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Examination of the Effects of Increasing G_s Protein on β_2-Adrenergic Receptor, G_s, and Adenylyl Cyclase Interactions

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ABSTRACT. We have examined the effect of increased G_s protein levels on the abilities of three different β_2-agonists to induce GTP shifts and stimulate adenylyl cyclase response in an effort to investigate the kinetic association between the β_2-adrenergic receptor G_s and adenylyl cyclase. Agonist competition binding analysis and adenylyl cyclase concentration–response assays revealed that increases in G_s protein resulted in proportional increases in the areas of the GTP shift and adenylyl cyclase activity. Changes in the magnitude of the GTP shift were evaluated with a novel and straightforward approach for analyzing the GTP shift data that allowed us to determine the proportion of high agonist affinity binding receptor population and the apparent dissociation constant between the agonist bound receptor and G_s, regardless of the G_s protein level or the type of β_2-agonist. Using this method, we concluded that increased G_s results in the accumulation of the receptor population displaying high affinity towards agonist (HRG_s) by increasing the number of receptor–G_s complexes (to a receptor:G_s protein ratio of about 0.7 at maximal G_s expression) without affecting the affinity between hormone bound receptor and G_s. Using the G_s protein levels determined with our novel analysis, we ran simulations using the theoretical shuttle model equation that relates the EC_{50} to available G_s. Fitting the simulations to experimental data required a receptor to catalytic unit ratio of 0.45 and revealed at least two distinct stages for β_2-agonist-stimulated adenylyl cyclase activity, namely, the activation of G_s by the β_2-adrenergic receptor (a step whose rate is dependent on the type of agonist used to stimulate activity), and the activation of adenylyl cyclase by active G_s (a step whose rate is independent of the type of agonist). BIOCHEM PHARMACOL **54**;1:61–72, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. G protein; β_2-adrenergic receptor; adenylyl cyclase; stoichiometry; two-state model; GTP shift

The β_2-adrenergic receptor stimulation of adenylyl cyclase requires the presence of the stimulatory guanine nucleotide-binding protein G_s†. The exact nature of the interaction of the heterotrimeric G protein with respect to the receptor and adenylyl cyclase plays a critical role in predicting the response under conditions where G_s levels may vary [1–4]. It has been widely accepted that the ternary complex between agonist, receptor, and G_s [5, 6] is a key intermediate in the activation of adenylyl cyclase. This intermediate accumulates in the absence of GTP, which, when present, replaces GDP on the G_s protein and causes the release of the agonist receptor complex. However, the extent to which C is involved in the complex (making it a quaternary complex) has not been explored extensively. Over a decade ago, Tolkovsky and Levitzki [7] advanced kinetic arguments as to why G_s and C should be precoupled. With the assumption that G_s and C are precoupled, excellent phenomenological equations describing adenylyl cyclase activation in terms of agonist concentration can be derived, and there is no doubt that the system behaves in many ways as if it were precoupled. Since that time, the accumulation of related knowledge has made the exact mode of the G_s–C interaction more important. First, as measured immunochemically [8], there seems to be a vast excess of G_s over both receptor and C; although this is not inconsistent with the precoupling model, it seems strange in the light of it. Second, aberrant G_s proteins that are associated with tumors have been described [9]. In the heterozygous state, do aberrant G proteins compete with normal G proteins, and can their effects be mitigated by overexpressing normal G proteins? Such questions can only be satisfactorily approached with some knowledge of the strength and the duration of the G_s to C interaction.

Quantitation of the G_s to C interaction requires a knowledge of the amount of G_s that is actually present and

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†*Abbreviations:* C, catalytic unit of adenylyl cyclase; DMEM, Dulbecco's modified Eagle's medium; EC_{50}, the concentration of agonist required to elicit 50% of a maximal response; G proteins, hetrotrimeric gunine nucleotide-binding proteins; G_s, stimulatory G protein; $G_s\alpha_{long}$, long splice variant of GTP binding subunit for G_s; GTPγS, guanosine 5′-O-3-thiotriphosphate; ^{125}ICYP, [^{125}I]iodocyanopindolol; K_{RG}, dissociation constant between R and G_s; MMTV LTR, mouse mammary tumor virus long terminal repeat promoter; and R, β_2-adrenergic receptor.

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available for receptor interaction in the plasma membrane. Radioimmunoassay and cholera toxin labeling of G_s gives G_s levels in S49 cells that are many times greater than the level of the receptor [10, 11]. Nevertheless, GTP shift curves strongly suggest that the amount of G_s that is actually available to interact with the adenylyl cyclase system is present at a lower molar concentration than receptor [5]. Current means for analyzing GTP shift curves involve the use of computer programs that describe the ternary complex model via the solution to a quadratic equation or a mathematical approximation of the ternary complex model which assumes two independent receptors. These programs generally yield good estimates for the ternary complex interactions; however, Abramson *et al.* [12] suggest that caution be used in the interpretation of the affinity constants related to the formation of the ternary complex as they are not reliable. In the present paper, we introduce a novel method for analyzing binding data that gives a direct read of the G_s to R ratio and an apparent dissociation constant between hormone receptor complex (HR) and G_s without the need for curve fitting to computer programs. This adds a good deal of confidence to the quantitative basis for our analysis.

Determination of the G_s to R levels with our novel analysis allowed us to kinetically examine the G_s to C interaction in S49 cyc^- cells expressing G_s at various levels. G_s levels were varied successfully by stably transfecting S49 cyc^- cells (S49*cyc^-) with a vector containing the gene for $G_s\alpha_{long}$ under the control of the dexamethasone-inducible mouse mammary tumor virus promoter. Induction of G_s with dexamethasone for various times allowed us to measure adenylyl cyclase response at various G_s levels and thus to test alternative hypotheses of G_s to C interaction. The two broadest categories of hypothetical mechanisms are those in which G_s and C are precoupled [7, 13, 14] and those in which G_s is activated and the active G_s subsequently binds with and activates C [5, 15, 16]. It has been shown by us in the accompanying paper [4] that the existence of a shift in the concentration–response curves of S49*cyc^- cells to epinephrine to lower values in the presence of increased G_s is incompatible with a purely precoupled model. In the present paper, we look at a quantitative model for G_s to C interactions, which describes and explains the response of adenylyl cyclase to three different β_2-adrenergic agonists.

MATERIALS AND METHODS

Materials

Molecular biology reagents, DMEM and Geneticin were from Gibco BRL (Grand Island, NY); Tris base, GTP, and GTPγS from Boehringer Mannheim (Indianapolis, IN); and [α^{32}P]ATP, Na-^{125}I, and [2,8-^{3}H]cyclic AMP from NEN/DuPont (Boston, MA). Dexamethasone and the remaining reagents were from Sigma (St. Louis, MO).

Stable Transfection and Cell Isolation of an Inducible $G_s\alpha$ Cell Line

The pMMTV · $G_s\alpha$ · neo vector used to generate a unique cell line capable of varying $G_s\alpha_{long}$ protein levels was a gift from J. Gonzales and is described in detail in Ref. 17. Briefly, the 7.7 kb vector contains the cDNA encoding rat $G_s\alpha_{long}$ linked downstream of the dexamethasone-inducible MMTV LTR promoter. The vector also contains the selection marker for neomycin resistance, neomycin phosphotransferase, constitutively driven by the human β-globin promoter.

Batch clones of stable transfectants were established by electroporating 2 μg of *Pvu*I linearized plasmid into S49 cyc^- cells. The procedure and parameters for electroporation, 25 μF and 750 mV (BioRad Electroporation Unit), were similar to those described by O'Donnell *et al.* [18]. Briefly, 1×10^7 S49 cyc^- cells were prepared for electroporation by two washes in 15 mL of PBS buffer (2.68 mM KCl, 1.47 mM KH_2PO_4, 136.8 mM NaCl, and 8.06 mM Na_2HPO_4) followed by resuspension in 1 mL of "Cytomix" [19], and the addition of the linearized vector. After electroporation, the cells were allowed to recover on ice for 10 min before being diluted into 5 mL of DMEM containing 10% horse serum. The transfected cells were placed in a 37° incubator with 5% CO_2 for 24 h prior to cell isolation.

Transfected cells were isolated using a protocol similar to that of Gonzales *et al.* [17]. The day following electroporation, the cells were diluted into 20 mL of DMEM containing 500 μg/mL Geneticin (G418, final concentration 400 μg/mL). The diluted cells were pipetted as 200-μL aliquots (~10,000–40,000 cells per well) into 96-well round-bottom plates and maintained in a 37° incubator with 5% CO_2. Every 2–3 days ~100 μL of medium was replaced with fresh medium containing 400 μg/mL G418. After 2 months of selection, resistant colonies displaying G_s protein activity were selected for further analysis and are herein denoted as S49*cyc^-.

Cell Culture

Drug-resistant S49*cyc^- cell lines were maintained in stock tissue culture flasks (Corning) at 37° in HEPES-buffered DMEM supplemented with penicillin, streptomycin, 10% heat-treated horse serum, and 200 μg/mL of G418 to maintain selective pressure. Cells were expanded for $G_s\alpha$ gene induction experiments into eight individual preconditioned 1-L roller bottles (Corning) from a single stock source. The final cell density was 1×10^6 cells/mL when the volume was made up to 1 L with fresh DMEM + 10% horse serum medium. $G_s\alpha$ protein induction was initiated when the S49*cyc^- cells were incubated with 5 μM dexamethasone (final concentration, added from a 10 mg/mL stock prepared in 95% EtOH) for times that ranged from 1 to 14 hr. Transfected control cells underwent no treatment.

Membrane Preparations

Cell membranes were prepared and isolated as follows. Cells were washed twice with an excess of buffer A (137 mM NaCl, 5.36 mM KCl, 1.1 mM KH_2PO_4, and 1.08 mM Na_2HPO_4, pH 7.2) by centrifugation at 600*g*. The cells were then resuspended in ice-cold cell lysis buffer B (20 mM HEPES, 150 mM NaCl, 5 mM NaH_2PO_4, 1 mM EDTA, and 1 mM benzamidine, pH 7.4; buffer B also contained 10 μg/mL trypsin inhibitor, and 10 μg/mL leupeptin in order to protect $G_s\alpha$ from possible proteolysis) and placed in a Parr bomb (at 500 p.s.i.) for 25 min. The disrupted cells were centrifuged for 5 min at 600*g* to pellet nuclear debris. The supernatant was layered onto a 23 and 43% sucrose step gradient in HE buffer (20 mM HEPES and 1 mM EDTA, pH 8.0) and centrifuged at 85,000 *g* in a Beckman SW 28 rotor for 45 min at 4°. The membrane fraction was collected as a band at the sucrose interface. The membranes were immediately frozen in liquid N_2 and stored at −80°. Membrane concentrations were determined using the BioRad assay [20].

β_2-Agonist Competition Binding: GTP Shift Analysis

GTP shift binding analyses were used to analyze the functional coupling between the β_2-adrenergic receptor and the G_s protein. Binding analyses were carried out in 500-μL reactions with a single 80 pM concentration of the radiolabeled β_2-antagonist ^{125}ICYP (prepared by iodinating cyanopindolol, employing the protocol of Barovsky and Brooker [21] with modifications by Hoyer *et al.* [22]), in the presence of increasing β_2-agonist concentrations and the following final concentrations of the reagents: 1 mM EDTA, pH 7.4; 20 mM HEPES, pH 7.4; 10 μM phentolamine; 0.3 mM $MgCl_2$; and 20–50 μg cell membranes (diluted with HE, pH 8.0). Binding experiments were conducted in the presence and absence of 10 μM GTPγS, and non-specific ^{125}ICYP binding was determined in the presence of 10 μM alprenolol. The binding reaction took place for 55 min at 30° and was terminated with the addition of 2.5 mL of ice-cold stop buffer (50 mM Tris–Cl, pH 7.4; 10 mM $MgCl_2$). The nonhydrolyzable guanine nucleotide GTPγS was selected in lieu of GTP because preliminary experiments conducted with 10 μM GTP, albeit in the absence of $MgCl_2$, revealed a leftward shift for the +GTP curve in membranes with higher G_s levels, suggesting that a significant amount of GTP was hydrolyzed during the course of the assay. The reaction was immediately poured onto a Whatman GF/C filter, and filtered with a vacuum filtration apparatus (Millipore model 1225). An additional 2.5 mL of ice-cold stop buffer was used to rinse out the reaction tube. The GF/C filters were washed 4–5 times with 2.5 mL of stop buffer. The filters were removed to scintillation vials, and ^{125}ICYP activity was counted on a Beckman Gamma 4000 System Counter for 1 min. The activity, measured as counts per minute, was converted into units of femtomoles per milligram with the aid of a spreadsheet program using Lotus. The specific binding of ^{125}ICYP for each concentration was determined as mean values of triplicate measurements for total binding less the mean values of triplicate measurements for non-specific binding. Data were analyzed using a spreadsheet program on Excel using the equations described below for GTP shift analysis. The data points were plotted as log [agonist] versus the normalized fraction of agonist bound, using Graph-Pad. The area of the GTP shift for each set of curves was calculated by the trapezoid method. Comparisons of data ± range were performed using a two-sample Student's *t*-test ($P < 0.05$).

Adenylyl Cyclase Assay

Adenylyl cyclase assays were conducted as described by Salomon *et al.* [23], with some modifications by Clark *et al.* [24]. Briefly, assays were carried out in 100 μL volumes with the following reagents (given as final concentrations): 40 mM HEPES, pH 7.7; 1 mM EDTA; 0.3 mM $MgCl_2$; 8 mM creatine phosphate; 16 U/mL creatine phosphokinase; 0.2 mM ATP; 10 μM GTP; 0.1 mM 3-isobutyl-l-methylxanthine; and 2×10^6 cpm [α-^{32}P]ATP. Reactions included agonist, solubilized in thiourea-ascorbic acid, and were initiated with the addition of cell membranes (20–30 μg of cell membranes diluted in HE buffer, pH 8.0). Triplicate points were measured for each agonist concentration. Adenylyl cyclase concentration–response curves were fit initially to a sigmoidal least squares non-linear regression procedure (Graph-Pad, ISI Software) to determine EC_{50} values. Comparisons of EC_{50} values were performed using analysis of variance (Excel). Curves were also analyzed using the equation for cyclase response in a two-step system, shown below, using an Excel spreadsheet.

Analysis of Adenylyl Cyclase Concentration Response via a Two-Step Process

The steady-state fraction of adenylyl cyclase activity is best described as the solution to the quadratic equation shown below [4]:

$$G_s^*C = \frac{\left(C_{total}+\frac{k_{-1}}{k_3}+[G_s]_{total}\theta\right) - \sqrt{\left(C_{total}+\frac{k_{-1}}{k_3}+[G_s]_{total}\theta\right)^2 - 4C_{total}[G_s]_{total}\theta}}{2}$$

where $\theta = k_2[HR]/(k_2[HR] + k_{-1})$. However, a simplified version of the equation can be approximated by a rectangular hyperbola as shown below:

$$\frac{[G_s^*C]}{[C]_{total}} = \frac{\left(\frac{(k_2r)([G_s]_{total})}{\left([C]_{total} + \frac{k_{-1}}{k_3}\right)(k_2r + k_{-1}) + (k_2r)([G_s]_{total})}\right)[H]}{\left(\frac{\left([C]_{total} + \frac{k_{-1}}{k_3}\right)(k_{-1})}{\left([C]_{total} + \frac{k_{-1}}{k_3}\right)(k_2r + k_{-1}) + (k_2r)([G_s]_{total})}\right)K_d + [H]}$$

where r = total receptor density, $[G_s]_{total}$ = total G_s protein, $[C]_{total}$ = total amount of activatable adenylyl cyclase catalytic unit, $[G_s^*C]$ = activated cyclase, k_{-1} represents the rate constant for inactivation, a first-order GTPase process intrinsic to the G_s protein (independent of association to C [25, 26]), k_2 the activation rate constant for [HR] dependent activation of $[G_s]$, and k_3, the rate constant for the association between activated G_s^* and inactive C.

To analyze the accuracy of the quadratic equation above, experimental EC_{50} data obtained from adenylyl cyclase assays for each of the agonists were compared to EC_{50} curves calculated using the quadratic equation. Although EC_{50} values from Michaelian responses can be read directly from the Michaelian equation when it is arranged as $V_{max}[H]/(EC_{50} + [H])$, the calculation of EC_{50} values from the quadratic equation is more elaborate. The procedure for determining the EC_{50} in terms of the amounts of G_s protein, catalytic unit, and the rate constants (k_2, k_3, and k_1) is to solve the quadratic:

$$[G_s^*C]^2_{max} - \left([C]_{total} + \frac{k_{-1}}{k_3} + [G_s]_{total}\theta_{max}\right)[G_s^*C]_{max} + [C]_{total}[G_s]_{total}\theta_{max} = 0$$

in terms of $[C]_{total}$, $[G_s]_{total}$, k_{-1}, k_2, and k_3 with agonist concentration set at infinity [i.e. saturating concentrations so that $\theta_{max} = k_2r/(k_2r + k_{-1})$], then substitute this solution for $[G_s^*C]_{max}$ in the expression:

$$\left(\frac{[G_s^*C]_{max}}{2}\right)^2 - \left([C]_{total} + \frac{k_{-1}}{k_3} + [G_s]_{total}\theta\right)\frac{[G_s^*C]_{max}}{2} + [C]_{total}[G_s]_{total}\theta = 0$$

where the value of θ is determined in order to satisfy the equation. Finally, the EC_{50} of the reaction is determined using the fact that since $[HR] = [H]r/(K_d + [H])$, θ is (by definition):

$$\theta = \frac{\left(\frac{k_2r}{k_2r + k_{-1}}\right)[H]}{\left(\frac{k_{-1}}{k_2r + k_{-1}}\right)K_d + [H]}$$

enabling us to calculate the value of [H] (i.e. EC_{50}) which gives a value of $[G_s^*C]$.

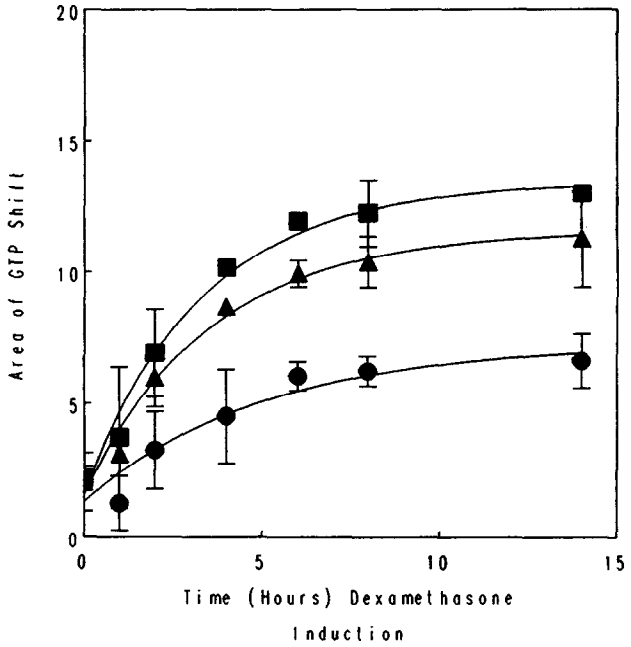

FIG. 1. Comparison of the areas of the GTP shifts for epinephrine, fenoterol, and dobutamine to S49*cyc⁻ membranes. The data representing areas of the GTP shift for epinephrine (■), fenoterol (▲), and dobutamine (●) were determined as discussed in Materials and Methods and are plotted relative to the time of dexamethasone induction. Each point on the curves represents the mean ± range of two area determinations from independent membrane preparations treated for similar times with 5 μM dexamethasone. The data were plotted using Graph-Pad.

RESULTS

Agonist-Induced GTP Shift

The ability of different β_2-agonists to produce the functional coupling between the β_2-adrenergic receptor with increasing G_s levels was examined using GTP shift analysis. Induction of the S49*cyc⁻ cells with 5 μM dexamethasone resulted in a time-dependent increase in the area of the GTP shift for all three β_2-adrenergic agonists examined: the strong agonist epinephrine, the moderate agonist fenoterol, and the weak agonist dobutamine (Fig. 1). The uninduced control membranes displayed a small, but detectable, GTP shift, suggesting the existence of low basal $G_s\alpha$ protein expression. Following 2 hr of dexamethasone induction, the area between the curves increased proportionally for all three agonists with respect to time. The absolute differences in the areas of the GTP shift at each G_s protein level reflected the anticipated efficacy of the β_2-agonist (i.e. epinephrine > fenoterol > dobutamine). The change in the area of the GTP shifts with dobutamine was consistently less than that for the stronger agonists approaching a maximum shift ~60% of that for epinephrine. The increases in the areas were not associated with an overall increase in the β_2-adrenergic receptor number (data not shown) or a change in the K_d for agonist binding in the presence of GTPγS to the receptor following dexamethasone induction (Cheng and Prusoff [27] K_i for epineph-

rine = 1.10 ± 0.12 μM, fenoterol = 0.41 ± 0.07 μM, and dobutamine = 1.47 ± 0.18 μM).

Novel Scatchard Analysis of GTP Shift Binding Data

The functional interaction between the β_2-adrenergic receptor and induced G_s protein was examined using a novel method for calculating the percentage of receptors displaying high affinity towards agonist and the dissociation constant, K_{RG}, between HR and G_s proteins. The method involved the use of Scatchard plots and required the determination of the amount of receptor bound to agonist ([HR]) and receptor–G_s complex bound to agonist ([HRG_s]) in the absence of GTP. The resulting Scatchard plots of [HRG_s] versus [HRG_s]/[HR] yielded the percentage of receptors found in the high affinity agonist bound form from the X-intercept, and the dissociation constant K_{RG} between HR and G_s from the negative inverse of the slope of the line.

The values for [HR] and [HRG_s] used in the Scatchard plots were determined by first conducting agonist competition binding studies using the radiolabeled antagonist ^{125}ICYP, in the presence and absence of GTP. Binding data were transformed from the experimentally measured values of radiolabeled antagonist bound as a function of agonist concentration to calculated values of agonist bound as a function of agonist concentration (Fig. 2). The fraction of receptors occupied by agonist is given directly in a ^{125}ICYP competition profile by the fractional decrease in the binding of the ^{125}ICYP. This is because the ^{125}ICYP, at a given concentration, occupied a constant fraction of receptors not bound by the competing ligand, so that a (say) 50% reduction in the ^{125}ICYP binding requires a 50% occupancy by the competing ligand. The fraction of receptors occupied by agonist is therefore obtained simply from the expression (Bound $^{125}ICYP_{control}$ − Bound $^{125}ICYP_{agonist}$)/(Bound $^{125}ICYP_{control}$). The argument which follows seeks to determine the amounts of agonist/receptor complex (HR) and of agonist/receptor/G_s complex (HRG_s) from GTP shift curves by making use of the above relationship and of a second assumption (considered further in the Discussion), namely that in the −GTP case receptor unbound by agonist is not precoupled significantly to G_s. The consideration of the units of the concentrations of the receptor complexes and the free energy of the receptor to G protein interaction is discussed below.

The amount of [HR] and [HRG_s] along the −GTP curve was determined in the following manner: at some fraction of total receptor bound by agonist, say α, the liganded receptors are found associated ([HRG_s]) and unassociated ([HR]) with G_s proteins such that the fraction of the total binding population can be denoted as:

$$\alpha = [HR] + [HRG_s] \qquad (1)$$

where α is the fraction of maximal agonist binding achieved at the agonist concentration under consideration.

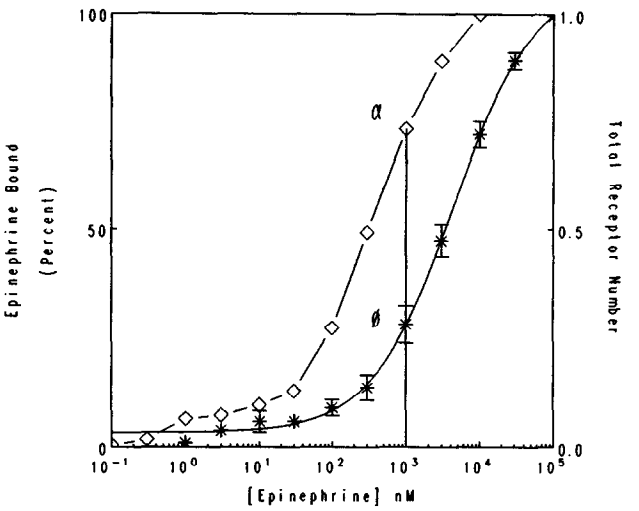

FIG. 2. Representation of agonist binding to β_2-adrenergic receptors. Data from a competition binding study using S49*cyc$^-$ membranes induced for 6 hr with 5 μM dexamethasone are replotted as the amount of epinephrine bound to the β_2-adrenergic receptor in the presence of increasing epinephrine concentrations. The binding isotherms were generated by competitive binding between an ~80 pM concentration of the radiolabeled antagonist ^{125}ICYP in the presence of increasing concentrations (0.3 nM to 100 μM) of agonist. The percentage of receptors bound to agonist in the presence (*) and absence (◇) of 10 μM GTPγS (the y-axis) was determined using the following relationship: % agonist bound = 100%—normalized ^{125}ICYP bound. At a constant concentration of epinephrine, indicated by the vertical line, the two points, α and ϕ, represent the amount of epinephrine bound to receptor alone (giving HR) plus receptor complexed to G_s (giving HRG_s); and to receptor alone (HR), respectively. The data were plotted using GraphPad.

In this equation and subsequent equations in this section, [HR] is the *fraction* of total receptor that is bound to agonist and [HRG_s] is the *fraction* of receptor bound to agonist and G_s. These quantities are, therefore, without dimension.

While the [HR] to $[R]_{total}$ ratio is Michaelian with respect to [H], the [HR] to $[R]_{free}$ ratio is always exactly equal to $[H]/K_d$, whether or not [HRG_s] is present; therefore, the proportion of [HR] relative to the total receptor population in the absence of GTP (where $[R]_{total} = [R]_{free} + [HR] + [HRG_s]$, and $[R]_{total}$ by definition is equal to 1) at point α is given by the following relationship:

$$[HR] = (1 - [HRG_s])\phi \qquad (2)$$

where ϕ represents the fraction of receptor bound to hormone [HR] in the presence of GTP and at the same concentration of agonist required to produce α.

By substituting [HR] from equation (2) into equation (1), the amount of [HRG_s] for any given concentration of hormone can be given as:

$$[HRG_s] = \frac{\alpha - \phi}{1 - \phi} \qquad (3)$$

By substituting $[HRG_s]$ from equation (3) into equation (2), the amount of [HR] for any given concentration of epinephrine on the −GTP curve can finally be defined as:

$$[HR] = \frac{\phi(1 - \alpha)}{(1 - \phi)} \tag{4}$$

(A second method for calculating ϕ, which reduces the amount of error from any individual experimental point on the +GTP curve, requires the determination of the experimental dissociation constant K_{de} (a unique value for each experiment where agonist displaces 50% of the labeled antagonist—not to be confused with the Cheng–Prusoff K_i) and the Hill coefficient, n, for any particular +GTP curve by using a Sigmoid non-linear regression analysis. The values for K_{de} and n are substituted into the Langmuir binding isotherm equation $[\phi = [H]^n/(Kde^n + [H]^n)]$, and the amounts of [HR] and $[HRG_s]$, at any given hormone concentration, are then calculated using equations 3 and 4 above.) With corresponding values for $[HRG_s]$ and [HR], one can further determine the dissociation constant between the receptor and G_s, K_{RG}, using the following relationship:

$$K_{RG} = \frac{[HR][G_s]}{[HRG_s]} \tag{5}$$

The conservation of mass applied to the scheme above reveals that the amount of G_s in the system is equivalent to:

$$[G_s] = [G_s]_{total} - [HRG_s] \tag{6}$$

Substitution of $[G_s]$ from equation (6) into equation (5), followed by rearrangement yields a linear equation:

$$\frac{[HRG_s]}{[HR]} = \frac{[G_s]_{total}}{K_{RG}} - \frac{[HRG_s]}{K_{RG}} \tag{7}$$

where the x-intercept represents the percentage of hormone binding receptors that display high affinity towards the agonist, and −1/slope yields the value for the dissociation constant, K_{RG}, between the receptor and G_s.

The method of determining the quantities we label as [HR] and $[HRG_s]$, in fact, determines the fraction of total receptor in these forms. This is equivalent to defining the total concentration of receptors as unity, and giving HR and HRG_s in terms of that concentration. Similarly, the concentrations of G_s are then automatically given as a concentration ratio to the total receptor. It should also be noted that this approach defines the "standard state" in the thermodynamic sense as to whatever the initial concentration of receptors happens to be. The free energy for the reaction using the standard relationship $\Delta G = -RT\ln K$ gives the free energy change when 1 mol of HR, at a concentration equal to the total receptor concentration, interacts with 1 mol of G_s at that same concentration to produce 1 mol of HRG_s at the same concentration. This seems to be a more useful approach since the free energy calculations use concentrations of the same order of magnitude as those actually present in the system, rather than with reference to a hypothetical standard state (such as 1 pmol/mg membrane protein), which would give a standard free energy change bearing little relationship to experimental values.

Scatchard Analysis of Agonist Binding Curves

The data from GTP shift experiments conducted with three different β_2-agonists on S49*cyc$^-$ membranes induced for increasing times with dexamethasone (data not shown) were transformed to Scatchard plots using the method described above. Figure 3A–C represents a typical depiction of the transformed binding data for epinephrine, fenoterol, and dobutamine in plasma membranes containing the same level of G_s. The transformed data were readily fit to a linear function with R^2 values typically >0.90, regardless of the type of agonist used to induce the GTP shift or the G_s protein level present in the plasma membrane preparation. The parameters obtained from the Scatchard plots were in excellent agreement with values obtained using non-linear regression analysis of HRG formation as a function of HR concentration. Note that this is quite different from the non-linear regression fitting of HR and HRG as a function of agonist concentration as estimated by a number of commercial curve-fitting programs.

The x-intercept values of the Scatchard plots, representing both the proportion of total receptors displaying high agonist affinity and the total G_s protein level capable of coupling to the β_2-adrenergic receptor, increased from 0.24 for uninduced control membranes to a maximum value of 0.69 following 14 hr of dexamethasone induction. (The proportion of receptors displaying high agonist affinity after 14 hr of dexamethasone induction (0.69) agreed with the 0.60 to 0.80 values demonstrated for S49 wild type membranes (data not shown) and previously published observations [28].) The maximum x-intercept, i.e. $[HRG_s]_{max}$, of ~0.7 also suggests that it is possible (or likely) that other G_s protein in the system is not available to the receptor, and hence 0.7:1 may not reflect the total G_s levels in the preparation as determined immunochemically. The range of the x-intercept also increased in concert with changes in magnitude of agonist-stimulated adenylyl cyclase response.

The −1/slope values, representing the thermodynamic dissociation constant between agonist bound receptor and the G_s protein, were 0.007 ± 0.002 for epinephrine, 0.007 ± 0.003 for fenoterol, and 0.029 ± 0.016 for dobutamine. (These values represent the average ± the SD of the negative inverse slopes from Scatchard plots derived from a series of eight membrane preparations produced from two independent dexamethasone induction time–course experiments.) For these plots $[R]_{total}$ is arbitrarily set at unity; therefore, the units for the dissociation constant are relative to the concentration of the receptor in the mem-

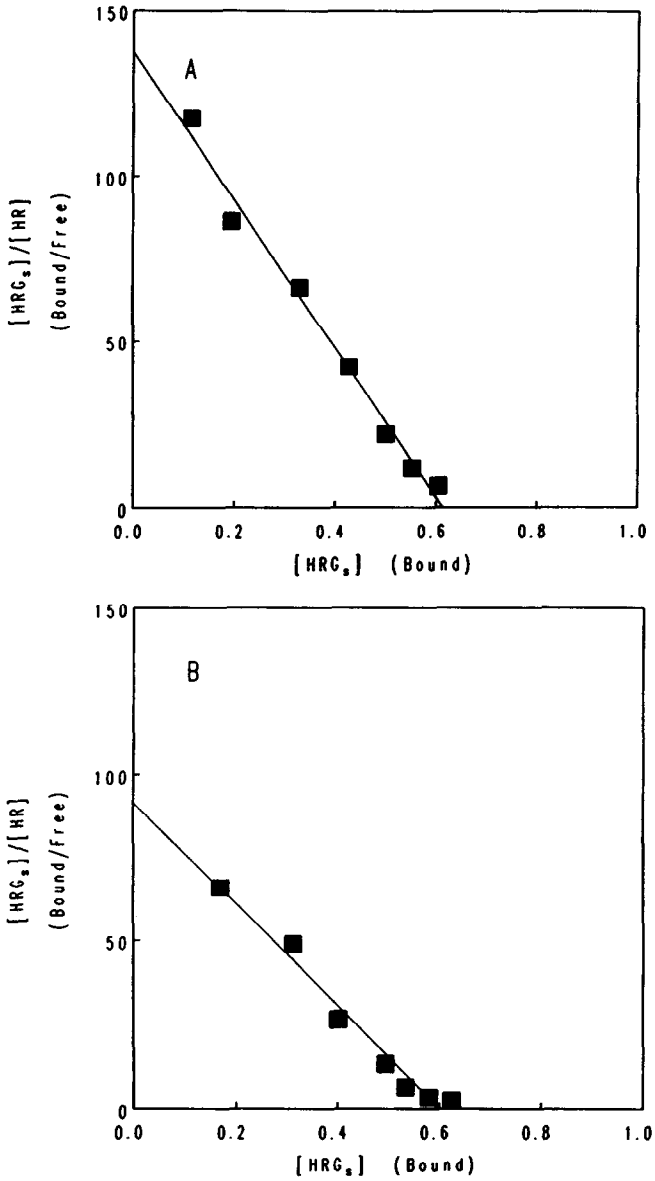

FIG. 3. Scatchard plots of (A) epinephrine, (B) fenoterol, and (C) dobutamine binding to the β_2-adrenergic receptor. Data representing the 6-hr 5 μM dexamethasone-induced membrane preparation from GTP shift analyses were transformed and replotted as Scatchard plots as discussed in Materials and Methods. The HRG axis is in receptor units with total receptor defined as 1.0 (see Discussion). The data were generated using a spreadsheet on Excel and plotted using Graph-Pad.

brane. Since the average $[R]_{total}$ for this membrane preparation was 0.2 ± 0.01 pmol/mg, the final K_{RG} values in terms of pmol/mg of protein would be 0.0014, 0.0014, and 0.0058, respectively, for epinephrine, fenoterol, and dobutamine. The differences in the magnitude of the K_{RG} values for each of the agonists are consistent with either a multi-state model for receptor conformation where the agonist induces different conformations of the receptor that alter the rate of HRG formation, or a two-state model for receptor conformation where weaker agonists produce the same active form of the receptor as stronger agonists, albeit at lower levels.

The dissociation constant for the HRG complex into HR and G was also determined using the Graph-Pad non-linear fit for a rectangular hyperbola with [HR] as the independent variable and [HRG] as the dependent variable. This extra analysis was made because the dependent and independent variables in a Scatchard plot ([HRG]/[HR] and [HRG], respectively) are not actually independent of each other. The values we obtained for $[HRG]_{max}$ on no occasion differed by more than 2.5% from the two approaches. We therefore retained the values for $[G_s]_{total}$ obtained from the Scatchard plot for subsequent calculations since there was no significant difference in any case. The data are subsequently presented in the form of Scatchard plots, instead of being directly plotted as [HRG] versus [HR], because this type of plot allows one to see more easily that the data are well described by a rectangular hyperbola.

Agonist-Stimulated Adenylyl Cyclase Activity

To examine the effects of increased G_s levels on partial agonist activation of adenylyl cyclase, three different β_2-agonists (epinephrine, fenoterol, and dobutamine) were used to stimulate adenylyl cyclase activity in membranes prepared from dexamethasone-induced S49*cyc$^-$ cells (Fig.

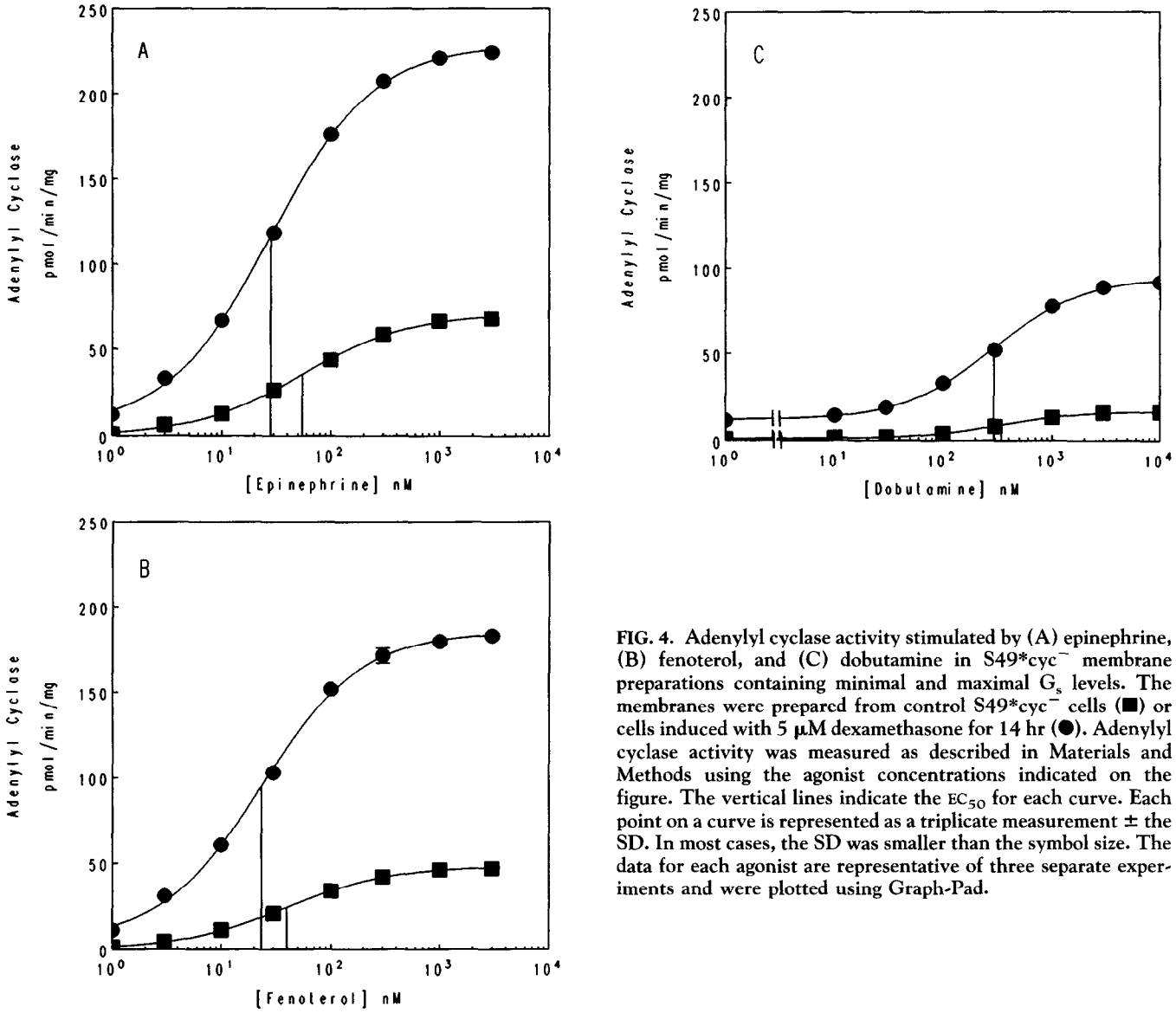

FIG. 4. Adenylyl cyclase activity stimulated by (A) epinephrine, (B) fenoterol, and (C) dobutamine in S49*cyc$^-$ membrane preparations containing minimal and maximal G_s levels. The membranes were prepared from control S49*cyc$^-$ cells (■) or cells induced with 5 μM dexamethasone for 14 hr (●). Adenylyl cyclase activity was measured as described in Materials and Methods using the agonist concentrations indicated on the figure. The vertical lines indicate the EC_{50} for each curve. Each point on a curve is represented as a triplicate measurement ± the SD. In most cases, the SD was smaller than the symbol size. The data for each agonist are representative of three separate experiments and were plotted using Graph-Pad.

4A–C). Comparisons of the agonist/response curves for the three agonists revealed similar increases in dexamethasone-induced basal cyclase activity [from 1.0 ± 0.4 pmol/min/mg for uninduced cells to 11.5 ± 0.5 pmol/min/mg for 14-hr induced membranes (N = 3)] when basal activity was examined in a particular membrane preparation. Agonist stimulation of uninduced control membranes revealed the presence of some basal G_s protein activity, in agreement with the agonist competition binding analysis. Moreover, agonist/response curves were always Michaelian and first order regardless of the G_s protein level and/or the type of agonist used to stimulate activity. Increases in G_s protein levels following dexamethasone induction resulted in agonist-dependent differences for V_{max}, when compared in membrane preparations containing the same level of G_s (e.g. epinephrine, fenoterol, and dobutamine generated maximum activities of 224.2 ± 1.3, 182.9 ± 3.4, and 91.6 ± 0.6 pmol/min/mg, respectively, following 14 hr of dexamethasone induction). Furthermore, the different agonists exhibited different abilities to cause a reduction in the relative EC_{50} for adenylyl cyclase activation in the presence of increasing G_s. An analysis of variance demonstrated a significant difference in the relative decreases in EC_{50} in the presence of high G_s levels compared with low G_s levels with stronger agonists inducing larger decreases in EC_{50} than weaker agonists [epinephrine −44.3 ± 9% (N = 3), fenoterol −29.2 ± 11% (N = 3), and dobutamine −14.0 ± 2% (N = 3)].

Quantitative Analysis of β_2-Agonist Activity via a Shuttle Mechanism

The effects of the three different β_2-agonists on adenylyl cyclase response were examined quantitatively by comparing the EC_{50} values for each agonist at increasing G_s levels to the EC_{50} values calculated from the quadratic equation

shown in Materials and Methods. The calculated EC_{50} values were fit to the experimental data using 4 input values for the parameters involved in hormone stimulated adenylyl cyclase activity; these were the R:G_s ratio, the R:C ratio, the k_2:k_{-1} ratio, and the k_3:k_{-1} ratio, where k_2 was the rate constant for G_s activation by the receptor, k_3, the rate constant for C activation by G_s^*, and k_{-1}, the rate constant for G_s^* and G_s^*–C inactivation. The R:G_s ratios were determined by Scatchard analysis for each membrane preparation, leaving the R:C ratio, the k_2:k_{-1} ratio, and the k_3:k_{-1} ratio as arbitrary inputs. Of these last three inputs, the R:C ratio and the k_3:k_{-1} ratio must be the same for all agonists, while the k_2:k_{-1} ratio will vary since it describes the agonist dependent step. Figure 5 demonstrates the good agreement between experimental and simulated data, where the relative k_2:k_{-1} values used in these analyses were 18 for epinephrine, 8 for fenoterol, and 2.3 for dobutamine, reflecting the anticipated effectiveness of the agonist when agonist effectiveness is defined by its "coupling efficiency" [29].

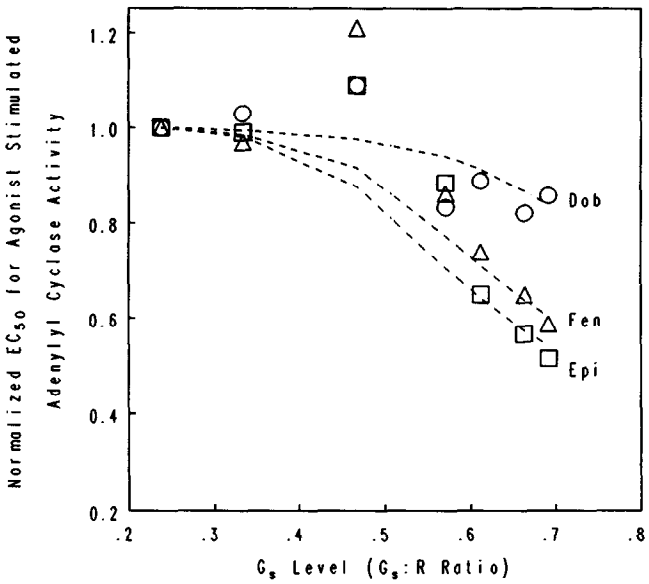

FIG. 5. Comparison between experimental EC_{50} values obtained from β_2-adrenergic agonist stimulation of adenylyl cyclase and EC_{50} values calculated from the quadratic solution to a simple shuttle mechanism for adenylyl cyclase activation. The experimental EC_{50} values for epinephrine (□), fenoterol (△), and dobutamine (○) were determined by fitting adenylyl cyclase concentration–response data to a sigmoidal non-linear regression analysis (Graph-Pad). The EC_{50} values were normalized with respect to the EC_{50} at low G_s for each agonist. All experimental EC_{50} values can be obtained by multiplying the respective normalized values by 54.8 nM for epinephrine, 39.8 nM for fenoterol, and 341.5 nM for dobutamine. The $[G_s]_{total}$ values were determined for each membrane preparation using the G_s to R ratio yielded from the Scatchard analysis of GTP shift binding data and were as follows: control (0.24), 1 hr dexamethasone induction (0.33), 2 hr (0.47), 4 hr (0.57), 6 hr (0.61), 8 hr (0.66), and 14 hr (0.69). The data shown here are representative of three separate experiments conducted on independent membrane preparations where G_s levels have varied. The very high values for the EC_{50} of all three agonists at $[G_s]_{total}$ = 0.47 were not replicated in any other preparation. The predicted EC_{50} values (indicated as dashed lines for each agonist) were determined as described in Materials and Methods, using the following parameters as constants: k_{-1} (1.0), k_3 (200.0), $[R]_{total}$ (1.0), and $[C]_{total}$ (0.45). The values for K_d, the dissociation constant for the agonist, were determined experimentally (K_d = 1100 nM for epinephrine, 400 nM for fenoterol, and 1500 nM for dobutamine). The k_2 values were allowed to vary in order to find a good fit. The final k_2 values were epinephrine, 18; fenoterol, 8; and dobutamine, 2.3.

DISCUSSION

HR and G_s Interactions in the Presence of Increasing G_s

This study was initiated in order to quantitatively examine the R, G_s, and C interactions in a plasma membrane system where the G_s levels have been varied. Our accompanying manuscript [4] demonstrated that a pure G_s–C precoupled mechanism was incompatible with a decrease in EC_{50} in the presence of increasing G_s. Here we have examined the abilities of three different β_2-agonists (epinephrine, fenoterol, and dobutamine) to stimulate adenylyl cyclase activity in order to compare the effect of increased G_s levels on EC_{50} so as to further examine the G_s to C shuttle model. To compare experimental EC_{50} values for the different agonists in the presence of increasing G_s to EC_{50} values simulated from a shuttle model, it was necessary to ascertain the G_s level capable of interacting with the β_2-adrenergic receptor. Therefore, a novel method for analyzing competition binding data was developed that not only allowed us to determine the G_s levels in each membrane preparation, but also provided the additional opportunity to examine the interaction between agonist/receptor and G_s.

The interaction between the β_2-adrenergic receptor and the heterotrimeric G_s protein was examined by transforming competition binding data to a more useful Scatchard method of analysis. Our method contains advantages over the current computer models that attempt to fit agonist competition binding data to either the quadratic solution of the ternary complex model or to a two-independent receptor model because our method enables us to arrive at the HR to G_s interaction parameters in a more objective way than has been possible previously. If the equilibrium concentration of the RG_s binary complex in a membrane is low, then straightforward measurements from GTP shift data give the concentration of HR and HRG_s at any agonist concentration. The dissociation of HRG_s into HR and G_s can then be described by $K_{RG} = [HR][G_s]/[HRG_s]$. The dissociation equation can be arranged into Scatchard plot form and a plot of $[HRG_s]/[HR]$ against $[HRG_s]$ should give a straight line with slope equal to $-1/K_{RG}$. Current computer programs that analyze agonist binding to R and RG_s do not allow for accurate HR and G_s affinity constants. Moreover, the x-intercept in such a plot gives an accurate and objective analysis of the amount of G_s protein interacting with the R as a G_s:R ratio. It can be measured with more precision than any technique that relies on antibody

procedures and measures only the interacting G_s protein, rather than total cellular G_s protein, much of which may be irrelevant (discussed in more detail below).

To evaluate the new model, agonist competition binding studies were performed with three different β_2-agonists ranging in coupling efficiencies [29] from the strong agonist epinephrine to the moderate agonist fenoterol and the weak agonist dobutamine. Results from the GTP shift analysis (Fig. 1) revealed that the area of the GTP shift was dependent on the G_s protein level and the type of agonist used to induce the shift. Scatchard transformations of the agonist competition binding isotherms demonstrated the expected proportional increases in the high agonist affinity receptor binding population (i.e. HRG_s) relative to the increases in the areas of the GTP shifts. Because the x-intercept values that represent the high agonist affinity receptor binding population also represent the total G_s protein level that can couple to the receptor (see equation 7 of the Results), the x-intercept values for a particular membrane preparation should be identical regardless of the type of β_2-agonist used to induce the GTP shift (Fig. 3A–C). This means that the differences between the agonist-induced areas of the GTP shift (Fig. 1) should be attributable to the apparent dissociation constant between HR and G_s. In the Samama *et al.* extended two-state model for receptor conformation [6], the receptor exists in either an inactive R state or an active R* state, where only the R* state is capable of interacting with G_s. Under these conditions, the difference in apparent affinities between HR and G_s can be accounted for by the distribution of hormone bound to R and R*, i.e. HR $\leftrightarrow$ HR* as predicted by Richardson's analysis of the ternary complex model [30]. The binding of epinephrine would more likely favor the HR* conformation than, say, dobutamine. Initial studies appear to support this prediction, as weaker agonists appear to have a reduced apparent affinity (measured as the -1/slope of the Scatchard plot) between HR and G_s relative to epinephrine (Fig. 3A–C). (An alternative hypothesis to the two-state model, which could account for the reduced apparent affinity between HR and G_s for weaker agonists, is a multi-state model that would attribute the differences in the rate of HRG_s formation to the different agonist stabilized receptor conformations.)

The presence of RG_s in the equilibrium mixture along with HRG_s, HR, and R in the $-$GTP case in no way affects the thermodynamic consideration of the interaction between HR and free G_s to give HRG_s. Moreover, a large fraction of an RG_s interaction is precluded if there is veracity to the two-state receptor model of DeLean *et al.* [5]. However, as our estimate of the amount of HR in the agonist bound fraction is calculated from the assumption that the fraction not bound by agonist is R only, the presence of a significant amount of RG_s would cause a small error in our estimates for HRG_s and potentially much larger ones for HR. This potential error would be greatest when the ratio of G_s to R is high and should, if it were significant at any time, be greatest at low agonist concentrations in the membrane preparations where the G_s to R ratio is about 0.70. It should be clear that as $[HRG_s]$ approaches saturation (at high agonist concentrations) the amount of G_s not bound up as HRG_s is small and, therefore, becomes negligible irrespective of the conditions at low agonist (or no agonist) concentration. For this reason, whatever the initial conditions of R to G_s interaction, we expect the approach given in Results to determine the equilibrium constant between HR and G_s to work well as $[HRG_s]$ approaches saturation.

The demonstration of a G_s:R ratio of $\sim$0.7:1 in S49*cyc$^-$ membranes containing high levels of G_s agrees well with the findings from competition binding studies for other G-protein coupled receptors [5, 31, 32]. These data would suggest that G_s is limiting with respect to R; however, Ransnäs and Insel [33] have demonstrated with the use of an enzyme-linked immunosorbent assay that the molecular ratio of G_s:R in S49 WT cells is $\sim$40:1. This apparent paradox between the molecular G_s levels and those G_s levels that interact with the β_2-adrenergic receptor has led Neubig [34] to suggest that the cell restricts receptor access to a portion of G_s by: (1) organizing protein components into physically separate compartments, and (2) establishing biochemical differences between the receptor and G_s. This also suggests that the excess G_s may be kept in reserve or serve other functions within the cell, such as interacting with microtubules, as has been intimated by Leiber *et al.* [35].

Agonist-Stimulated Adenylyl Cyclase Activation

We have demonstrated previously that the EC_{50} value for epinephrine-stimulated adenylyl cyclase activity decreases with increasing G_s levels, confirming the prediction of a shuttle model (in general terms) [4]. With good estimates for the amount of G_s protein actually interacting with the β_2-adrenergic receptor (obtained from Scatchard analysis), we have now been able to test more specific predictions of a simple shuttle model. This was done by comparing simulated EC_{50} data with experimental EC_{50} data for three different β_2-agonists in the presence of increasing G_s levels.

In fitting the EC_{50} values derived from the model to the experimental data, there are only limited ranges of parameter values that allow for a reasonable fit. Thus, if the proposed interaction sequences that are used to generate the model are a good description of reality, then the stoichiometry required for the fits should represent the stoichiometry within the membrane preparations. The scheme for the simple shuttle model, as shown in Materials and Methods of the accompanying manuscript [4], suggests that the one variable that differs from agonist to agonist should be k_2 (the rate constant that determines the activation of G_s by GTP/GDP exchange). All other parameters, such as k_{-1} (the rate constant for inactivation), k_3 (the rate of C activation by G_s^*), $[C]_{total}$, and $[G_s]_{total}$,

should be constant for all agonists for any particular membrane preparation.

Figure 5 demonstrates that it is possible to get good fits for all three of the β_2-adrenergic agonists used in this study by varying only $k_2{:}k_{-1}$. The inactivation constant (k_{-1}) only appears in the quadratic equation as a quotient with k_2 or k_3, and it can, therefore, be arbitrarily set to 1, and k_2 and k_3 are then set as multiples of it. (This procedure is always possible when dealing with a steady state.) The value chosen for $k_2{:}k_{-1}$ determines the value of EC_{50} when $[G_s]_{total}$ is small compared with R. That is, it determines the initial horizontal part of the EC_{50} versus G_s:R plot shown in Fig. 5. The $k_2{:}k_{-1}$ values are, therefore, forced by the data, and only the present values (epinephrine, 18; fenoterol, 8; and dobutamine, 2.3) are acceptable, i.e. only a very small variation is possible without destroying any sort of fit.

A good simulation of the experimental data is only possible if a large value for $k_3{:}k_{-1}$ is chosen, but any value over 20 gives the same fit. In mechanistic terms, this is equivalent to the G_s^* to C interaction being very fast compared to G_s^* and G_s^*C hydrolysis of GTP. The relatively large value for the $k_3{:}k_{-1}$ ratio also helps to explain why kinetic equations assuming a G_s–C precoupled mechanism, in a system where G_s levels are kept constant, have generally given excellent *kinetic* predictions.

The final parameter required to simulate the data was $[C]_{total}$. The $[C]_{total}$:R level of 0.45, which agrees well with measurements of the adenylyl cyclase levels in S49 cell membranes based on forskolin binding [8, 36], is forced by the point of departure of the EC_{50} versus $[G_s]_{total}$ plot from the horizontal. The greater steepness of the decline in EC_{50} for the stronger agonists relative to dobutamine at G_s:R values greater than 0.45 (Fig. 5) did not have to be specifically fitted, but arose automatically from the values of the parameters forced by the considerations discussed above. The experimental changes in EC_{50} for the different agonists (i.e. stronger β_2-agonists produce greater relative decreases in EC_{50} with increasing G_s levels than weaker agonists) are, in fact, predicted by the simple shuttle model (see equation 13 in the accompanying manuscript [4]) and only become obvious when $G_s > C$, where following agonist stimulation a more obvious spare pool of G_s^* is built up. This gives some credence to the appropriateness of the shuttle model.

Every feature of the shuttle model has not been proven by the quantitative fit of the adenylyl cyclase response data, and alternative, but similar, models could give similar fits. For instance, if there were different rates of GTP hydrolysis for G_s^* and G_s^*C, the fits with the experimental data would be at least as good. It would also be possible to use a mixed shuttle/precoupled model to quantitatively describe our data. The decline in EC_{50} at high G_s levels; however, means that a pure precoupled mechanism is not possible, but the very rapid rate of interaction between active G_s^* and C means that many of the predictions of the precoupled model are borne out in practice. It is only when $[G_s]$ is greater than [C] that it becomes clear that G_s activated separately from C can subsequently interact with C in such a way as to give a reduction in EC_{50}, a seminal feature for a shuttle mechanism. In our quantitative calculations, we have assumed no precoupling between G_s and C at all because the present scheme is the simplest model that incorporates all aspects of the shuttle model, and as it is also sufficient for a good description, we have not gratuitously complicated things by including a precoupled aspect.

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