S]GTPγS bound was determined as described. Each data point represents the increment in GTPγS bound that was caused by pretreatment with agonist, and the data shown are the average of duplicate determinations. Each sample contained 30 fmol of receptor and 60 fmol of receptor-sensitive G_s. This experiment was repeated 3 times with similar results.

($k_{obsd} \sim 0.05$ min^{-1}). Furthermore, little if any stimulation of binding was observed when the binding reaction itself was carried out in the presence of isoproterenol (compare the two lower curves in Figure 2). As was observed at 30 °C (Figure 1), the principal effect of preincubating the vesicles with agonist was the relatively rapid burst of GTPγS binding that was observed in the first 15 s of incubation with nucleotide and that was essentially complete by 1 min ($k_{obsd} > 3$ min^{-1}). The burst at 0 °C was also followed by a slower second phase of binding ($k_{obsd} \sim 0.1$ min^{-1}), but this second phase was much slower than that seen at 30 °C (Brandt & Ross, 1986) and was only slightly faster than the binding that occurred in the vesicles that had not been preexposed to agonist.

In order to minimize receptor-stimulated GTPγS binding subsequent to the burst, the experimental protocol was modified as follows. After incubation at 0 °C with 10 μM isoproterenol, the vesicles were diluted with 1.4 volumes of cold buffer A that contained 50 μM *l*-alprenolol, a potent β-adrenergic antagonist. After a further 60 min at 0 °C, the [^{35}S]GTPγS binding reaction was initiated at 0 °C by the addition of vesicles to a reaction mixture that also contained 50 μM *l*-alprenolol. Under these conditions, the burst was still observed, although it was somewhat diminished in magnitude (see below). Thus, G_s that had been primed by the receptor for the rapid binding of nucleotide was stable to incubation in the presence of antagonist. The experiments described below characterize the formation of this rapidly binding fraction of G_s, referred to as the burst complex, and its properties.

Formation of the burst complex at 0 °C proceeded with a $t_{1/2}$ of about 15 min and was essentially complete at 1 h (Figure 3). Preincubation with isoproterenol for up to 24 h resulted in no further increase in the magnitude of the burst (not shown). During this prolonged incubation, there was no loss of receptor or G_s binding activity. The rate of appearance of the burst was much slower that that expected for the binding of isoproterenol to the receptor, especially given that the concentration of isoproterenol used was 10 μM. These data suggest that some reaction other than agonist binding is involved in the formation of the G_s species capable of rapid nucleotide binding. Candidates for such a slow step include the formation of an agonist–receptor–G_s complex in the bilayer or the receptor-mediated dissociation of either GDP or the βγ subunits from the α subunit of G_s.

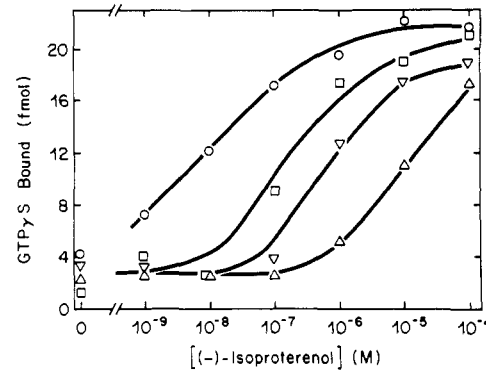

FIGURE 4: Effect of increasing concentrations of isoproterenol and inhibition by alprenolol. Vesicles (26 fmol of receptor, 54 fmol of receptor-coupled G_s) were incubated at 0 °C for 2 h in buffer A with the indicated concentrations of (−)-isoproterenol, either alone (○) or in the presence of 0.5 μM (□), 5 μM (▽), or 50 μM (△) *l*-alprenolol at 0 °C. [^{35}S]GTPγS binding reactions were then initiated at 0 °C by adding vesicles to a reaction mixture containing 0.2 μM [^{35}S]-GTPγS, 50 μM *l*-alprenolol, and the indicated isoproterenol concentration. The reaction was stopped after 2 min, and bound [^{35}S]GTPγS was determined. Data shown are the mean of triplicate determinations.

The concentration of isoproterenol needed for formation of the burst complex approximated that needed for binding to the receptor. When the isoproterenol concentration was varied during the initial incubation at 0 °C, the EC_{50} was approximately 10^{-8} M (Figure 4). This value is somewhat lower than the K_d of the receptor for isoproterenol determined from competitive ligand binding assays at 30 °C ($\sim 1 \times 10^{-7}$ M; Asano et al., 1984). It is also lower than the EC_{50} of isoproterenol for stimulation of steady-state GTP hydrolysis or of the rate of binding of GTP or GTPγS to receptor–G_s vesicles at 30 °C [$(1-2) \times 10^{-7}$ M; Brandt et al., 1983; Brandt & Ross, 1986; Asano et al., 1984]. This small discrepancy may reflect the increased affinity of the receptor for agonist at low temperatures (Weiland et al., 1979). Also shown in Figure 4 is the ability of alprenolol to inhibit the formation of the burst in competition with agonist. Vesicles were incubated with the indicated concentrations of isoproterenol and

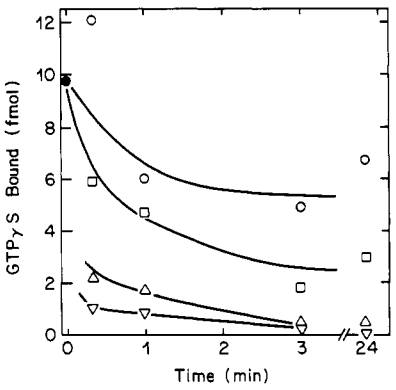

FIGURE 5: Effect of antagonist and detergent solubilization on the agonist-induced burst [^{35}S]GTPγS binding. Vesicles were incubated in buffer A with or without 10 μM (−)-isoproterenol for 90 min at 0 °C. An aliquot was removed, and [^{35}S]GTPγS binding was assayed for 2 min at 0 °C (shown as zero time, ●). The remaining vesicles were then diluted at 0 °C with 1 volume of either buffer A (○) or buffer A plus additives to yield final concentrations of 50 μM *l*-alprenolol (□), 1% CHAPS plus 50 μM *l*-alprenolol (△), or 1% digitonin plus 50 μM *l*-alprenolol (▽). Diluted samples were held at 0 °C. At the indicated times, [^{35}S]GTPγS binding reactions were initiated at 0 °C by adding vesicles to a reaction mixture containing 0.2 μM [^{35}S]GTPγS and 50 μM *l*-alprenolol with no detergent, 0.1% CHAPS, or 0.1% digitonin as appropriate. Reactions were carried out for 2 min, the reaction was stopped, and the amount of bound [^{35}S]GTPγS was determined. Each data point represents vesicles that contained 62 fmol of receptor and 134 fmol of hormone-sensitive G_s. Data shown are averages of duplicate measurements.

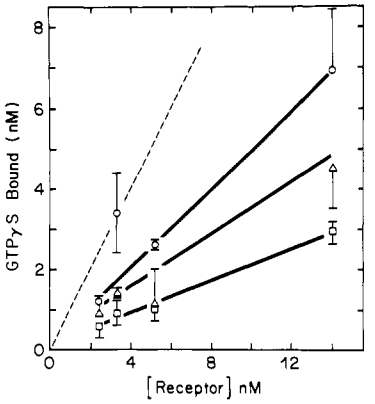

FIGURE 6: Relationship between the receptor concentration and burst size in receptor–G_s vesicles. Data are from experiments using four different vesicle preparations. The concentrations of receptor in the assay were 2.4, 3.3, 5.2, and 14 nM, and the concentrations of receptor-sensitive G_s were 8.4, 12.8, 14, and 14.5 nM, respectively. Vesicles were incubated at 0 °C in 10 μM isoproterenol for 1–2 h. Following isoproterenol treatment, vesicles were diluted with 0.1 volume of buffer A (○) (1 h), 0.5 mM alprenolol (△) (1 h), or 10% CHAPS/0.5 mM alprenolol (□) (15 min) prior to the initiation of 2-min nucleotide binding reactions at 0 °C. The binding reactions contained 0.2 μM [^{35}S]GTPγS and 50 μM alprenolol (as well as 0.1% CHAPS in vesicles treated with CHAPS/alprenolol). Data points represent the mean of three to six determinations. Error bars represent the standard deviation.

alprenolol for 2 h at 0 °C prior to assay of the burst of GTPγS binding. The family of curves appeared to demonstrate a competitive relationship between isoproterenol and alprenolol. However, the possibility of a poorly competable component of isoproterenol action was suggested by the relatively weak inhibition displayed by 0.5 μM alprenolol. At this concentration, over 100 times its K_d, alprenolol only increased the EC_{50} for isoproterenol by a factor of about 10, significantly less than predicted by simple competitive binding arguments. This anomaly also points to a kinetic component in the apparent concentration dependence on agonist and suggests that the burst complex, once formed, may be relatively stable in the time frame of the incubation.

A direct estimate of the stability of the burst complex is provided by data of the sort shown in Figure 5. In this experiment, vesicles that had been preincubated with isoproterenol were exposed to the antagonist alprenolol at 0 °C before initiation of the GTPγS binding assay. As shown, the ability to rapidly bind nucleotide decayed by about 70% over the first few minutes and reached a plateau level that was consistent with competition between agonist and antagonist for occupancy of the receptor (compare with Figure 4). The burst complex then remained stable at that level for at least 24 h. Similar results have been observed with propranolol instead of alprenolol. If the preincubated vesicles were exposed to detergent at concentrations sufficient to solubilize the receptor, there was a greater loss of the burst than was seen when antagonist alone was added (Figure 5). The magnitude of the burst decreased by 90% after 15 min of exposure to detergent, and by ~97% in 3 h. Loss of the burst was similar whether or not a β-adrenergic antagonist was added with the detergent, and similar results were observed with CHAPS, digitonin, and octyl glucoside. The addition of Lubrol 12A9 or cholate, which caused denaturation of the β-adrenergic receptor under these conditions, completely eliminated the burst at the earliest time points examined (not shown).

In several preliminary experiments, the quantitative relationship between the magnitude of the burst and the concentrations of either receptor or G_s was probed by using vesicles that contained varying amounts of one or the other protein. We found essentially no quantitative correlation between the size of the burst and the amount of G_s in the vesicles, but the extent of the burst did appear to correlate with the quantity of receptor. The relationship between GTPγS bound during the burst and the concentration of receptor is shown in Figure 6. For this experiment, four batches of vesicles that contained different amounts of receptor were prepared and their capacity to form the burst complex was measured according to three different protocols. In this and similar experiments, there was a roughly linear relationship between the receptor concentration and the magnitude of the burst. When vesicles were treated with 10 μM isoproterenol for 2 h and assayed immediately, the burst accounted for 49% of the receptors present. In similar experiments, the relationship between the burst and the receptor concentration was also linear, with the burst ranging from 45% to 90% of total receptors. In no case has the size of the burst exceeded the amount of receptors, even when G_s far exceeded the number of receptors present. When isoproterenol-primed vesicles were exposed to antagonist or to antagonist plus detergent before initiation of the GTPγS binding reaction, the size of the burst was reduced, reflecting competition between isoproterenol and alprenolol as shown above (Figure 6). However, the general correlation between burst and receptor content was maintained.

In previous studies of receptor-catalyzed binding of guanine nucleotides to reconstituted G_s, we found that the rate of binding was apparently first order with respect to the concentration of G_s but saturable with respect to the concentration of GTPγS (Asano & Ross, 1984). As shown above, the burst of GTPγS binding that occurred after preincubation of receptor–G_s vesicles with agonist was considerably faster than that observed previously. Its dependence on the concentration of GTPγS was also different from that observed in the standard agonist-stimulated reaction. As shown in Figure 7A, B, the rate of binding during the burst was pseudo first order over a reasonably wide range of nucleotide concentrations. The apparent first-order rate constants (k_{obsd}) for GTPγS binding

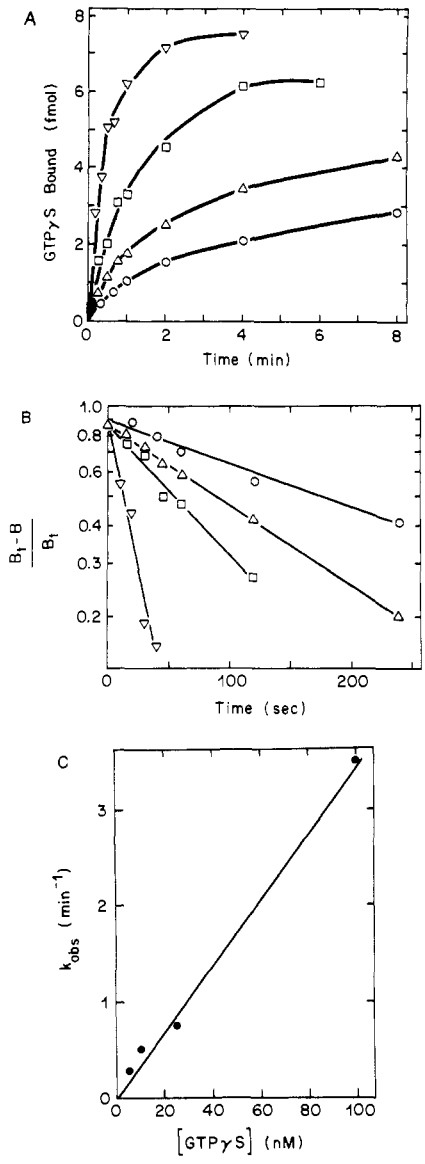

FIGURE 7: (A) Effect of nucleotide concentration on the rate of GTPγS binding to G_s in agonist-primed vesicles. Receptor–vesicles were incubated for 1 h at 0 °C in buffer A with or without 10 μM (−)-isoproterenol. *l*-Alprenolol (0.01 volume of 5 mM) was added and incubation was continued for 1 h. Nucleotide binding reactions were then initiated by the addition of vesicles to a reaction mixture containing 50 μM *l*-alprenolol and the indicated concentration of [^{35}S]GTPγS. Reactions were carried out at 0 °C for the indicated times. Each reaction volume contained 39 fmol of receptor and 51 fmol of receptor-sensitive G_s. Data points represent the average of duplicate measurements and reflect specific isoproterenol-stimulated nucleotide binding. (B) Data from part A, shown as first-order replots where B is the amount of GTPγS bound at each time point and B_t is the amount of [^{35}S]GTPγS bound at long times. These values were as follows: 5 nM, 3.5 fmol at 16 min; 10 nM, 4.3 fmol at 8 min; 25 nM, 6.25 fmol at 6 min; and 100 nM, 8 fmol at 4 min [see Asano and Ross (1984)]. The apparent first-order rate constants were determined from a linear least-squares fit of each replot. (c) Dependence on nucleotide concentration of the apparent first-order rate constants, k_{obsd}, determined in part B. The apparent second-order rate constant determined from a linear fit of these data was 3.3 × 10^7 (M·min)$^{-1}$. This experiment has been repeated 3 times with similar results.

during the burst phase increased linearly with increasing nucleotide concentration and yielded a second-order rate constant of 3.3 × 10^7 (M·min)$^{-1}$ (Figure 7C). In three similar experiments, second-order rate constants of (2.4–3.3) × 10^7 (M·min)$^{-1}$ were measured. Second-order kinetics and the high rate constant that was obtained here are consistent with the burst's reflecting a diffusion-limited, bimolecular binding reaction between GTPγS and a pool of G_s that has been primed for rapid binding during the initial incubation of the vesicles with agonist.

Discussion

The binding of GTP by G proteins and their concomitant activation has generally been described as a slow and highly regulated reaction. Reported rate constants for the binding of GTP or GTP analogues to various G proteins has ranged from 0.01 min^{-1} to approximately 5 min^{-1}, a range that is far slower than would be predicted for a simple bimolecular binding interaction. These rates can be altered as much as 100-fold by changes in the physical composition of the medium (micelle, bilayer, or solution), by an agonist-liganded hormone receptor, or by the presence of Mg^{2+}, Cl^-, or the common βγ subunits [see Gilman (1987) for review]. In contrast to these studies, G_i or G_o that has been freed of bound GDP are able to bind GTPγS with apparently diffusion-controlled kinetics (Ferguson et al., 1986). The data presented here indicate that the receptor-stimulated binding of GTPγS to G_s can be resolved temporally into an initial, relatively slow event and a subsequent, diffusion-controlled, second-order binding reaction similar to that described by Ferguson et al. (1986). This finding clarifies the order of events in the receptor-stimulated binding reaction by placing a rate-limiting step before the high-affinity binding of nucleotide. It raises the questions of the composition of the primed "burst complex" and the steps that lead to its formation.

Both the formation (Figure 3) and the dissociation (Figure 5) of the burst complex are slower than what would be expected for the reversible binding of 10 μM agonist to receptor, even at 0 °C [for [^{3}H]hydroxybenzylisoproterenol, $k_{assoc} \sim$ (3–5) × 10^7 (min·M)$^{-1}$ at 37 °C; Heidenreich et al., 1980]. This discrepancy suggests the existence of a discrete step subsequent to binding. This slow step may represent, among other possibilities, the diffusional collision of agonist-liganded receptor with G_s [see Hanski et al. (1979)]. This mechanism predicts that the rate of formation of the burst complex, as measured in the experiment of Figure 3, should increase linearly with the concentrations of G_s and receptor. Alternative candidates for the rate-limiting process are the release of either GDP or of the βγ subunits of G_s from a receptor–G_s complex [see Smigel et al. (1984)]. In any case, the product would be some complex of agonist, receptor, and the α subunit of G_s with its nucleotide binding site in an open, readily exchangeable state.

It is likely that the G_s species that rapidly binds GTPγS represents an agonist–receptor–G_s complex from which GDP has dissociated. The existence of such a complex that rapidly exchanges guanine nucleotides has been suggested by the work of Cassel and Selinger (1978), Levitzki and co-workers (Braun et al., 1982), Brandt and Ross (1986), and others. An apparent agonist–receptor–G_s complex has been resolved from a detergent extract of rat reticulocyte membranes and identified according to its size and stable binding of agonist (Limbird & Lefkowitz, 1978; Limbird et al., 1980). The dissociation of this complex by guanine nucleotide was also consistent with its role as an intermediate in the nucleotide binding reaction [reviewed by Stadel et al. (1982); see also Smigel et al., (1984)]. We have not yet succeeded in solubilizing, reisolating, and analyzing the burst complex described here because of its limited stability in solution (Figure 5). This stability is less than that predicted by the work of Limbird and Lefkowitz (1980). However, the limited effect of guanine nucleotides on the turkey erythrocyte receptor suggests that

its binding to G_s may be of lower affinity than that of receptor from mammalian cells [see Kelleher et al. (1983) and Lad et al. (1980)].

It seems unlikely that the primed form of G_s reflects merely isolated G_s, free from receptor, from which bound GDP has been displaced, although its binding characteristics are similar to those described for solubilized, unliganded G_i or G_o (Ferguson et al., 1986). Were this the case, the decay of the burst complex upon the addition of antagonist would be expected to be slower than that observed (Figure 5) because the concentrations of unliganded G_s and released GDP would be at or below the nanomolar level. Rebinding of GDP should thus be slow. Furthermore, experiments performed with a constant amount of vesicles in a total volume of 0.5 mL did not yield higher levels of burst than those performed in the usual 0.05-mL volume, even though the concentrations of unliganded G_s and of free GDP would each have been 10-fold decreased in the larger volume. The amount of burst complex was also always less than equal to the molar amount of receptor, regardless of the concentration of G_s (Figure 6), suggesting a stoichiometric involvement of receptor with the burst complex. Regardless, it will be necessary to test this possibility and the exact role of GDP release in the formation of the burst complex by preparing, reconstituting, and using nucleotide-free G_s in experiments similar to these. However, quantitative removal of GDP from G_s has so far been relatively difficult due to the instability of the unliganded protein.

In general, the results presented here are consistent with the ability of the agonist-liganded receptor to promote a conversion of the nucleotide binding site on G_s from a slowly exchanging to a rapidly exchanging form. There is apparently a sequential pattern of initial binding of agonist to receptor and interaction of the agonist–receptor complex with G protein to yield an exchangeable form of the nucleotide binding site, followed by a diffusion-controlled, second-order binding of nucleotide to this complex. The number and identity of the rate-limiting steps and their possible dependence on lateral motion of the proteins remain to be determined.

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