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Evidence for the Shuttle Model for $G_s\alpha$ Activation of Adenylyl Cyclase

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ABSTRACT. Knowledge of the nature of the interaction between the stimulatory G protein (G_s) and the adenylyl cyclase catalytic unit (C) is essential for interpreting the effects of G_s mutations and expression levels on cellular response to a wide variety of hormones, drugs, and neurotransmitters. It has been proposed that β-adrenergic receptor activation of adenylyl cyclase occurs either by a two-step "shuttle" mechanism where the receptor activates G_s independently of cyclase followed by $G_s\alpha$ activation of cyclase independent of the receptor; or the receptor activates a "precoupled" G_s–C complex in a single step. Simulations of the two models revealed that the two forms of activation are distinguishable by the effect of G_s levels on epinephrine-stimulated EC_{50} values for cyclase activation; specifically, the shuttle model predicts an increased potency of epinephrine stimulation as levels of $G_s\alpha$ increase. To address this problem, S49 cyc$^-$ cells were stably transfected with the gene for $G_s\alpha_{long}$ regulated by the MMTV LTR promoter, which allowed for an induction of $G_s\alpha_{long}$ expression levels over a 40-fold range by incubation of the cells for various times with 5 μM dexamethasone. Expression of $G_s\alpha$ was strongly correlated to the appearance of GTP shifts in the competitive binding of epinephrine with [^{125}I]iodocyanopindolol to the β-adrenergic receptors and epinephrine-stimulated adenylyl cyclase activity. Most importantly, high expression of $G_s\alpha$ resulted in lower EC_{50} values for epinephrine and prostaglandin E_1 stimulation of adenylyl cyclase activity. The decrease in EC_{50} did not occur as a result of a change in β_2-adrenergic receptor, $G_i\alpha$, $G\beta\gamma$, or adenylyl cyclase levels. These novel findings demonstrate that a change in the level of a protein downstream of a plasma membrane receptor can influence hormone potency. We explain these results by using kinetic arguments to suggest that some fraction of hormone-activated adenylyl cyclase occurs via a shuttle mechanism, and not a purely precoupled mechanism. BIOCHEM PHARMACOL **54**;1:43–59, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. G proteins; β_2-adrenergic receptor; adenylyl cyclase; activation; kinetics; stoichiometry

Activation of adenylyl cyclase (C§) by hormone proceeds via a cascade that is initiated by hormonal activation of a stimulatory receptor. Activated receptors interact with, and subsequently stimulate, the heterotrimeric membrane bound stimulatory guanine nucleotide binding protein (G_s). The activated G_s protein consequently stimulates adenylyl cyclase to synthesize the second messenger cAMP from ATP.

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§ *Abbreviations:* C, catalytic subunit of adenylyl cyclase; cAMP, 3′,5′-cyclic AMP; EC_{50}, concentration of agonist necessary to elicit 50% of maximal response; G proteins, heterotrimeric guanine nucleotide-binding proteins; $G_i\alpha$, inhibitory GTP binding subunit; G_s, stimulatory G protein; $G_s\alpha$, GTP binding subunit of G_s; G_s–C, preformed G_s and adenylyl cyclase complex; GTPγS, guanosine 5′-O-(3-thiotriphosphate); G418, geneticin sulfate; HSV-1 TK, herpes simplex virus 1 thymidine kinase; ^{125}ICYP, [^{125}I]iodocyanopindolol; MMTV LTR, mouse mammary tumor virus long terminal repeat; MTI, mouse metallothionein I; PGE_1, prostaglandin E_1; PKA, protein kinase A; PVDF, polyvinylidene difluoride; and R_p-CPT-cAMPS, 8-(4-chlorophenylthio)-adenosine-3′,5′-cyclic monophosphate.

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While many kinetic models of adenylyl cyclase activation have been proposed in terms of the Cassel–Selinger "on–off" cycle [1], all of the models fall into two distinct classes with respect to G_s and C coupling. The "shuttle" model proposes that G_s is loosely associated with adenylyl cyclase in the inactive state and upon activation diffuses to, and subsequently activates, adenylyl cyclase [2–4]. The second model, the "G_s–C pre-coupled" model is based on kinetic constraints instituted within the "collision-coupling" model, which proposes that G_s is in close association with adenylyl cyclase regardless of the activation state [5–7]. Kinetically speaking, the term "in close association" refers to the kinetic constraint that the duration of the G_s and adenylyl cyclase association must be longer than the interval required for cyclase activation. This constraint does not imply that the association between G_s and adenylyl cyclase is permanent. The activation and accumulation of active G_s–C complexes occur as a result of transient collisions between hormone-activated receptor and the G_s–C complex [5]. The general shuttle and G_s–C precoupled models are each, in fact, classes of models for

which other assumptions need to be made for the complete definition of a specific model.

Experiments supporting a shuttle model have relied on the use of the non-hydrolyzable GTP analogue GTPγS in order to enhance cyclase response [8, 9]. Critics of the shuttle have pointed out that GTPγS not only activates G_s but leads to G_s dissociation into $G_s\alpha$ and $G\beta\gamma$ subunits, an event that has not been demonstrated with GTP [10]. More importantly, one would have expected a complex, non-Michaelian dependence of the rate of activation on the concentration of the non-hydrolyzable analogue with respect to the kinetics of adenylyl cyclase activation [11]. Tolkovsky *et al.* [6], however, demonstrated that hormone stimulation of turkey erythrocyte membranes yielded simple first-order Michaelian responses independent of the non-hydrolyzable GTP concentration. These authors also demonstrated that the rate constant for adenylyl cyclase activation was dependent on the β-adrenergic receptor number but apparently independent of the G_s protein and adenylyl cyclase concentrations. These findings led the authors to conclude that hormonally stimulated adenylyl cyclase activation in plasma membranes is incompatible with a shuttle mechanism.

Kinetic models of the simple first-order Michaelian adenylyl cyclase responses demonstrated in hormonally stimulated plasma membranes have served as the foundation for the G_s–C precoupled model [5]. Unfortunately, attempts to support the kinetic arguments by measuring cyclase activity in the presence of varying G_s and adenylyl cyclase levels have relied on non-specific alkylating agents [6, 12] and irreversible photoreactive GTP analogues [13] to change these "substrate" levels. These treatments may have resulted in suppressing the shuttle process.

We demonstrate that the two models can be distinguished experimentally by measurement of the change in the EC_{50} (the concentration of epinephrine at which half-maximal cyclase activity is observed) for epinephrine-stimulated adenylyl cyclase activity under conditions where the levels of G_s or adenylyl cyclase are varied. A decrease in EC_{50} levels with increasing $G_s\alpha$ levels is compatible with a shuttle mechanism for the activation of adenylyl cyclase, but not with a G_s–C precoupled mechanism.

A range of $G_s\alpha$ levels was produced in an S49 cyc^- transfected cell system utilizing the dexamethasone-inducible MMTV LTR promoter to increase $G_s\alpha$. Expression of the $G_s\alpha$ protein over a ~40- to 60-fold range was proportional to adenylyl cyclase stimulation and the area of the GTP shift, demonstrating that the expressed $G_s\alpha$ could functionally couple with the β_2-adrenergic receptor and adenylyl cyclase. We demonstrate for the first time that increased G_s proteins reduce the EC_{50} for epinephrine-activated adenylyl cyclase. In an attempt to describe the mechanism of hormone-stimulated adenylyl cyclase activity, we also examined the usefulness of a set of kinetic equations developed from a simple shuttle model. We subject the quantitative model to a more rigorous quantitative assessment in the accompanying manuscript [14].

MATERIALS AND METHODS

Materials

Molecular biology reagents, Dulbecco's modified Eagle's medium (DMEM), and Geneticin were from Gibco BRL (Grand Island, NY); Tris base, GTP, and GTPγS were from Boehringer Mannheim (Indianapolis, IN); [α^{32}P]ATP, Na-^{125}I, and [2,8-^{3}H]cAMP were from NEN/DuPont (Boston, MA); Rp-CPT-cAMPS was from the BioLog Life Science Group (La Jolla, CA); and dexamethasone and the remaining reagents were from Sigma (St. Louis, MO).

Vectors Used to Establish Inducible $G_s\alpha$ Cell Lines

The two vectors used to generate unique cell lines capable of varying $G_s\alpha$ protein levels each contain different inducible promoters linked to cDNA encoding rat $G_s\alpha_{long}$ [15]. The first vector, pMMTV · $G_s\alpha$ · neo (a gift from J. Gonzales and described in detail in Ref. 16) is described briefly here. 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.

The second vector, pMTI · αs · neo, was subcloned, amplified, and isolated according to standard protocols detailed by Sambrook *et al.* [17]. The pMTI · αs · neo vector was produced by ligating the heavy metal (Zn^{2+}) inducible mouse metallothionein I promoter (from pMT · SV · neo supplied by R. Kataoka in the laboratory of Dr. Lanier [18]) to the $G_s\alpha_{long}$ gene, which encodes the 395 amino acid long form of the protein (from pMV7 supplied by H. Bourne [19]). The 1500 bp cDNA insert, encoding the rat $G_s\alpha_{long}$ gene, was isolated from the pMV7 vector using flanking *Hin*dIII restriction sites. The 7.2 kb pMTI · neo backbone was linearized subsequently at a unique *Hin*dIII restriction site downstream of the metallothionein promoter. The linear products were ligated and examined by restriction analysis with *Eco*RI and *Hin*dIII. The pMTI · αs · neo vector contains the gene for neomycin phosphotransferase driven by the constitutive HSV-1 TK promoter.

Stable Transfection (Electroporation) and Cell Isolation (Limiting Dilution)

Batch clones of stable transfectants were established by electroporating 2 μg of *Pvu*I linearized plasmids, pMMTV · $G_s\alpha$ · neo or pMTIαs · neo, 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.* [20]. 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" [21], 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 hr prior to cell isolation.

Transfected cells were isolated using a protocol similar to that of Gonzales *et al.* [16]. The day following electroporation, the cells were diluted into 20 mL of DMEM + 10% heat-treated horse serum 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/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 were screened for G_s activity using western blot analysis and adenylyl cyclase assays. Resistant colonies displaying G_s protein activity are denoted as either S49*cyc$^-$ or MTI 2.12 for cells transfected with pMMTV · $G_s\alpha$ · neo or pMTI · αs · neo, respectively.

Cell Culture

All of the S49 murine T cell lymphoma cell lines, including mutant and transfected cells, were maintained in stock tissue culture flasks (Corning) at 37° in HEPES-buffered DMEM supplemented with penicillin, streptomycin, and 10% heat-treated horse serum. Transfected cell lines were incubated with 200 μg/mL of G418 to maintain selective pressure. When necessary, cells were expanded in 1-L roller bottles (Corning).

Cell expansion for gene induction experiments involved the use of eight individual 1-L roller bottles, coinciding with the eight different time points of inducer treatment. The cells were distributed from a single stock source to preconditioned roller bottles, so that 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$^-$ and MTI 2.12 cell lines were incubated with either 5 μM dexamethasone (final concentration, added from a 10 mg/mL stock prepared in 95% EtOH) or 50 μM $ZnCl_2$ (final concentration), respectively, for times that ranged from 1 to 24 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 frozen immediately in liquid N_2 and stored at −80°. Membrane concentrations were determined using the BioRad assay [22].

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-1-methylxanthine; and 2×10^6 cpm [α-^{32}P]ATP. Reactions included epinephrine (solubilized in 10 mM thiourea and 1 mM ascorbic acid) or prostaglandin E_1 (solubilized in 8% EtOH in HEPES, pH 7.4, and NaCl), 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. Reactions examining maximal forskolin stimulation of adenylyl cyclase contained 200 μM forskolin, 20 mM $MnCl_2$ and 10 mM $MgCl_2$, in lieu of hormones, to functionally determine the relative levels of adenylyl cyclase within the membrane preparations. These conditions were selected to stimulate adenylyl cyclase independently of G_s [25].

k_{-1} *Determinations*

Time–course assays examining the decay rate for active adenylyl cyclase were used to determine the rate constant for inactivation, k_{-1}, as described by Cassel *et al.* [26]. Briefly, three separate conditions for examining cAMP accumulation were used to assess the basal activity (reaction #1), epinephrine-stimulated activity (reaction #2), and the rate of decay for cyclase activity following epinephrine-stimulated activity (reaction #3). The assays were initiated when 100 μL of cell membranes (~5 μg/μL, initial) were incubated with 1700 μL of the typical cyclase reagents described above, in 15 × 100 mm borosilicate glass tubes, in the presence of 3 μM epinephrine (final concentration) and the absence of [α-^{32}P]ATP. (Basal cAMP accumulation was examined in the absence of 3 μM epinephrine stimulation.) The membranes were preincubated for 3 min at 30° in order to allow the cyclase reactions to come to steady state. Data collection was initiated when a 200-μL aliquot of [α-^{32}P]ATP, cyclase reagents (at the appropriate final concentrations), and the ligands for either reaction #1 (20 μM propranolol, final), #2 (3 μM epinephrine, final), or #3 (3 μM epinephrine + 20 μM propranolol, final) were added to the preincubating membrane samples. The reac-

tions were quenched subsequently when 100-μL aliquots were removed from the reaction every 5–10 sec and placed into 12 × 75 mm glass tubes containing 500 μL of ice-cold stop buffer. Activity for each time point was determined as described above for adenylyl cyclase assays. The k_{-1} value for each membrane tested was determined graphically [27]. Linear equations for the epinephrine-stimulated curve (reaction #2) and the linear portion of the propranolol-antagonized epinephrine curve (reaction #3) were generated using Graph-Pad. The two linear equations were solved simultaneously in order to find the x-intercept for the two curves, which represents the mean time (t) for adenylyl cyclase inactivation. The relationship between the calculated mean time and k_{-1} is, $t = 1/k_{-1}$.

Western Blots: Detection of Proteins Involved in the Adenylyl Cyclase Response

Western analysis was used as the primary means for detecting the levels of the protein components involved in regulating adenylyl cyclase activity. Specific proteins in plasma membrane preparations were analyzed with the aid of rabbit polyclonal antibodies and enhanced chemiluminescent detection. Membrane concentrations for S49 cyc^- (negative control), S49 WT (positive control), and S49*cyc^- (test sample) were determined using the BioRad protein assay prior to sample preparation. Samples were prepared by adding 100 μL of 2 × Laemmli sample buffer [28] to 200 μL of membranes diluted in HE buffer and heating for 3 min in a boiling water bath. The volume of each sample loaded onto the gel was adjusted to achieve the desired amount of membranes (typically 20, 50, or 100 μg per well). The SDS–PAGE gel consisted of a 12% separating gel with a 1% stacking gel, cast and run in a BioRad Protean II apparatus in electrode buffer consisting of 192 mM glycine, 25 mM Tris, and 0.1% SDS.

The separated proteins were transferred from the gel onto PVDF membrane (NEN/DuPont) using a TransBlot apparatus (BioRad). Transfers were routinely conducted at a constant current of 500 mA for 2 hr at 4° in transfer buffer (3.03 g Tris, 14.4 g glycine, and 20% methanol per L). Upon completion of the transfer, the PVDF membrane was blocked for 1 hr at room temperature in 5% Blotto solution [10 g of Carnation Instant Milk in 200 mL of TBS buffer (50 mM Tris–Cl, pH 8.0; 200 mM NaCl)]. The blots were washed briefly in antibody buffer (0.1% Blotto, and 0.05% Tween-20 in TBS) prior to overnight incubation with commercially available primary antibody [anti-$G_s\alpha$ sera RM/1 (NEN/DuPont); anti-$G_i\alpha$ common antisera P-960 (a gift from S. Mumby; also available from Calbiochem); and anti-Gβ sera SW/1 (NEN/DuPont)] at 4°. The specificities of these antibodies have been demonstrated previously [29–31]. Antibody dilutions were typically 1:5000 unless otherwise listed. Blots were washed subsequently in antibody buffer prior to incubation with goat anti-rabbit horseradish peroxidase (GAR-HRP) conjugated secondary antibodies (BioRad). Secondary (GAR-HRP) antibodies were diluted 1:30,000–35,000 in antibody buffer and incubated with the blots for 1 hr at room temperature. The blots were washed extensively in TTBS (0.05% Tween-20 in TBS) and rinsed one time in TBS before being subjected to the Enhanced Chemiluminescent reaction (Amersham, Arlington Heights, IL). Blots were then exposed to Hyperfilm (Amersham) for times of 5 sec, 10 sec, 30 sec, 1 min, 2 min, and 5 min, developed, and quantified by densitometric scanning with an LKB Bromma 2202 Ultroscan Densitometer. The data for the relative intensities were generated with the aid of a Gelscan software package loaded on an Apple IIe computer system. The absorption curves, made by a printer, were cut and weighed. Data are presented as relative values, of the mean of two curve weights ± range, normalized to the control peak, except in cases where no reliable control peaks were detected.

β_2-Receptor Density and GTP Shift Analysis

Scatchard binding analyses were used to determine the β_2-adrenergic receptor density in plasma membrane preparations. Binding analyses were carried out in 500 μL reactions with the radiolabeled β_2-antagonist ^{125}ICYP (prepared by iodinating cyanopindolol, employing the protocol of Barovsky and Brooker [32] with modifications by Hoyer [33]), 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 $MgCl_2$; ^{125}ICYP concentrations ranging from 25 to 500 pM; and 20 to 50 μg cell membranes (diluted with HE, pH 8.0). Non-specific ^{125}ICYP binding was determined in the presence of 10 μM alprenolol. GTP shift-binding analyses were conducted as described above using a single 80 pM ^{125}ICYP concentration with increasing epinephrine concentrations (0.3 nM to 100 mM) in the presence and absence of 10 μM GTPγS. 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 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.

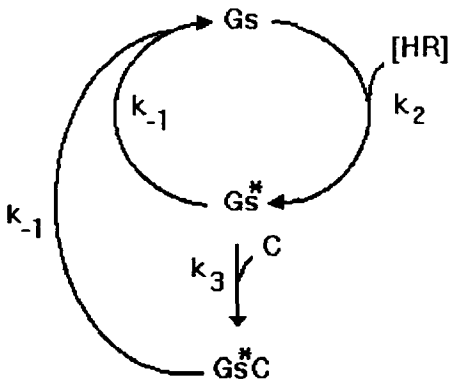
Statistical Methods

Adenylyl cyclase concentration–response curves were either transformed to Eadie–Hofstee plots or fit to a least

squares, non-linear regression procedure (Graph-Pad, ISI Software), for a sigmoid curve, in order to determine EC_{50} values. Curves for epinephrine competition binding to the β_2-adrenergic receptors, in the presence of GTPγS, were fit to sigmoid non-linear regression analysis (Graph-Pad), in order to determine the K_i for epinephrine binding [34]. Comparisons of EC_{50} values and binding data (± SD or SEM) were performed using single-factor ANOVA or a two-sample Student's t-test ($P < 0.05$).

Predicted Quantitative Cyclase Concentration Responses for the Shuttle and G_s–C Precoupled Models

It has been established over wide ranges of receptor availability that the rate of adenylyl cyclase activation is directly proportional to the concentration of agonist/receptor complex [5, 35, 36]. This greatly restricts the sub-models that can be used to describe adenylyl cyclase activation. The above observation when combined with the shuttle model in the simplest possible scheme gives:

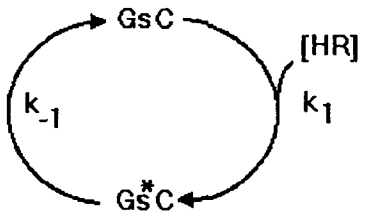


where HR = hormone receptor complex, G_s = free inactive G_s, G_s^* = activated G_s, C = free adenylyl cyclase catalyst, and G_s^*C = activated cyclase; k_{-1} represents the rate constant for inactivation, a first-order GTPase process intrinsic to the G_s protein (independent of association to C [37, 38]). The quantitative relationship between EC_{50} and G_s levels for the shuttle mechanism (developed in the Appendix) is:

$$EC_{50} = \frac{k_{-1}}{k_{-1} + k_2 r\left(1 + \dfrac{[G_s]_{total}}{[C]_{total} + \dfrac{k_{-1}}{k_3}}\right)} K_d \qquad (1)$$

where K_d represents the dissociation constant between the receptor and agonist, r the total receptor number per milligram of membrane protein, k_2, the activation rate constant for G_s activation, dependent on [HR], and k_3, the rate constant for the association between activated G_s^* and C. This relationship demonstrates that if k_3 is relatively large compared to k_{-1}, i.e. the association between G_s^* and C is large, then as $[G_s]_{total}$ increases, the EC_{50} decreases. Thus, a decrease in EC_{50} with increasing G_s is more compatible with a shuttle mechanism in the activation of adenylyl cyclase.

The relationship between adenylyl cyclase response and increasing G_s levels for the G_s–C precoupled model is quantitatively developed in Ref. 36, based on the activation scheme shown below:



where k_1 and k_{-1} correspond to the rate constants for G_s–C activation by [HR], and G_s^*C inactivation to G_s–C, respectively. Note that, in this model, the EC_{50} is independent of G_s levels:

$$EC_{50} = \frac{k_{-1}}{k_{-1} + k_1 r} K_d \qquad (2)$$

where r represents the total receptor number, and K_d the dissociation constant between the agonist and the receptor. A change in EC_{50} with a change in G_s level, therefore, would very strongly suggest that the precoupled model is invalid.

RESULTS

Adenylyl Cyclase Response

Epinephrine-stimulated adenylyl cyclase activity was examined in a clonal population of pMMTV · $G_s\alpha$ · neo transfected S49 cyc^- cells (denoted as S49*cyc^-), following dexamethasone treatment. We found that dexamethasone induction increased epinephrine-stimulated adenylyl cyclase activity in a time-dependent manner. Eadie–Hofstee transformations of adenylyl cyclase concentration–response curves demonstrated a significant 40% decrease in the EC_{50} with increasing time of dexamethasone induction (Fig. 1A). The transformations, performed on the set of curves shown in the *inset* of Fig. 1A, yielded correlation values > 0.98 for all curves and EC_{50} values similar to sigmoid non-linear curve-fitting analysis.

The epinephrine-stimulated activity appeared to plateau (approaching 183.1 ± 4.5 pmol/min/mg) after 8 hr of induction (Fig. 1A *inset*). The membranes prepared from uninduced cells revealed the presence of some basal MMTV promoter activity that resulted in a small hormone-dependent cyclase activity (23 ± 0.6 pmol/min/mg with 3 μM epinephrine). Dexamethasone induction also resulted in a time-dependent increase in basal adenylyl cyclase

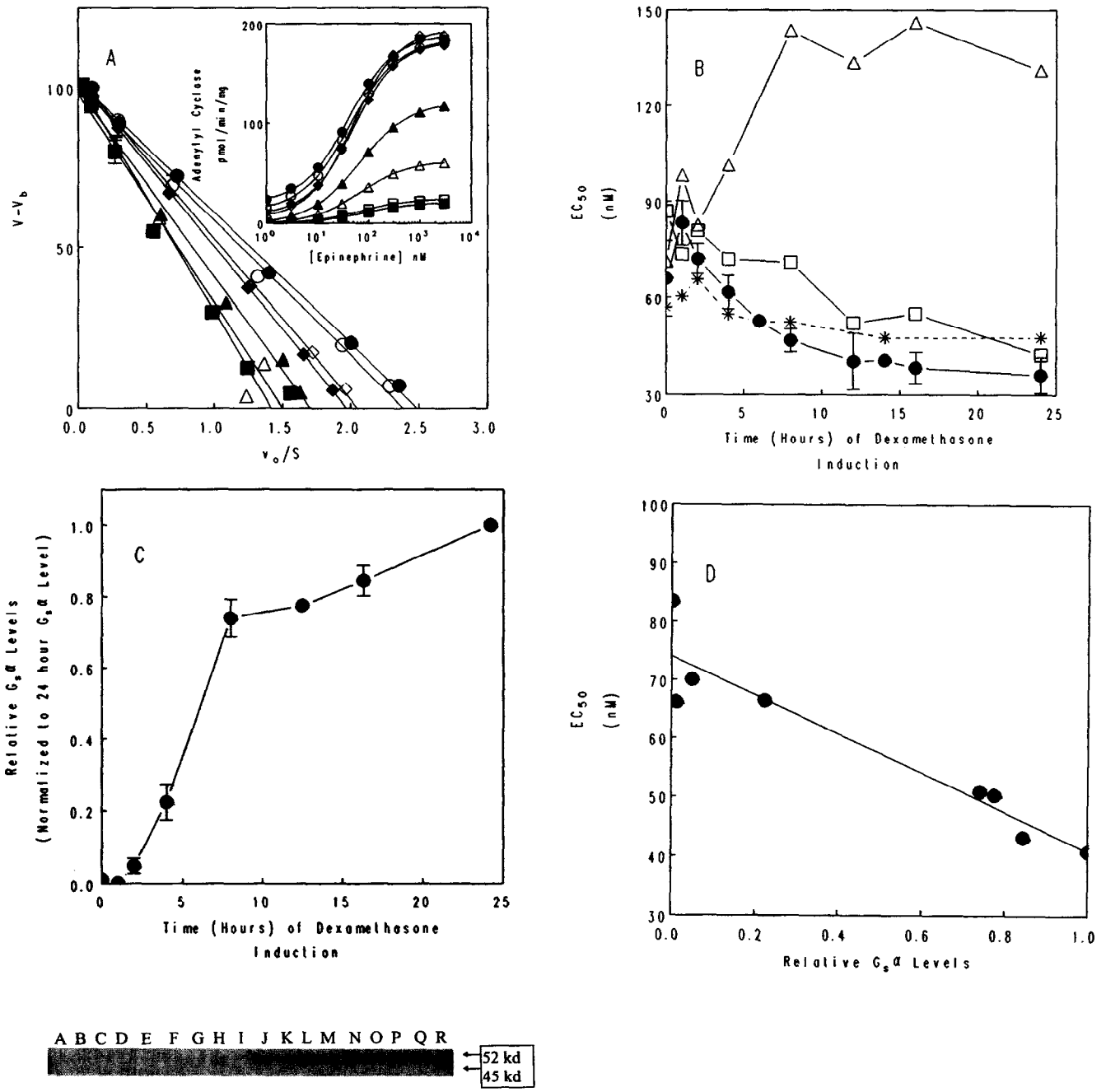


FIG. 1. (A) Eadie–Hofstee representation of epinephrine-stimulated adenylyl cyclase concentration–response data in membranes prepared from pMMTV · $G_s\alpha$ · neo transfected S49 cyc^- cells (denoted as S49*cyc^-). The membranes were prepared from cells treated with 5 μM dexamethasone for the following times: 1 hr = ■, 2 hr = △, 4 hr = ▲, 8 hr = ◇, 12.5 hr = ◆, 16.3 hr = ○, and 24.2 hr = ●. The linear correlations for all of the curves was >0.98. The -slope values, representing the EC_{50} (nM) values, were as follows: 1 hr = 65.2 ± 4.14, 2 hr = 71.4 ± 4.59, 4 hr = 58.2 ± 1.9, 8 hr = 50.0 ± 0.99, 12 hr = 51.8 ± 0.18, 16 hr = 41.4 ± 0.84, and 24 hr = 40.8 ± 0.77. The data are plotted using the experimental results shown in the *inset*. *Inset*: Epinephrine-stimulated adenylyl cyclase activity in S49*cyc^- membranes. The conditions employed for this assay are described in Materials and Methods using the following final epinephrine concentrations: 0, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM, 1 μM, and 3 μM. Data are plotted using the same symbols for each time of dexamethasone induction as mentioned above, with S49*cyc^- control = □. (The response for S49 cyc^- membranes was 0 pmol/min/mg regardless of the time of 5 μM dexamethasone treatment.) Each point on a curve is represented as a triplicate measurement ± the SD. The concentration–response curves, representing a single experiment selected from five separate experiments, were plotted using Graph-Pad. (B) EC_{50} Values for epinephrine-stimulated adenylyl cyclase following treatment with dexamethasone or $ZnCl_2$ in transfected and non-transfected S49 cell lines. Changes in EC_{50} values for adenylyl cyclase activation in membranes prepared from dexamethasone-inducible $G_s\alpha$ S49 cells (● = S49*cyc^-), zinc-inducible $G_s\alpha$ S49 cells (□ = MTI 2.12), wild type S49 cells (△ = S49 WT), and PKA activity deficient S49 cells (--*-- = S49 kin^-). All membranes were prepared from cells treated with 5 μM dexamethasone over time (in hours), except for MTI 2.12 which were treated with 50 μM $ZnCl_2$. The EC_{50} values from S49*cyc^- concentration–response assays are represented as the mean ± SD of five independent membrane preparations. The EC_{50}

activity (from 0.0 pmol/min/mg for control to 23.4 ± 0.29 pmol/min/mg for 24-hr induced membranes) without affecting the maximum forskolin-stimulated adenylyl cyclase activity in S49*cyc$^-$ membranes (data not shown). Two hundred micromolar forskolin-stimulated activities in uninduced and 24-hr induced membranes were 907.0 ± 16.4 and 906.8 ± 6.3 pmol/min/mg, respectively.

Adenylyl cyclase activity was also examined in other S49 cell lines in order to dissociate $G_s\alpha$ specific effects on the EC_{50} from the non-specific effects of dexamethasone. Figure 1B compares the change in EC_{50} values in a cell line transfected with a zinc-inducible promoter ligated upstream of $G_s\alpha_{long}$ cDNA (denoted as MTI 2.12) with those for the dexamethasone-inducible S49*cyc$^-$ membranes. The figure demonstrates that the EC_{50} values also decreased ~40–50% for epinephrine-stimulated activity following 50 μM $ZnCl_2$ induction. Dexamethasone-dependent effects on EC_{50} values in the S49*cyc$^-$ cell line were also compared with EC_{50} values in the S49 wild type (S49 WT) and the PKA mutant (S49 kin$^-$) cell variants, where 5 μM dexamethasone treatment over time resulted in a 2- to 3-fold increase and no change in EC_{50} values, respectively.

Detection of Varied G_s Levels: Western Blotting Analysis

The increase in adenylyl cyclase activity demonstrated in Fig. 1A coincided with the appearance of immunodetectable $G_s\alpha$ protein. The data shown in Fig. 1C revealed a progressive increase in a 52,000-Da immunoreactive $G_s\alpha$ protein band in membranes prepared from S49*cyc$^-$ cells treated with 5 μM dexamethasone (lanes B–Q). The blot revealed no immunodetectable bands for $G_s\alpha$ protein in control and 1-hr dexamethasone-treated preparations; however, after 2 hr a 52-kDa immunoreactive band, consistent with $G_s\alpha$, was detected. The intensity of this band increased with the time of dexamethasone induction and approached a ~40- to 60-fold increase over control levels following 24 hr of treatment. The absence of consistently reliable immunodetectable bands for $G_s\alpha$ protein on western blots for the control and 1-hr dexamethasone-treated time points made it difficult to reliably assess the fold increase of $G_s\alpha$ protein induction. The relationship between the immunodetectable $G_s\alpha$ levels and EC_{50} values for adenylyl cyclase activity is demonstrated in Fig. 1D.

*Appearance of a GTP Shift for S49*cyc$^-$ Membranes*

Changes in the area of the GTP shift following dexamethasone induction were used to analyze the functional coupling between the β_2-adrenergic receptor and the G_s protein [41]. The appearance of functional $G_s\alpha$ proteins in S49*cyc$^-$ membranes following dexamethasone induction resulted in the concomitant appearance of a GTPγS shift (Fig. 2). The uninduced membranes displayed a small, but detectable, GTPγS shift, in agreement with adenylyl cyclase results demonstrating low basal $G_s\alpha$ protein expression. The area between the GTPγS shift increased after 2 hr of dexamethasone treatment corresponding with the first immunodetectable $G_s\alpha$ proteins by western analysis. The shift increased proportionally with the expressed level of $G_s\alpha$ proteins until a maximum ~2 log unit separation between +GTPγS and −GTPγS curves was achieved 12–16 hr after dexamethasone treatment.

PGE_1 Stimulated Adenylyl Cyclase Activity

The PGE_1 receptor stimulates adenylyl cyclase activity in S49 WT cell membranes; however, the PGE_1 stimulation of adenylyl cyclase is less well characterized than β_2-adrenergic stimulation. We therefore examined the effects of increased G_s levels on PGE_1-stimulated adenylyl cyclase activity in the S49*cyc$^-$ membranes. Figure 3 demonstrates that increases in G_s levels led to proportional increases in PGE_1-stimulated activity. The absolute activities did not approach the same level of activity as that for β_2-adrenergic

values (± SEM) for the remaining cell types were determined from single experiments. The data were generated by fitting the data points from epinephrine-stimulated concentration–response curves to a sigmoid curve using the Graph-Pad computer program. (C) Relative amounts of $G_s\alpha$ in 100-μg samples of membranes prepared from S49*cyc$^-$ cells treated with 5 μM dexamethasone. The data were generated by scanning the western blot shown at the bottom of the panel, using a Bromma LKB densitometer, and are represented as the mean weight of two densitometric absorption curves per sample ± range. The levels of $G_s\alpha$ are plotted, as a function of time, relative to maximum levels of $G_s\alpha$ absorbance, i.e. the 24-hr dexamethasone-treated point. The data are representative of three different blots and were plotted using Graph-Pad. Bottom of panel: Western blot analysis for the appearance of $G_s\alpha$ protein in S49*cyc$^-$ membranes prepared from cells treated with 5 μM dexamethasone. Lanes on a 12.5% SDS–PAGE gel were loaded with 100 μg of membranes prepared from S49 cyc$^-$ cells (negative control = lane A), S49*cyc$^-$ cells (treated with 5 μM dexamethasone for the following times: 0 hr = lanes B and C; 1 hr = lanes D and E; 2 hr = lanes F and G; 4 hr = lanes H and I; 8 hr = lanes J and K; 12.5 hr = lanes L and M; 16.3 hr = lanes N and O; and, 24.2 hr = lanes P and Q), and S49 WT cells (positive control = lane R). The specificity of the $G_s\alpha$ antisera (RM/1) is indicated by examining the control lanes loaded with 100 μg of S49 cyc$^-$ and S49 WT membranes. Immunoreactive $G_s\alpha$ protein is absent in lane A corresponding to S49 cyc$^-$ membranes, whereas S49 WT (lane R) has two immunoreactive bands corresponding to the 52 and 45 kDa splice variants of $G_s\alpha$ [39]. The western blot was developed as described in Materials and Methods. In some high resolution blots, an immunoreactive band appearing just below the 52 kDa band coincided with the emergence of the 52 kDa band. These faster migrating bands may represent either the proteolytic digestion of $G_s\alpha$, or the state of $G_s\alpha$ palmitoylation [40]. Adenylyl cyclase response was not affected when membranes were prepared with trypsin inhibitors. (D) Correlation of EC_{50} for epinephrine-stimulated adenylyl cyclase activity to relative $G_s\alpha$ levels in S49*cyc$^-$ cell membranes. The EC_{50} values determined from the concentration–response curves shown in panel A are plotted versus the relative $G_s\alpha$ levels shown in panel C. The extrapolated y-intercept value, when $G_s\alpha$ levels approach zero, is 74–80 nM. The data are representative of five separate assays. The curves were plotted using Graph-Pad.

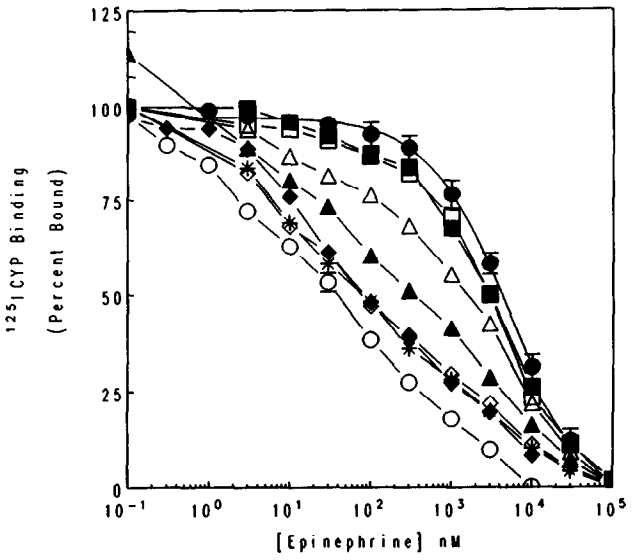


FIG. 2. Collection of eight GTPγS shift curves for S49*cyc$^-$ membranes. Eight separate binding curves (treated with 5 μM dexamethasone for 0 hr, □; 1 hr, ■; 2 hr, △; 4 hr, ▲; 8 hr, ◇; 12.5 hr, ◆; 16.3 hr, ○; and 24.2 hr, *) were generated by competitive binding with an ~80 pM concentration of the radiolabeled antagonist ^{125}ICYP in the presence of increasing (0.3 nM to 100 μM) concentrations of epinephrine. Two curves were generated for each membrane preparation in the presence and absence of 10 μM GTPγS. The amount of ^{125}ICYP binding at each concentration of epinephrine was normalized to control (no epinephrine). The +GTPγS curve (●) represents a collection of all eight +GTPγS curves from each membrane preparation, plotted as a single curve (mean ± SD). Each point on the −GTPγS curves represents the mean ± SD of three points. The data were plotted using Graph-Pad.

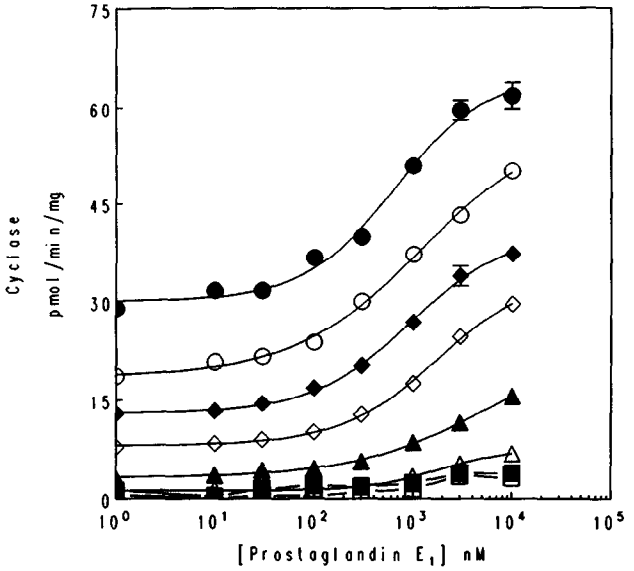


FIG. 3. PGE_1-Stimulated adenylyl cyclase activity in S49*cyc$^-$ membranes. The membranes were prepared from S49*cyc$^-$ cells exposed for and subjected to the same conditions as described in Fig. 1A, with the exception that epinephrine was substituted with the agonist PGE_1. For different times with 5 μM dexamethasone (control = □, 1 hr = ■, 2 hr = △, 4 hr = ▲, 8 hr = ◇, 12.5 hr = ◆, 16.3 hr = ○, 24.2 hr = ●). Each point on the curve represents the mean ± SD of three measurements. The curves represent a single experiment and were plotted using Graph-Pad.

stimulated activity, consistent with previous observations when maximum concentrations of PGE_1 were used to stimulate activity [42]; nevertheless, the EC_{50} decreased ~60% (from 1.8 μM for 4-hr treated membranes to 0.65 μM for 24-hr treated membranes) as G_s levels increased. Sigmoidal non-linear regression analysis of the EC_{50} did not converge for responses from membranes incubated with dexamethasone for less than 4 hr. The differences in the magnitude of response between PGE_1 and epinephrine stimulation of the same membranes may reflect fewer PGE_1 receptors relative to the β_2-adrenergic receptor and/or a reduced capacity of PGE_1 receptors to activate G_s.

Determination of β_2-Adrenergic Receptor Number

Whaley *et al.* [36] have demonstrated that the β_2-adrenergic receptor density has an inverse effect on the EC_{50} for adenylyl cyclase activity, i.e. as receptor density increases, the EC_{50} decreases. To determine the potential effect of dexamethasone on β_2-adrenergic receptor levels, the B_{max} was measured as a function of time of dexamethasone treatment. The β_2-adrenergic receptor level in membranes prepared from S49*cyc$^-$ cells markedly declined from a control value of 136 ± 13 fmol/mg (a level similar to S49 cyc$^-$ membranes) to 92 ± 8 fmol/mg (a level similar to S49 WT membranes) after 24 hr of dexamethasone treatment (Fig. 4). The reduction in receptor number occurred without a concomitant change in β_2-receptor affinity for the ^{125}ICYP antagonist (K_d = 20.8 ± 1.03 pM when averaged over all eight membrane preparations). Dexamethasone neither altered the low affinity of the β_2-receptor for epinephrine (Cheng and Prusoff [34] K_i = 1.10 ± 0.13 μM) nor did it significantly change the Hill coefficient for low affinity binding. The decrease in β_2-adrenergic receptor levels that we observed would have predictably caused an increase in EC_{50}.

In contrast to the effects of dexamethasone on receptor density in S49*cyc$^-$ cells, dexamethasone had little effect on receptor density or antagonist (^{125}ICYP) affinity in membranes prepared from treated S49 cyc$^-$ and S49 WT cells (data not shown). The overall receptor number was found to be greater in S49 cyc$^-$ membranes (116–133 fmol/mg) than in S49 WT membranes (71–85 fmol/mg), but the receptor affinity for ^{125}ICYP remained the same for the two cell lines (S49 cyc$^-$ K_d = 18.6 ± 1.4 pM vs S49 WT K_d = 21.4 ± 2.4 pM), even after dexamethasone treatment. The difference in receptor densities for S49 cyc$^-$ and S49 WT are consistent with those demonstrated for S49 cells by Abramson and Molinoff [43].

Influence of Dexamethasone on $G_i\alpha$ and Gβ Levels

The $G_i\alpha$ levels in the S49 WT, S49 cyc$^-$, and S49*cyc$^-$ membranes, prepared from cells treated with dexametha-

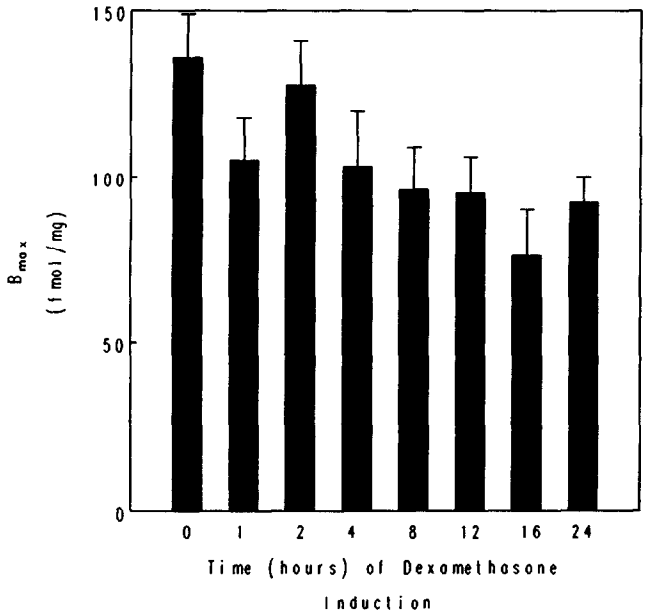


FIG. 4. Bar graph depicting the β_2-adrenergic receptor number (as B_{max}) in S49*cyc$^-$ membranes. The assay was conducted as described in Materials and Methods. The membranes used in this binding assay were of the same preparation used to examine adenylyl cyclase activity in Fig. 1A. The data represent the mean B_{max} ± SEM from a representative of four separate Scatchard assays. The data were plotted using Graph-Pad.

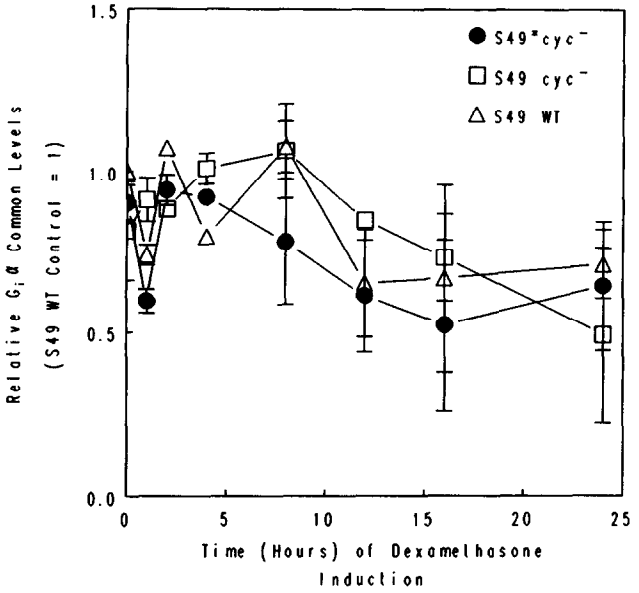


FIG. 5. Relative amounts of immunodetectable $G_i\alpha$ common in membranes prepared from wild type S49 (S49 WT), S49 cyc$^-$, and S49*cyc$^-$ cells. The levels of $G_i\alpha$ are plotted, as a function of time, relative to $G_i\alpha$ levels in S49 WT control membranes. The data were generated by scanning western blots, probed with P-960 anti-$G_i\alpha$ antibody, as described in Materials and Methods, with a Bromma LKB densitometer set on low resolution. The absorbance curves, generated with the aid of a Gelscan program loaded on an Apple IIe computer system, were cut and weighed. The points on the curve represent the mean of two curve weights ± range. The data were plotted using Graph-Pad.

sone, were determined by western analysis (as described in Materials and Methods) followed by densitometric quantitation. Dexamethasone treatment resulted in a slight but gradual decrease in immunodetectable $G_i\alpha$ levels prior to 24 hr; however, 24-hr dexamethasone treatment induced a 30% decrease in immunodetectable $G_i\alpha$ common levels in each of the three cell lines tested (Fig. 5). There appeared to be no correlation between $G_i\alpha$ protein levels and EC_{50} values ($r = 0.41$) obtained from cyclase assays. Quantification of the immunoreactive Gβ bands by densitometry (data not shown) revealed that the relative levels of immunodetectable Gβ changed in concert with the relative amount of $G_i\alpha$ common in S49 cyc$^-$ and S49*cyc$^-$ membranes.

Effect of Increasing $G_s\alpha$ Levels on the Rate Constant for Cyclase Inactivation: k_{-1}

The effect of increasing $G_s\alpha$ levels in S49*cyc$^-$ membranes on the rate of adenylyl cyclase inactivation, k_{-1}, was assessed as described in Materials and Methods (Fig. 6). The mean time (t) for adenylyl cyclase inactivation, where $t = 1/k_{-1}$, was measured in S49*cyc$^-$ membranes prepared from dexamethasone-treated cells and compared with the mean inactivation time for S49 WT control membranes. A two-sample Student's t-test ($P < 0.05$) revealed no statistical difference between the calculated t values ± the SEM versus the group mean ± the SD (0.309 ± 0.05 min), except for the 4-hr dexamethasone-treated S49*cyc$^-$ membranes. However, no statistical difference for these membranes and the group mean was found ($P < 0.025$). These data demonstrate that neither dexamethasone nor the increased level of $G_s\alpha$ proteins affected the first-order rate constant for inactivation, k_{-1}.

DISCUSSION

*Adenylyl Cyclase Response in S49*cyc$^-$ Membranes*

The interaction between G_s and adenylyl cyclase, prior to hormonal activation, has important implications for predicting the potency of the cyclase response to hormones when G_s levels are varied. The two most accepted models for hormonal activation of adenylyl cyclase, the shuttle model and the G_s–C precoupled model, predict entirely different outcomes for hormone potency in the presence of varied G_s levels. The shuttle model predicts an increase in epinephrine potency (measured as a decrease in EC_{50}) with increasing $G_s\alpha$ levels, whereas epinephrine potency remains independent of $G_s\alpha$ levels for the G_s–C precoupled model.

The importance of the G_s protein in the epinephrine stimulation of adenylyl cyclase activity is shown in Fig. 1A *inset* where the absence of G_s proteins in S49 cyc$^-$ cell membranes precluded hormonal activation of adenylyl cyclase. In S49 cyc$^-$ cells that have been transfected with

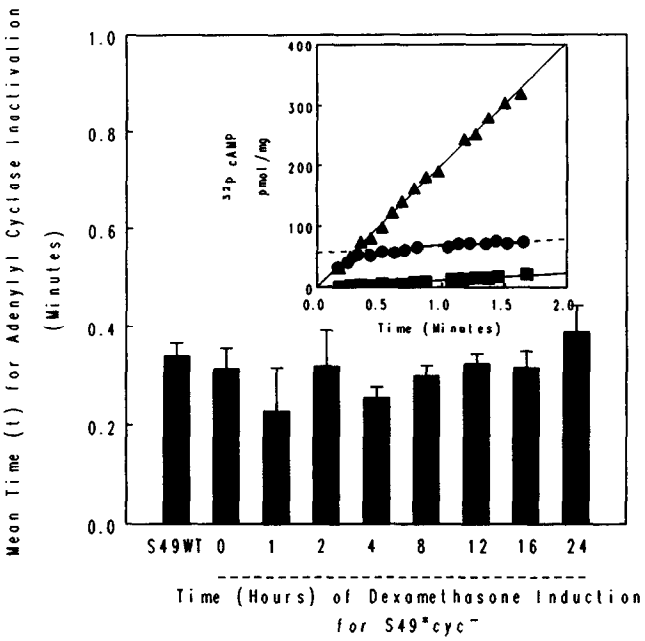


FIG. 6. Comparison of the mean time required for adenylyl cyclase inactivation in S49 WT control and S49*cyc$^-$ membranes. The membranes were prepared from cells treated with 5 μM dexamethasone for the indicated times. Each bar on the graph represents the mean time for inactivation (t) ± the SEM for three independent experiments calculated from assays similar to the representative assay depicted in the *inset*. *Inset*: Determination of the mean time (t) for adenylyl cyclase inactivation in 8-hr dexamethasone-treated S49*cyc$^-$ membranes. Each mean time (t) determination required three separate reactions as described in Materials and Methods. Reaction #1 (■) contained 20 μM propranolol and was used to assess basal adenylyl cyclase activity. Reaction #2 (▲) contained 3 μM epinephrine and examined the linearity of the hormone-stimulated response during the time–course. Reaction #3 (●) contained 3 μM epinephrine + 20 μM propranolol and was used to observe the rate of adenylyl cyclase activity decay. The values for t (where $t = 1/k_{-1}$) were determined graphically [27]. The data were plotted using Graph-Pad.

pMMTV · $G_s\alpha$ · neo (denoted as S49*cyc$^-$), increased $G_s\alpha$ expression resulted in the proportional increase in epinephrine- and PGE_1-stimulated cyclase response (Figs. 1A and 3). Hormone-stimulated cyclase responses were Michaelian and first order. A comparison of the magnitudes in adenylyl cyclase response generated by epinephrine and PGE_1 revealed that both systems appear to behave differently with respect to the saturation of V_{max} (reached 8 hr following dexamethasone induction for epinephrine while remaining unsaturated following 24 hr for PGE_1) even though these responses were determined in the same membrane preparation. The differences in the level of response in the presence of saturating epinephrine and PGE_1 concentrations have been observed previously in S49 WT cell membranes [42] and may represent differences in total receptor number and/or the rate of HRG formation, the rate-limiting step of adenylyl cyclase activation. Another possibility concerns the differential availability of G protein to different systems in the same membrane [44].

Adenylyl cyclase concentration–response data were subjected to sigmoid non-linear regression analysis to determine EC_{50} values for each membrane preparation containing increasing G_s levels. The analyses resulted in the demonstration of an overall ~40–50% decrease in EC_{50} ($N = 5$, $P = 9.57 \times 10^{-12}$) for epinephrine-stimulated activity (Fig. 1B) and a ~60% decrease for PGE_1-stimulated activity (Fig. 3) when EC_{50} values were compared in membranes containing low G_s levels to those containing high G_s levels. The decreases in EC_{50} associated with increased G_s levels demonstrate, for the first time in plasma membranes, that the level of signal transducing proteins downstream of the receptor can influence hormone potency. The decrease in EC_{50} correlated linearly with the increasing $G_s\alpha$ levels ($r = -0.95$, Fig. 1D) and did not appear to be a result of non-specific effects of dexamethasone on adenylyl cyclase activity since S49 cyc$^-$ cells transfected with a vector containing the rat $G_s\alpha_{long}$ cDNA driven by a zinc-inducible mouse metallothionein I promoter (Fig. 1B, cell line denoted as MTI 2.12) also resulted in a ~50% decrease in EC_{50} following $G_s\alpha$ induction. The S49*cyc$^-$ cells were eventually selected over the MTI 2.12 cell line as the primary model cell line for detailed analysis because of the greater range of expressed $G_s\alpha$ activity, i.e. due to the leaky metallothionein promoter in control MTI 2.12 cells, zinc induction of $G_s\alpha$ expression resulted in a 2- to 3-fold increase in cyclase activity versus an 8-fold increase for S49*cyc$^-$.

The observed decreases in EC_{50} with increasing G_s levels are not consistent with a pure G_s–C precoupled mechanism for activation, and therefore activation by some form of a shuttle mechanism is more likely. The observations for EC_{50} and V_{max} are predicted by the simplified shuttle equations and can be explained in the following manner. When G_s levels are limiting with respect to adenylyl cyclase, increases in G_s lead to proportional increases in adenylyl cyclase activity. However, when G_s levels exceed adenylyl cyclase levels, further increases in G_s lead to an accumulation of a pool of active G_s that serves to increase the rate at which inactive adenylyl cyclase is activated.

*Comparison of S49*cyc$^-$ and S49 WT*

The functionality of the induced G_s protein in the S49*cyc$^-$ membranes was assessed by comparing the β_2-receptor and G_s levels, GTP shifts, and adenylyl cyclase responses with S49 WT membranes prepared from dexamethasone-treated cells. The β_2-adrenergic receptor levels in S49*cyc$^-$ membranes (92 ± 8 fmol/mg) were similar to the levels in S49 WT membranes (71–85 fmol/mg) following 24 hr of dexamethasone treatment. In comparison to the immunodetectable $G_s\alpha$ levels in S49 WT control membranes, the maximum levels of induced $G_s\alpha$ protein in the S49*cyc$^-$ cell membranes represented ~70% of the immunoreactive $G_s\alpha$ protein present in control S49 WT membranes. However, dexamethasone induced a significant 32 ± 3% decrease in the immunoreactive $G_s\alpha$ levels

in S49 WT membranes (data not shown), as assessed by densitometry, demonstrating that $G_s\alpha$ levels in the two cell types were similar after 24 hr of dexamethasone treatment.

Since membranes prepared from 24-hr dexamethasone-treated S49 WT and S49*cyc$^-$ cells contained similar receptor and G_s levels, it was expected that GTP shift assays would reveal comparable areas of the shift if the functional interaction between the receptor and G_s was similar in the two cell lines. Comparison of the maximum area of the GTP shift for 24-hr dexamethasone-treated S49*cyc$^-$ and S49 WT membranes revealed that the inducible $G_s\alpha$ membranes displayed 93% of the shift seen in S49 WT membranes. (No high affinity agonist receptors were produced as a result of treating S49 cyc$^-$ cells with dexamethasone.) The increase in high affinity agonist receptor in S49*cyc$^-$ membranes was not associated with either an increase in the β_2-adrenergic receptor number (Fig. 4) or a change in epinephrine affinity for the receptor (HR) (Cheng and Prusoff [34] $K_i = 1.04 \pm 0.04$, 1.10 ± 0.13, and 1.04 ± 0.26 μM for epinephrine binding to S49 cyc$^-$, S49*cyc$^-$, and S49 WT membranes treated with dexamethasone, respectively). Comparisons of epinephrine-stimulated adenylyl cyclase data also revealed that the V_{max} activity approached ~200 pmol/min/mg for both membrane preparations following 24 hr of dexamethasone treatment. These data, in conjunction with the relative levels of $G_s\alpha$ determined by western blot analysis, suggested that induced $G_s\alpha$ coupled to the β_2-adrenergic receptor as well as S49 wild-type $G_s\alpha$.

Osawa *et al.* [45] have demonstrated that the presence of "naked" $G_s\alpha$ proteins, without the concomitant appearance of $G\beta\gamma$, leads to unregulated cyclase activity. We were concerned, therefore, that the difference in EC_{50} values demonstrated for the S49*cyc$^-$ and S49 WT membranes were generated as a result of "naked" $G_s\alpha$ expression in the plasma membrane. Increases in basal adenylyl cyclase activity for $G_s\alpha$-inducible membranes paralleled the increase in basal activity in S49 WT membranes implying a common mechanism, independent of Gβ subunit levels. Additionally, our results for the agonist stimulation of adenylyl cyclase via the β_2-adrenergic receptor accounted for 85% of the total hormone activatable catalytic activity in both S49 WT and S49*cyc$^-$ membranes (data not shown), suggesting that both cell types functioned similarly. Finally, Katada *et al.* [46] have reported that the amount of $G\beta\gamma$ in S49 cell membranes is of sufficient quantity to produce G_s from $G_s\alpha$. It is therefore likely that the decrease in EC_{50} in the $G_s\alpha$-inducible cell line occurred as a direct result of G_s protein increases in the plasma membrane.

Dexamethasone-Induced Basal Adenylyl Cyclase Activity

It is currently not quite clear by what mechanism dexamethasone induces a change in basal activity (denoted in the Results and illustrated in Fig. 1A *inset*), but this effect on adenylyl cyclase has been demonstrated by Bian *et al.* [47]. Hadcock and Malbon [48] have suggested that the increase in activity is a result of dexamethasone-induced increases in β_2-adrenergic receptor number, but our results (Fig. 4) and those of Gonzales *et al.* [16] do not show increases in β_2-receptor number upon dexamethasone treatment. It is proposed that dexamethasone may induce the expression of a particular adenylyl cyclase subtype that is more sensitive to spontaneous G_s activation, or reduce the number of ancillary molecules involved in regulating cyclase activity (e.g. G_i or β-arrestin). In any case, we speculate that the increased basal activity desensitized S49 WT membrane responsiveness to epinephrine in a time-dependent manner after dexamethasone induction.

To evaluate whether the dexamethasone-dependent desensitization may have been due to an increase in PKA activity as a result of increased basal cAMP levels, we examined the effects of dexamethasone on the EC_{50} for epinephrine-stimulated adenylyl cyclase activity in S49 kin$^-$ and S49*cyc$^-$ membranes prepared from cells treated with dexamethasone in the presence and absence of the PKA inhibitor Rp-CPT-cAMPS. Dexamethasone treatment of S49 kin$^-$ cells revealed little change in EC_{50} (Fig. 1B). Our studies in the S49*cyc$^-$ cell line treated with dexamethasone and the PKA inhibitor Rp-CPT-cAMPS revealed that the EC_{50} decreased to a lower final value than in the absence of the inhibitor (data not shown), suggesting that PKA may play a role in blunting the "true" EC_{50} decrease, perhaps via PKA phosphorylation of type VII adenylyl cyclase [49] and/or β_2-receptor level phosphorylation [24].

Activation Mechanism Inconsistent with Precoupled Model

The argument developed below makes clear that the observed changes in EC_{50} for the hormone activation of adenylyl cyclase are inconsistent with a "pure" G_s–C precoupled mechanism as proposed by Tolkovsky and Levitzki [5]. Assuming that activation in membranes proceeded via a "pure" G_s–C precoupled mechanism, then the measured decrease in EC_{50} could only be attributed to a decrease in K_d and/or k_{-1}, and/or an increase in r and/or k_1 (see equation 2 in Materials and Methods). Experiments conducted in this study have demonstrated that K_d and k_{-1} (Fig. 6) remained unaffected by increasing $G_s\alpha$ levels or dexamethasone treatment, and therefore these terms were not responsible for the decrease in EC_{50}. The β_2-adrenergic receptor number, r, decreased in our inducible $G_s\alpha$ system with dexamethasone treatment (Fig. 4) and therefore could not account for the decrease in EC_{50}. In fact, the decrease in r would increase the EC_{50} as reported by Whaley *et al.* [36]; therefore, the observed decrease in r may have blunted the "true" decrease in EC_{50} with increasing $G_s\alpha$ levels, and, if "corrected," the decrease in EC_{50} may have been more like 75–100%. By the process of elimination, only an increase in the rate constant for adenylyl cyclase activation, k_1, could

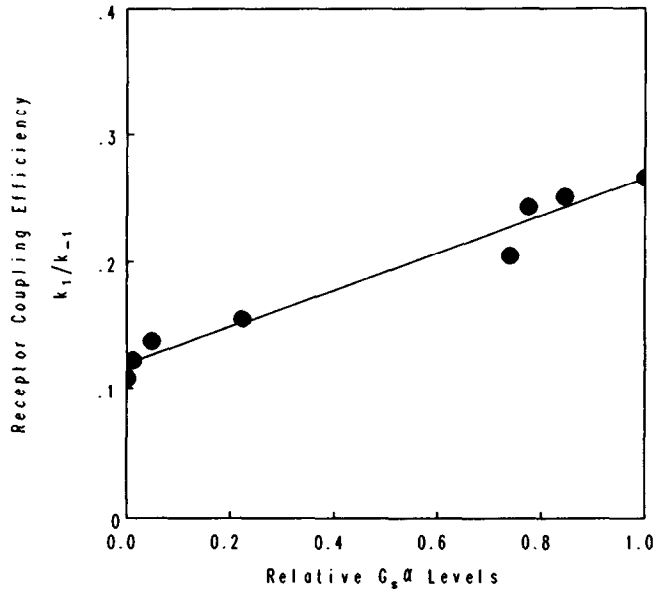


FIG. 7. Receptor coupling efficiencies, k_1/k_{-1}, in S49*cyc$^-$ membranes as a function of relative $G_s\alpha$ levels. The receptor coupling efficiencies were calculated using the following equation [36]:

$$\frac{k_1}{k_{-1}} = \frac{K_d - EC_{50}}{(EC_{50})r}$$

where K_d and EC_{50} and r, the total receptor number, were experimentally determined using data from GTP shift curves, adenylyl cyclase concentration–response curves, and Scatchard analyses, respectively, for each membrane preparation. The coupling efficiency can be used as a direct measure for the change in the k_1 rate constant as experiments have revealed that k_{-1} is constant with increasing $G_s\alpha$ levels. The data were plotted using Graph-Pad.

account for the decrease in EC_{50}, and this is very unlikely if a "pure" G_s–C precoupled mechanism was functioning.

Whaley *et al.* [36] have demonstrated that for a single receptor subtype (e.g. the β_2-adrenergic receptor), under conditions where the G_s level is constant, the coupling efficiency, defined as k_1/k_{-1}, dictates the extent of the decrease in EC_{50} shift with increasing receptor number. For our system, we have used the coupling efficiency as an indirect method for examining the effect of varying $G_s\alpha$ levels on the rate constant for activation, k_1. Because the inactivation rate constant k_{-1} did not change with increasing $G_s\alpha$ levels (Fig. 6), any change in coupling efficiency, k_1/k_{-1}, can be attributed to a change in k_1. Figure 7 reveals that the coupling efficiency (calculated as described in the legend of Fig. 7) increased 2- to 3-fold with increasing $G_s\alpha$ levels ($r = 0.98$). The increase in the coupling efficiency, or k_1, was not due to a non-specific effect of dexamethasone because the coupling efficiency decreased ~50% in S49 WT membranes (data not shown). These results for G_s-dependent increases in k_1 are not consistent with a single-step activation process, like the G_s–C precoupled model, but are consistent with the shuttle model [50].

Our results regarding the rate constant for activation, k_1, differ from those of Tolkovsky *et al.* [6], who have argued that a shuttle model is incompatible with their kinetic data, which show an invariant rate constant for activation in the presence of varied G_s and adenylyl cyclase levels, and first-order Michaelian responses for hormone activation of adenylyl cyclase. However, their experiments demonstrating an invariant rate constant for activation relied on the use of the non-specific alkylating agent *N*-ethylmaleimide in order to modify adenylyl cyclase levels, a treatment that may have suppressed the G_s to C shuttling [6]. In contrast, our results demonstrate that increased G_s levels augmented the rate at which adenylyl cyclase is activated without affecting the first-order kinetics for activation.

Experimental agonist concentration–response curves for adenylyl cyclase are indeed well described by Michaelian equations. The equation that describes the shuttle model used in this manuscript (or indeed to any shuttle model) is the solution to a quadratic equation. We have therefore spent a good deal of effort in showing that such a solution, in general, approximates rather well to a Michaelian representation. This was done in order to show, in as simple a manner as possible, the dependence of the EC_{50} on $[G_s]_{total}$. The appendix gives a formal conversion of a quadratic solution to an approximate Michaelian equivalent. The estimates of the amount of error involved in the use of such an approximation will generally be quite small. They can be estimated by calculating a concentration response to agonist using the full quadratic equation (equation 9) and comparing this with the response calculated for a rectangular hyperbola with the same EC_{50} and V_{max}. The amount of divergence between the two solutions depends on the values chosen for the rate constants and the molar ratios of $[G_s]_{total}$ and $[C]_{total}$. When the $[G_s]_{total}$ to $[C]_{total}$ ratio was less than 1, the divergence was $\leq 3\%$ for any values of k_{-1}, k_2, and k_3. Using a $[G_s]_{total}$ to $[C]_{total}$ ratio of 1 to 1.44, which corresponds to the G_s levels determined in the accompanying manuscript [14] for wild type S49 cells, the divergence was <25% at any part of the adenylyl cyclase concentration response. At adenylyl cyclase concentration responses greater than 20% of the maximum, the divergence was <15%. Thus, it is not surprising that experimental concentration–response curves are generally well described by a Michaelian fit, even though a shuttle model is operating.

The results of this study, however, do not rule out the possibility that a proportion of cyclase is activated via preformed G_s–C complexes. The existence of both mechanisms is embodied into a complicated series of interactions, defined as the rate constant for activation, and the extent to which either the shuttle or G_s–C mechanism contributes to adenylyl cyclase activity may simply be a function of the laws of mass action, or perhaps the different affinities that G_s may have for the different subtypes of adenylyl cyclase in S49 cells [51]. The answer to the question of which mechanism predominates under what set of circumstances, lies in the determination of the associa-

tion constant between the active G_s^* and inactive adenylyl cyclase. In the accompanying paper [14], we examine the relative rate of this reaction and offer some insight as to why the G_s–C precoupled mechanism yields excellent kinetic predictions when G_s levels are constant. It is clear, however, that much work remains to be done to fully elucidate the mechanism for agonist-stimulated adenylyl cyclase activity.

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APPENDIX

Derivation of the Relationship Between EC_{50} and $G_s\alpha$ Levels for a Shuttle Model

The relationship between EC_{50} and $G_s\alpha$ levels for the shuttle model was based on the cyclase activation scheme shown in Materials and Methods. The equations derived below seek to determine the effect of changes in total G_s protein levels on the concentration–response curves that give adenylyl cyclase activity as a function of agonist concentration. They are based on the idea that the shuttle model requires (1) the activation of G_s to G_s^*, followed by (2) the association of G_s^* and C resulting in G_s^*C activation. At steady state, the amount of active G_s^*C in the system is, therefore, dependent on the amount of active G_s^*, which is determined in the following manner:

$k_2[HR][G_s]$ = rate at which G_s is activated to G_s^*

$k_{-1}[G_s^*] + k_{-1}[G_s^*C]$

= rate at which G_s^* is removed from the system

where the magnitude of k_2 depends on the identity of the agonist. This same result is achieved if different agonists induce different receptor conformations that then interact with G_s at different rates or if the same active conformation is induced. In both cases, the activation rate is linear with total [HR] and can be expressed as $k_2[HR][G_s]$. At the steady state, the rate of G_s activation and the rate of G_s^* deactivation are set equal to one another:

$$k_2[HR][G_s] = k_{-1}[G_s^*] + k_{-1}[G_s^*C] \quad (1)$$

The amount of active adenylyl cyclase, G_s^*C, in the system, is now a function of the amount of G_s^* in the system, and the rate constant, k_3, for G_s^*C formation from G_s^* and C:

$k_3[G_s^*][C]$ = rate of active G_s^*C complex formation

$k_{-1}[G_s^*C]$ = rate of G_s^*C inactivation

at the steady state:

$$k_3[G_s^*][C] = k_{-1}[G_s^*C] \quad (2)$$

The conservation of mass, applied to the activation scheme illustrated in Materials and Methods, reveals that $[G_s]_{total}$ and C in the shuttle system are, respectively:

$$[G_s]_{total} = [G_s] + [G_s^*] + [G_s^*C] \quad (3)$$

$$[C] = [C]_{total} - [G_s^*C] \quad (4)$$

Therefore, the amount of active adenylyl cyclase, G_s^*C, that is produced for any given concentration of $[G_s^*]$ is

determined by substituting equation (4) for C into equation (2) and solving for G_s^*C:

$$\frac{[G_s^*C]}{[C]_{total}} = \frac{[G_s^*]}{\left(\frac{k_{-1}}{k_3}\right) + [G_s^*]} \tag{5}$$

where G_s^*C/C_{total} represents the fraction of active adenylyl cyclase within the system. Because the concentrations of inactive $[G_s]$ and active $[G_s^*]$ within the system are extremely difficult to measure with any accuracy, the term for G_s^* in equation (5) is substituted with terms that can be experimentally determined, for example, $[G_s]_{total}$ and [HR]. This is accomplished by first substituting G_s from equation (1) into equation (3) followed by rearranging to yield:

$$[G_s^*] = \frac{[G_s]_{total} - [G_s^*C]\left(1 + \frac{k_{-1}}{k_2[HR]}\right)}{1 + \frac{k_{-1}}{k_2[HR]}} \tag{6}$$

The unknown G_s^* values from equation (5) can be subsequently eliminated by substituting G_s^* from equation (6) into equation (5). After some rearranging, the fraction of active adenylyl cyclase is now:

$$\frac{[G_s^*C]}{[C]_{total}} = \frac{k_3\left(\frac{[G_s]_{total}}{\left(1 + \frac{k_{-1}}{k_2[HR]}\right)} - [G_s^*C]\right)}{k_{-1} + k_3\left(\frac{[G_s]_{total}}{\left(1 + \frac{k_{-1}}{k_2[HR]}\right)} - [G_s^*C]\right)} \tag{7}$$

Equation (7) can be manipulated more easily by substitution of the term $(1 + k_{-1}/k_2[HR])$ with A, and subsequent rearrangement gives a quadratic equation:

$$[G_s^*C]^2 - \left([C]_{total} + \frac{[G_s]_{total}}{A} + \frac{k_{-1}}{k_3}\right)[G_s^*C] + \frac{[C]_{total}[G_s]_{total}}{A} = 0 \tag{8}$$

Then writing 1/A as θ, the solution to the quadratic equation (8) is:

$$[G_s^*C] = \left(\left([C]_{total} + \frac{k_{-1}}{k_3} + [G_s]_{total}\theta\right) - \sqrt{\left([C]_{total} + \frac{k_{-1}}{k_3} + [G_s]_{total}\theta\right)^2 - 4[C]_{total}[G_s]_{total}\theta}\right)2 \tag{9}$$

Equation (9) gives G_s^*C as an explicit function of θ, which is, in turn, an explicit function of [HR] and hence the agonist concentration [H]. Thus, the activity of the adenylyl cyclase is known as a function of [H] and the EC_{50} can be calculated for any value of $[G_s]_{total}$. This equation describes agonist-stimulated activity and takes no account of any basal activity that may be present.

Considerable simplification of the above mathematical forms can be achieved under circumstances where the term $([C]_{total} + k_{-1}/k_3 + [G_s]_{total}\theta)^2$ is large compared to the term $4[C]_{total}[G_s]_{total}\theta$ or when k_{-1}/k_3 is small compared to $[C]_{total}$. Under both of these conditions, the quadratic solution can be approximated by rectangular hyperbolae as shown below.

Approximation when: $([C]_{total} + k_{-1}/k_3 + [G_s]_{total}\theta)^2 \gg 4[C]_{total}[G_s]_{total}\theta$

In physical terms, a Michaelian approach is possible if $[C]_{total} \gg [G_s]_{total}\theta$ or $[G_s]_{total}\theta \gg [C]_{total}$. The quadratic is developed when such physical assumptions are not made. Then the use of the mathematical approximation $([C]_{total} + k_{-1}/k_3 + [G_s]_{total}\theta)^2 > 4[C]_{total}[G_s]_{total}\theta$ takes both physical possibilities into account. Under these conditions, the quadratic solution can be approximated by a rectangular hyperbola as shown below. Equation (9) can be rearranged by isolation of the factor $([C]_{total} + k_{-1}/k_3 + [G_s]_{total}\theta)$ to give:

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

Then use of the Taylor expansion, $(1 + f(x))^{1/2} \approx (1 + f(x)/2)$ where $f(x) \ll 1$, gives:

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

After some rearrangement, the equation now becomes:

$$[G_s^*C] = \frac{[C]_{total}[G_s]_{total}\theta}{[C]_{total} + \frac{k_{-1}}{k_3} + [G_s]_{total}\theta} \tag{10}$$

To determine the fraction of active adenylyl cyclase, substitute θ with $k_2[HR]/(k_2[HR] + k_{-1})$ into equation

(10), where [HR] is defined as $r[H]/([H] + K_d)$ from the Langmuir binding isotherm, r represents the total number of receptors per milligram of membrane preparation, and K_d the dissociation constant between agonist and the receptor. Following some algebraic rearrangement, equation (10) now becomes:

$$\frac{[G_s^*C]}{[C]_{total}} = \frac{\left(\frac{(k_2r)([G_s]_{total})}{\left([C]_{total} + \frac{k_{-1}}{k_3}\right)(k_2r + k_{-1}) + (k_2r)([G_s]_{total})}\right)[H]}{\left(\frac{\left([C]_{total} + \frac{k_{-1}}{k_3}\right)(k_{-1})}{\left([C]_{total} + \frac{k_{-1}}{k_3}\right)(k_2r + k_{-1}) + (k_2r)([G_s])_{total}}\right)K_d + [H]} \tag{11}$$

The relationship between EC_{50} and $[G_s]_{total}$ is finally established for the shuttle model by realizing that the form of equation (11) resembles the empirical Michaelis–Menten equation for enzyme activity:

$$v = \frac{V_{max}[S]}{EC_{50} + [S]} \tag{12}$$

where v represents a fraction of the maximum activity, V_{max}, of the enzyme at any given concentration of substrate [S]. The EC_{50} value represents the concentration of [S] where activity is half-maximal. The relationship between EC_{50} and $G_s\alpha$ levels is established by setting the EC_{50} in equation (12) equal to the corresponding term in equation (11), and rearranging to yield:

$$EC_{50} = \frac{k_{-1}}{k_{-1} + (k_2r)\left(1 + \frac{[G_s]_{total}}{[C]_{total} + \frac{k_{-1}}{k_3}}\right)} K_d \tag{13}$$

The maximum activation of adenylyl cyclase for the shuttle model is determined by comparing equations (11) and (12), and recognizing that the numerators are equivalent:

$$[G_s^*C]_{max} = \frac{(k_2r)([G_s]_{total})}{\left([C]_{total} + \frac{k_{-1}}{k_3}\right)(k_2r + k_{-1}) + (k_2r)([G_s]_{total})} \tag{14}$$

Approximation when: $k_{-1}/k_3 \ll [C]_{total}$

If the rate at which G_s^* binds to C is very large compared to the rate at which it becomes inactivated by GTP hydrolysis, then $k_{-1}/k_3 \ll [C]_{total}$ and equation (9) is closely approximated by:

$$[G_s^*C] = \frac{([C]_{total} + [G_s]_{total}\theta) - \sqrt{([C]_{total} + [G_s]_{total}\theta)^2 - 4[C]_{total}[G_s]_{total}\theta}}{2} \tag{15}$$

Since

$$\sqrt{([C]_{total} + [G_s]_{total}\theta)^2 - 4[C]_{total}[G_s]_{total}\theta} = \sqrt{([C]_{total} - [G_s]_{total}\theta)^2} = ([C]_{total} - [G_s]_{total}\theta)$$

then

$$[G_s^*C] = \frac{[C]_{total} + [G_s]_{total}\theta - [C]_{total} + [G_s]_{total}\theta}{2} = [G_s]_{total}\theta \tag{16}$$

when $[C]_{total} > [G_s]_{total}\theta$. Alternatively, when $[G_s]_{total}\theta > [C]_{total}$, the solution is simply:

$$[G_s^*C] = [C]_{total} \tag{17}$$

As above, we note that $\theta = k_2[HR]/(k_2[HR] + k_{-1})$ and that $[HR] = r[H]/(K_d + [H])$, then when $[G_s]_{total} < [C]_{total}$ the level of adenylyl cyclase activity ($[G_s^*C]$) is given by:

$$[G_s^*C] = \frac{[G_s]_{total}\left(\frac{k_2r}{k_2r + k_{-1}}\right)[H]}{\left(\frac{k_{-1}}{k_2r + k_{-1}}\right)K_d + [H]} \tag{18}$$

which is an exact rectangular hyperbola. Maximal activity occurs at high agonist concentration with $[G_s^*C]$ equal to $[G_s]_{total}\, k_2r/(k_2r + k_{-1})$. The EC_{50} is $k_{-1}/(k_2r + k_{-1})\, K_d$.

When $[G_s]_{total} > [C]_{total}$ and the agonist is sufficiently strong that $[G_s]_{total}(k_2r)/(k_2r + k_{-1}) > [C]_{total}$, then the fraction of maximal activity at any agonist concentration is given by:

$$\frac{[G_s^*C]}{[C]_{total}} = \frac{[G_s]_{total}\left(\frac{k_2r}{k_2r + k_{-1}}\right)[H]}{\left(\frac{k_{-1}}{k_2r + k_{-1}}\right)K_d + [H]} \tag{19}$$

and the EC_{50} occurs when

$$\frac{[G_s]_{total}\left(\frac{k_2r}{k_2r + k_{-1}}\right)[H]}{\left(\frac{k_{-1}}{k_2r + k_{-1}}\right)K_d + [H]} = \frac{[C]_{total}}{2} \tag{20}$$

Rearrange and by definition EC_{50} = [H] at half-maximal response.

$$[G_s]_{total}\left(\frac{k_2 r}{k_2 r + k_{-1}}\right)EC_{50} = \frac{k_{-1}}{k_2 r + k_{-1}} K_d \frac{[C]_{total}}{2} + \frac{EC_{50}[C]_{total}}{2} \quad (21)$$

therefore

$$EC_{50}\left(\frac{[G_s]_{total}(k_2 r)}{k_2 r + k_{-1}} - \frac{[C]_{total}}{2}\right) = \frac{k_{-1}}{k_2 r + k_{-1}} K_d \frac{[C]_{total}}{2} \quad (22)$$

and

$$EC_{50} = \frac{\left(\frac{k_{-1}}{k_2 r + k_{-1}}\right) K_d}{\left(\frac{2[G_s]_{total}(k_2 r)}{[C]_{total}(k_2 r + k_{-1})} - 1\right)} \quad (23)$$

Equation 13 is the most appropriate approximation for the EC_{50} when k_{-1} and k_3 are of comparable magnitude or $k_{-1} > k_3$. When $k_3 \gg k_{-1}$, the system is best described by equations 18 and 20 according to whether or not $[G_s]_{total} > [C]_{total}$.