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Differential Expression of the β-Adrenergic Receptor Modifies Agonist Stimulation of Adenylyl Cyclase: A Quantitative Evaluation

BRENDA S. WHALEY, NANYONG YUAN, LUTZ BIRNBAUMER, RICHARD B. CLARK, and ROGER BARBER

Laboratories of Cyclic Nucleotide Research, University of Texas, Graduate School of Biomedical Sciences, Houston, Texas 77225 (B.S.W., N.Y., R.B.C., R.B.), and Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030 (L.B.)

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SUMMARY

The effect of varying levels of β_2-adrenergic receptor (βAR) expression on the capacity of the receptor to activate adenylyl cyclase through regulatory G proteins has been systematically explored in this paper, using differential expression of hamster and human βAR in L cells. Equations have been developed based on the cycle of G protein activation first proposed by Cassel and Selinger and the mobile receptor model, which assumes that hormone-bound βAR can stimulate a number of different adenylyl cyclase moieties through the G protein during a single cycle of activation. These equations predict the relationship of receptor number to the EC_{50} (potency) and V_{max} (efficacy) for adenylyl cyclase activation. L cell clones were selected with stable expression of the βAR over a 2000-fold range of levels (from 5 to 10,000 fmol/mg of membrane protein). Experimentally determined values for the EC_{50} and the V_{max} for epinephrine stimulation of adenylyl cyclase over the entire range of receptor levels were found to be in excellent agreement with predictions of the traditionally accepted models. A method is introduced that allows calculation of βAR coupling efficiency while taking into account the effect of variable receptor levels. The approach provides a quantitative means for the determination of coupling efficiency of the receptor/G protein/adenylyl cyclase system over wide variations in receptor levels and allows for a rational comparison of coupling efficiencies of wild-type and mutant receptors when receptor levels differ.

In recent years there has been an explosion of effort directed toward the definition of the domains of the heptahelical transmembrane receptor family, members of which are involved in ligand binding, coupling to regulatory G proteins, and desensitization (1-11). In these studies wild-type and mutant receptors are typically expressed stably or transiently in a model cell system over an enormous range of receptor densities. Over the past decade evidence has been accumulating from a variety of approaches that the number of βAR present in the membrane has dramatic effects on the kinetic parameters of adenylyl cyclase activation (12-18). However, because of the preliminary nature of the findings many of these reports have been mainly empirical and descriptive. Up to the present no effort has been made to fit the consequences of varying receptor levels into any currently accepted model for adenylyl cyclase activation. In many studies in which the activation of adenylyl cyclase by mutant and wild-type βAR was compared, the effect of different receptor levels remained largely unexplored (1-6). This practice may lead to erroneous conclusions concerning the ability of mutant and wild-type βAR to activate adenylyl cyclase when receptors are expressed at different levels within the membrane.

We have extended the previous studies of the consequences of varying βAR levels on the activation of adenylyl cyclase by measuring EC_{50} and V_{max} for epinephrine stimulation of adenylyl cyclase over a range of receptor levels of 5-10,000 fmol/mg of protein. In an attempt to more quantitatively define this relationship, we have used currently accepted models (13, 19) for βAR/G protein activation of adenylyl cyclase to derive expressions that predict changes in EC_{50} and V_{max} as receptor number varies. Comparisons between predicted results and actual measurements have been made to determine whether accepted models of adenylyl cyclase activity correlate with the observed variation of EC_{50} and V_{max} with receptor number. Finally, we introduce an equation that will allow investigators to accurately determine coupling efficiency from readily measured kinetic parameters (K_d, EC_{50}, and V_{max}) and to deal in a quantitative manner with the problems presented by variable receptor density.

Materials and Methods

Transfection of βAR into L cells and cell culture. The expression vector pSVLneo carrying mutant and wild-type hamster β_2AR was

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ABBREVIATIONS: βAR, β-adrenergic receptor(s); CYP, cyanopindolol; 5-HT, 5-hydroxytryptamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

transfected into Ltk⁻ cells by co-precipitation with calcium phosphate. Stable cell lines were selected and cultured at 37° with 5% CO_2 in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 200 μg/ml geneticin. For transfection with the human β_2AR, Ltk⁻ cells were co-transfected with plasmid pHSV-106, which is a pBR322-based plasmid containing the herpes simplex virus thymidine kinase gene (20). Clones containing wild-type hamster βAR included wild-type-1, wild-type-2, wild-type-A, wild-type-G, wild-type-H, wild-type-I, and wild-type-MB. Clones containing wild-type human βAR included Lβ2.2A, Lβ2.20, Lβ2.5, and Lβ2.7.

Preparation of mutant S261A. cDNA encoding hamster β_2AR was cloned into bacteriophage M_{13} to generate single-stranded DNA. Mutagenesis reactions were conducted following the Amersham mutagenesis protocol, to produce a mutation of serine-261 to alanine. Authenticity of the mutated base sequences was verified by direct sequencing using the dideoxynucleotide method. Mutated β_2AR was inserted into the mammalian expression vector pSVLneo, and plasmid DNA with high purity for *in vitro* transfection was obtained using Qiagen plasmid columns. Transfection into L cells was performed as described above.

Membrane preparation. Preconfluent cells were washed twice at 4° with HME buffer (20 mM HEPES, pH 8, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM benzamidine, 2 mM tetrasodium pyrophosphate, 10 μg/ml trypsin inhibitor, 0.1 mg/ml bovine serum albumin), scraped into HME plus 10 μg/ml leupeptin, and homogenized with seven strokes of a type B Dounce homogenizer. The homogenates were layered over gradients of 23% and 43% sucrose in 20 mM HEPES, pH 8, containing 1 mM EDTA (HE buffer) and were centrifuged for 45 min at 25,000 rpm in a Beckman SW28.1 rotor. The 23/43% interface bands were removed, quickly frozen in liquid nitrogen, and stored at −80°.

Measurement of EC_{50} and V_{max} for adenylyl cyclase activation. Adenylyl cyclase activity was assayed by a modification of the method of Salomon *et al.* (21). Membranes (50 μl, diluted in HE buffer to 0.2–0.5 mg/ml) from a single membrane preparation were incubated for 10 min at 30° in 40 mM HEPES, pH 7.7, 1 mM EDTA, 2.68 mM $MgCl_2$, 8 mM creatine phosphate, 16 units/ml creatine kinase, 50 μM ATP, 1 μM GTP, 0.1 mM 1-methyl-3-isobutylxanthine, with 2 μCi of [α-^{32}P]ATP (30 Ci/mmol; DuPont), in a total volume of 100 μl. The assays were performed in triplicate, using six to eight concentrations of epinephrine that bracketed the EC_{50} concentration. The ^{32}P-labeled cAMP that was produced during the 10-min incubation was purified on Dowex and alumina columns (22). Epinephrine dose-response curves and EC_{50} values were calculated using GraphPAD software.

The V_{max} value quoted in this paper is the absolute activity of adenylyl cyclase obtained with saturating levels of epinephrine, without subtraction of basal activity. We have done this for consistency because basal activities varied among clones. However, using V_{max} values with basal activity subtracted yielded basically the same results as those shown below. Each value of V_{max} obtained with the adenylyl cyclase assay was normalized to the adenylyl cyclase activity obtained with saturating levels of forskolin, by multiplying V_{max} by the average activity obtained for forskolin (80.1 pmol/mg/min, with a standard deviation of 21.1 pmol/mg/min) and dividing the result by the forskolin activity obtained for that same membrane preparation.

Determination of βAR levels. βAR levels were determined by binding of the radioligand ^{125}I-CYP. The ^{125}I-CYP was prepared by the method of Barovsky and Brooker (23), as modified by Hoyer *et al.* (24). Reactions were performed in triplicate in an incubation mixture containing 0–700 pM ^{125}I-CYP, 50 μM phentolamine, and 15–30 μg of membrane protein from a single membrane preparation, in the HE buffer described above, in a total volume of 0.2 ml. Nonspecific binding was assessed in the presence of 1 μM alprenolol. The binding reaction proceeded to equilibrium (50 min at 30°) and was then stopped by rapid dilution with 3 ml of buffer containing 50 mM Tris·HCl, pH 7.4, and 10 mM $MgCl_2$. The 3.2-ml volume was immediately filtered through Whatman GF/C paper on a Millipore model 1225 vacuum filtration manifold. The filter was washed six times with 3-ml volumes of the same buffer, and bound radiolabel was counted in a Beckman Gamma 4000 counter. Mean values were calculated for each triplicate and specific binding was determined as total cpm − cpm with alprenolol. Results were analyzed using a Scatchard plot (bound ligand/free ligand versus bound ligand), and the x-intercept of the resulting line determined total receptor number. Once several membrane preparations of each clone had been characterized by this method, the receptor level in subsequent preparations was determined by performing the binding assay as described above in the presence of a single concentration of 500–800 pM ^{125}I-CYP and using the specifically bound radioligand as the total receptor number. Values for receptor number were also normalized to the maximum forskolin activity, as described above for V_{max}. This was done to correct for any widespread protein denaturation or damage that may have occurred during membrane preparation.

Equations predicting EC_{50} and V_{max}. We derived the following equations based on the mobile-receptor model of Tolkovsky and Levitzki (13) and the GTPase model of Cassel and Selinger (19) for adenylyl cyclase activity. The response of adenylyl cyclase to an agonist concentration is given empirically by:

$$\frac{v}{V_{max}} = \alpha = \frac{[H]}{EC_{50} + [H]} \tag{1}$$

where v is the activity at agonist concentration [H], V_{max} is the maximal activity possible at saturating concentrations of that agonist, and EC_{50} is the concentration of the agonist required to produce half of the maximal activity.

The receptor occupancy by the same agonist is given by:

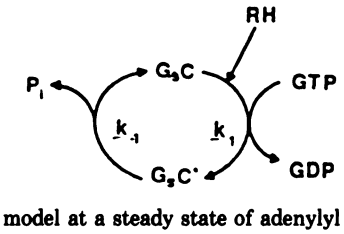
$$\Theta = \frac{[RH]}{r} = \frac{[H]}{K_d + [H]} \tag{2}$$

where Θ is the fraction of the receptors that are bound by the agonist (and is equal to $[RH]/r$, the ratio of the concentration of agonist-bound receptors to the total concentration of receptors) and K_d is the dissociation constant for the agonist and the receptor.

Combining eqs. 1 and 2, with elimination of the agonist concentration, we obtain adenylyl cyclase activity as a function of receptor occupancy:

$$v = V_{max} \frac{\frac{K_d}{K_d - EC_{50}}[RH]}{\frac{EC_{50}r}{K_d - EC_{50}} + [RH]} \tag{3}$$

which is a rectangular hyperbola with respect to [RH]. It can be noted at this stage that, as [H] approaches ∞, Θ approaches 1 and v approaches V_{max}. The above formulation is purely empirical and depends only on the knowledge that both binding and dose-response are Michaelian. However, these empirical formulations can be related specifically to the processes of activation and inactivation by the Cassel-Selinger model:



Because in this model at a steady state of adenylyl cyclase activation the rate of activation is equal to the rate of inactivation we have:

$$v = V_{100} \frac{k_1 f([RH])}{k_{-1} + k_1 f([RH])} = V_{100} \frac{f([RH])}{\frac{k_{-1}}{k_1} + f([RH])} \tag{4}$$

where V_{100} is the adenylyl cyclase activity with 100% of the receptors fully activated, k_1 is the rate constant for activation by GDP/GTP exchange, k_{-1} is the first-order rate constant for inactivation, which

corresponds to the activity of the endogenous GTPase, and $f([RH])$ is some function of the concentration of the receptor-agonist complex in the membrane. Note that V_{100} is the activity obtainable by 100% activation of an adenylyl cyclase system. This is different from the activity obtained with high levels of forskolin, which is thought to activate adenylyl cyclase directly, does not act through βAR, and may have a different turnover number for the catalyst.

The empirical description of adenylyl cyclase activity described in eq. 3 can be equated with that obtained from the Cassel-Selinger model in eq. 4, giving:

$$V_{max}\frac{\frac{K_d}{K_d - EC_{50}}[RH]}{\frac{EC_{50}r}{K_d - EC_{50}} + [RH]} = V_{100}\frac{f([RH])}{\frac{k_{-1}}{k_1} + f([RH])} \quad (5)$$

The left and right sides of eq. 5 can be identical only if

$$V_{max}\frac{K_d}{K_d - EC_{50}} = V_{100} \quad (6)$$

and

$$\frac{EC_{50}r}{K_d - EC_{50}} = \frac{k_{-1}}{k_1} \quad (7)$$

and if $f([RH])$ is identical to $[RH]$.

The above identities can be combined and rearranged into the following relationships, which are predictions for V_{max} and EC_{50} as a function of the receptor level, r:

$$V_{max} = V_{100}\frac{k_1 r}{k_1 r + k_{-1}} \quad (8)$$

and

$$EC_{50} = K_d\frac{k_{-1}}{k_1 r + k_{-1}} \quad (9)$$

It should be noted that in the last two expressions k_{-1} is determined by the activity of the endogenous GTPase and is independent of the nature of the agonist. The agonist-dependent parameter is k_1, which is the rate constant for activation of the adenylyl cyclase as catalyzed by the complex between the receptor and the agonist in question. For the purpose of simulating EC_{50} and V_{max}, it was not necessary to deduce exact values for k_1 and k_{-1} but to determine the ratio k_1/k_{-1}. For this reason, k_{-1} is set to 1 and k_1 is treated as a variable in all simulations.

Results

Variation of EC_{50} with receptor level. The βAR level and the EC_{50} for adenylyl cyclase activation by epinephrine were determined as described above for membranes prepared from seven clones of L cells transfected with wild-type hamster βAR. These clones expressed the receptor at a level of <300 fmol/mg of membrane protein. The results are shown in Table 1 and plotted in Fig. 1A. Each data point in Fig. 1A represents the results of measurements performed on an individual membrane preparation of a particular L cell clone, as shown in Table 1 and identified in the figure legend. Variation in receptor level for different membrane preparations of a single clone is commonly observed (14). The simulation shown in Fig. 1A represents the values predicted by eq. 9 for the EC_{50} as receptor level varies. The curve shown was obtained using the k_1/k_{-1} value set to 0.15 and the K_d set to 250 nM. Equation 9 represents a rectangular hyperbola in which the K_d is the maximum value approached by the EC_{50} as r approaches 0. A value of 250 nM for K_d is corroborated by the value of 306 ± 111 nM (with GTP) that we typically measure for epinephrine and the βAR with ^{125}I-CYP displacement in membranes from clones transfected with the hamster receptor (data not shown).

As demonstrated in Fig. 1A, the EC_{50} values we measured for adenylyl cyclase at βAR levels of <300 fmol/mg of membrane protein correlated well with the predictions made by equations derived from traditionally accepted models for adenylyl cyclase activity. To determine whether these relationships were valid at even higher densities of receptor, we measured the EC_{50} for adenylyl cyclase in the membranes of four additional L cell clones expressing βAR at 28 fmol/mg of membrane protein to as much as 10,000 fmol/mg of membrane protein in some individual preparations. These clones included L cells transfected with wild-type human βAR as described in Materials and Methods. The results are shown in Table 2. Fig. 1B is a plot of the average values of EC_{50} and receptor number obtained for the 11 wild-type clones studied. These averages, along with the associated standard deviations, are shown in Tables 1 and 2. The simulation shown in Fig. 1B was obtained using the same values for k_1/k_{-1} and K_d as was used in Fig. 1A, with the curve being extended to r values of 12,000 fmol/mg of membrane protein. Fig. 1C is a log/log plot of EC_{50} versus receptor number for the data obtained for both hamster and human receptors. In this form the EC_{50} values measured for receptor levels over 3 orders of magnitude can be viewed on a single unbroken plot. The simulation shown uses the log values predicted by the same equation used for the simulations in Fig. 1, A and B, using the same values for k_1/k_{-1} and K_d.

Variation of V_{max} with receptor level. The V_{max} for adenylyl cyclase activation by epinephrine was also measured for the seven clones shown in Fig. 1A. The results are shown in Table 1 and plotted in Fig. 2A. Once again, each data point represents the results of a single measurement performed on a unique membrane preparation, and all V_{max} values were normalized to the activity of adenylyl cyclase obtained with saturating amounts of forskolin, as described in Materials and Methods. The simulation represents the values predicted by eq. 8 for the V_{max} as receptor level varies. The curve shown in Fig. 1B was obtained using a k_1/k_{-1} value of 0.15 and a V_{100} value of 17 pmol/mg/min. Equation 8 indicates V_{100} as the maximum value approached by V_{max} as r becomes very large. Fig. 2 shows that 17 is a good estimate of the maximum value approached by the V_{max} measurements we obtained as r approached ∞. Note that the k_1/k_{-1} value used in this simulation is the same as that which was used in the simulation for EC_{50} in Fig. 1.

Once again, to determine whether predictions made by the mobile-receptor/GTPase model concerning V_{max} for adenylyl cyclase activity were valid for higher levels of βAR, we measured the V_{max} for the four additional L cell clones transfected with human βAR that were described above. The results are shown in Table 2. Average values and the associated standard deviations for V_{max} and receptor number are listed in Tables 1 and 2. Fig. 2A is a plot of the average values obtained for the 11 wild-type clones studied. The simulation shown in Fig. 2B was obtained using the same values for k_1/k_{-1} and V_{100} as were used in Fig. 1A, with the curve being extended to r values of 12,000 fmol/mg of membrane protein.

Relationship of V_{max} and EC_{50}. Dividing eq. 8 by eq. 9 yields the following prediction for the relationship of V_{max} and EC_{50} as the receptor number varies:

TABLE 1

Adenylyl cyclase activity and expression level for hamster β_2AR

The values are the kinetic parameters for adenylyl cyclase activation and B_{max} observed for wild-type hamster βAR expressed at different levels. For V_{max} and B_{max} the numbers in parentheses are original measurements and adjacent numbers have been normalized to forskolin activity as described in Materials and Methods.

Clone	Preparation	Basal cyclase activity	V_{max}	Forskolin-stimulated cyclase activity	Epinephrine EC_{50}	B_{max}
		pmol/mg/min	pmol/mg/min	pmol/mg/min	nM	fmol/mg
Wild-type-1 (n = 6)*	93-34	3.1	(20.9) 31.1	121.0	51.0	(34) 51
	93-39	1.6	(13.1) 11.5	91.6	43.0	(40) 35
	93-41	4.0	(27.3) 15.2	145.0	32.0	(120) 67
	93-53	2.0	(13.4) 14.3	75.7	81.0	(42) 45
	93-56	2.5	(14.5) 15.0	77.7	49.1	(30) 31
	93-60	2.5	(10.0) 12.4	64.8	35.0	(47) 58
	Average ± SD		16.6 ± 6.6		48.5 ± 16.1	48 ± 12
Wild-type-2 (n = 6)	93-39	2.6	(15.6) 15.2	82.6	31.0	(59) 58
	93-43	3.2	(23.7) 20.0	95.7	29.0	(72) 61
	93-45	3.0	(18.3) 20.5	72.1	27.0	(65) 73
	93-51	3.4	(12.3) 14.6	68.0	22.0	(51) 60
	93-57	3.6	(11.6) 13.0	72.0	42.0	(24) 27
	93-57B	3.6	(11.6) 12.8	72.8	42.0	(37) 41
	Average ± SD		16.0 ± 3.1		32.2 ± 7.5	53 ± 15
Wild-type-A (n = 3)	93-34	1.3	(6.7) 6.4	83.8	144.0	(5) 5
	93-39	0.9	(5.0) 5.4	74.6	173.0	(6) 7
	93-42	1.2	(6.3) 6.8	75.2	197.0	(6) 6
	Average ± SD		6.2 ± 0.6		171.3 ± 21.7	6 ± 1
Wild-type-G (n = 3)	93-34	1.5	(16.0) 9.5	136.0	48.2	(76) 45
	93-39	1.5	(17.0) 13.6	101.0	63.0	(36) 28
	93-54	1.7	(18.6) 19.2	78.2	31.0	(52) 54
	Average ± SD		14.1 ± 4.0		79.2 ± 13.1	42 ± 11
Wild-type-H (n = 5)	93-97	4.5	(19.5) 22.4	70.3	9.3	(210) 241
	93-115	2.5	(16.6) 15.5	75.9	4.5	(281) 262
	93-125	3.5	(22.8) 23.6	84.2	3.7	(275) 285
	93-151	4.0	(18.5) 17.5	77.1	4.6	(218) 207
	93-157	5.4	(17.7) 14.1	65.0	5.0	(266) 213
	Average ± SD		17.7 ± 4.2		4.5 ± 0.5	242 ± 38
Wild-type-I (n = 1)	93-39	5.2	(14.5) 11.5	102.0	9.4	(10) 8
Wild-type-MB (n = 2)	93-100	3.4	(13.8) 11.6	95.5	30.0	(103) 87
	93-105	2.5	(9.5) 11.2	68.6	47.0	(91) 107
	Average		11.4		38.5	97
	Range		0.4		17.0	20
S261A (n =5)	93-46	2.6	(8.6) 9.6	72.5	135.0	(24) 27
	93-47	1.5	(5.7) 9.1	50.4	42.0	(24) 39
	93-49	1.3	(6.3) 7.8	64.7	32.7	(35) 44
	93-69J	2.7	(23.7) 17.1	112.0	13.0	(393) 283
	93-69G	1.8	(18.4) 18.9	78.3	8.7	(371) 382
	Average ± SD		12.5 ± 4.6		46.3 ± 46.0	155 ± 148

* n, number of preparations.

$$\frac{V_{max}}{EC_{50}} = \frac{V_{100}k_1 r}{K_d k_{-1}} \quad (10)$$

A plot of V_{max}/EC_{50} versus receptor number is expected to be linear, with a slope equal to:

$$\frac{V_{100}k_1}{K_d k_{-1}}$$

We calculated V_{max}/EC_{50} using the data in Table 1 obtained from the seven clones transfected with wild-type hamster βAR. The results are plotted in Fig. 3. The simulation represents the values for V_{max}/EC_{50} predicted by eq. 10 using the values from the simulations for EC_{50} in Fig. 1 and for V_{max} in Fig. 2, i.e., k_1/k_{-1} is 0.15, V_{100} is 17 pmol/mg/min, and K_d is 250 nM. Linear regression analysis yielded an r^2 value of 0.84. We purposely did not include the V_{max}/EC_{50} calculated using the measurements from the four L cell clones transfected with human βAR in Fig. 3, in an attempt to ensure that all clones being considered had comparable values for k_1.

Comparison of the coupling efficiencies of clones with different receptor levels. The results shown in Figs. 1–3 indicate that the EC_{50} and V_{max} for adenylyl cyclase activation may vary dramatically with the level of βAR expression in membrane preparations with presumably identical or comparable abilities to couple to adenylyl cyclase. We define coupling efficiency quantitatively as the rate constant for activation in the Cassel-Selinger scheme (k_1), at a defined receptor density. Because k_1 is a measure of the ability of βAR to activate adenylyl cyclase, it is important to be able to accurately assess and compare k_1 values in two different clones that express βAR at different levels. We propose that eq. 7 would allow a calculation of k_1/k_{-1} for a particular receptor using K_d, EC_{50}, and r. Because k_{-1} is a measure of the intrinsic GTPase activity of $G_{s\alpha}$, it is independent of βAR and should be the same in two clones from identical cell lines. This allows the coupling efficiency, or k_1, of two different clones from identical cell lines to be compared by comparing the k_1/k_{-1} values obtained using eq. 7. This comparison may be made by calculation of the ratio of k_1/k_{-1} for one clone to k_1/k_{-1} obtained for a second clone. In this ratio k_{-1} would "cancel," because it is thought to be the

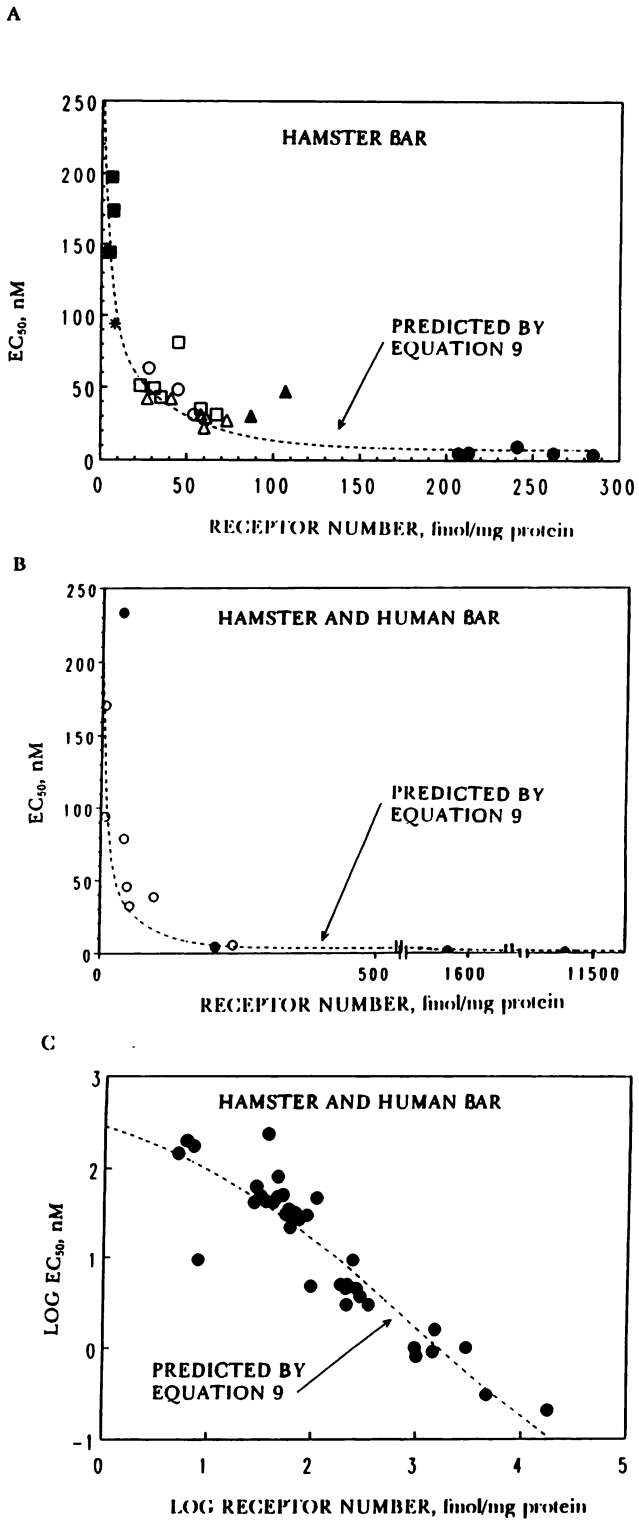


Fig. 1. Relationship of EC_{50} to receptor levels. The change in the EC_{50} for epinephrine as βAR level varies in the membranes of L cells is shown. The receptor numbers have been normalized as described in Materials and Methods. A, Plot of data for the wild-type hamster βAR expressed at different levels listed in Table 1. □, Wild-type-1; △, wild-type-2; ■, wild-type-A; ○, wild-type-G; ●, wild-type-H; *, wild-type-I; ▲, wild-type-MB. B, Each *point* represents the average EC_{50} and receptor level obtained for multiple membrane preparations of the 11 wild-type L cell clones listed in Tables 1 and 2. The *n* values and standard deviations for measurements are shown in Tables 1 and 2. ○, Hamster βAR; ●, human βAR. C, Log/log plot of data for the wild-type hamster and human βAR expressed at different levels listed in Tables 1 and 2.

same for both clones. The ratio of these two values can be thought of as a ratio of coupling efficiency when the two membrane preparations are compared.

We used eq. 7 to compare the k_1 values for eight of our L cell clones with the average k_1 value obtained for wild-type-1. We used the K_d value of 250 nM obtained in Fig. 1A for all of the clones in these calculations. The average values we obtained from these calculations are plotted in Fig. 4. A coupling efficiency ratio of approximately 1 was obtained for comparisons between wild-type-2, -A, -G, and -MB and the S261A mutant and wild-type-1, which were all transfected with hamster βAR. This indicates that k_1, or the rate constant for activation of adenylyl cyclase, is comparable in these systems. When we compared the coupling efficiency of clones transfected with human βAR with that of wild-type-1, Lβ2.5 and Lβ2.7 yielded coupling efficiency ratios of approximately 1. Lβ2.20, however, yielded a coupling efficiency ratio of >2. This indicates that the k_1 for adenylyl cyclase activation is slightly higher for this clone, compared with the other clones studied. Wild-type-1 was arbitrarily chosen as the standard for this comparison. As expected, use of another clone instead of wild-type-1 altered the absolute magnitude of the coupling efficiency ratios obtained for each clone but did not change the overall pattern of the relative magnitudes for the coupling efficiency ratios observed in Fig. 4 (data not shown).

Discussion

In recent years, studies of the activation of adenylyl cyclase by the βAR have resulted in considerable insight into the domains of βAR involved in the binding of agonists, in the interaction with G proteins, and in the various pathways of desensitization. In a typical study wild-type and mutant receptors are expressed in cells lacking the receptor, and activation parameters for βAR agonists, such as efficacy (V_{max}), potency (EC_{50}), and K_d, are determined. In an effort to better understand and interpret the results of studies involving these kinetic parameters for adenylyl cyclase activation, we have attempted to quantitatively examine the consequences of varied βAR levels on the V_{max} and EC_{50} for the activation of adenylyl cyclase. The equations presented in this paper predicting the relationship between V_{max} and EC_{50} and receptor density were derived from the mobile-receptor model for adenylyl cyclase activation and the Cassel-Selinger model of βAR interaction with G_s and adenylyl cyclase, two descriptions of the coupling that occurs between the βAR and adenylyl cyclase that have withstood the test of time. We have compared our measurements for V_{max} and EC_{50} with simulations obtained with these equations, to fit our observations into the framework of currently accepted models for adenylyl cyclase activation.

Our study of the EC_{50} for epinephrine activation of adenylyl cyclase at varying levels of receptor density shows a decrease from 200 to 0.2 nM as receptor density increases from 5 to 5000 fmol/mg of protein. This confirms and extends the results of two previous studies performed with Chinese hamster fibroblast membranes (15) and Chinese hamster ovary whole cells (17), in which a downward trend was observed for the EC_{50} as receptor number increased over a 10-fold and 300-fold change in βAR density, respectively, but disagrees with a third study performed over a small range of βAR densities in S49 lymphoma cells deficient in βAR (14), which showed no change in EC_{50} as receptor levels increased. Fig. 1 shows that the predictions

TABLE 2

Adenylyl cyclase activity and expression level for human β_2AR

The values are the kinetic parameters for adenylyl cyclase activation and B_{max} observed for wild-type human βAR expressed at different levels. For V_{max} and B_{max} the numbers in parentheses are original measurements and adjacent numbers have been normalized to forskolin activity, as described in Materials and Methods.

Clone	Preparation	Basal cyclase activity	V_{max}	Forskolin-stimulated cyclase activity	Epinephrine EC_{50}	B_{max}
		pmol/mg/min	pmol/mg/min	pmol/mg/min	nM	fmol/mg
Lβ2.2A (n = 1)	6-21	0.5	(4.7) 6.0	63.4	233	(28) 36
Lβ2.20 (n = 4)	93-67	18.5	(36.3) 31.1	94.2	4.8	(113) 97
	6-28	9.1	(23.0) 26.6	69.8	3.0	(300) 346
	7-20	17.7	(33.9) 26.6	102.8	5.0	(236) 185
	7-23	5.3	(15.5) 16.4	76.4	3.0	(201) 212
	Average ± SD		25.2 ± 5.4		4.0 ± 1.0	210 ± 89
Lβ2.5 (n = 5)	93-66	9.2	(21.8) 22.1	79.4	1.6	(1,490) 1,512
	6-16	5.3	(17.2) 17.5	79.1	1.0	(2,946) 3,002
	6-28	6.1	(13.3) 21.3	50.3	0.8	(630) 1,009
	7-20	12.3	(21.9) 21.7	81.4	0.9	(1,459) 1,445
	7-23	10.8	(24.0) 28.0	69.2	1.0	(833) 970
	Average ± SD		22.1 ± 3.4		1.1 ± 0.3	1,588 ± 741
Lβ2.7 (n = 2)	93-64	4.7	(21.6) 36.9	47.2	0.2	(10,546) 18,008
	7-20	4.0	(22.5) 32.2	56.3	0.3	(3,296) 4,718
	Average		34.5 ± 4.7		0.3 ± 0.1	11,363 ± 13,290
	Range					

made by eq. 9 for the variation of EC_{50} with receptor number closely fit the data obtained for EC_{50} values and receptor numbers in the 11 wild-type clones we studied.

The K_d value for epinephrine obtained when eq. 9 was used to simulate the data for EC_{50} was comparable to that obtained by equilibrium binding measurements. This is in spite of the limitations of the mobile-receptor model in membrane preparations. The mobile-receptor model specifically assumes that the available receptors are "shared" equally between all adenylyl cyclase moieties. Stickle and Barber (18) showed that in intact cells that assumption is not correct and the quantitative deviation from the predictions of the pure collision coupling model are most obvious at low receptor densities (25). In addition, in the preparation of membranes the homogenization procedure may cause the formation of small vesicles where, at low receptor densities, receptors, G proteins, and cyclase may not be present together. Vesicles containing no receptors are not observed in the adenylyl cyclase assay for epinephrine stimulation, which is dependent on the presence of βAR. Adenylyl cyclase activation requires at least one receptor per vesicle. Thus, in practice, receptor number does not extrapolate to zero but has a minimum value of one per vesicle. This could lead to an underestimation when the x-intercept of the plot in Fig. 1 is used to determine the K_d for epinephrine and βAR. The fact that the estimate of K_d from the plot is only slightly lower than the K_d measured directly indicates that the vesicle effect is not quantitatively important in this system.

Our measurements for V_{max} showed an increase from 5 to 20 pmol/mg/min as receptor level increased over a narrow range of 5–100 fmol/mg of protein. This corroborates previously published studies (14, 15, 17), which were in general agreement that V_{max} increases roughly proportionally to βAR levels, although one group of authors reported that V_{max} increased, reached a plateau, and then decreased as receptor density increased (15). Fig. 2 compares the values predicted for V_{max} by eq. 8 with our measurements for V_{max} as receptor level varied. The fit obtained describes the results well, but is less quantitative in nature than that obtained using eq. 9 to simulate the measurements of EC_{50} as receptor number varies.

As shown in Fig. 3, we observed a correlation between an increase in V_{max} and a decrease in EC_{50} for adenylyl cyclase activation as receptor level increases and found that the ratio of these kinetic parameters is linear with respect to receptor number. Equation 10 provided an accurate simulation of the change in V_{max}/EC_{50} as receptor level varied and was able to predict the slope of V_{max}/EC_{50} versus receptor number using values for V_{100}, k_1/k_{-1}, and K_d obtained with eqs. 8 and 9.

This systematic study of the kinetic parameters of adenylyl cyclase in relation to βAR levels in the membranes of L cells shows that V_{max} and EC_{50} are highly dependent on the level of βAR expression over the range that commonly occurs in wild-type cells. The variation observed for EC_{50} and V_{max} with varying receptor levels is predicted by accepted models for adenylyl cyclase activity and can be quantitated by equations derived from those models. Equations 8, 9, and 10 have been shown to closely predict the changes observed for EC_{50} of adenylyl cyclase and also to describe the V_{max} for adenylyl cyclase as βAR levels vary over 3 orders of magnitude.

Previously, the term "coupling efficiency" has been vaguely defined as the ability of a receptor to activate and/or interact with the G protein/adenylyl cyclase system. In this paper we have quantitatively defined coupling efficiency and introduced an equation that allows the comparison of coupling efficiency in the adenylyl cyclase system between two receptors that are expressed at different levels. Using eq. 7 we calculated and compared the coupling efficiency of eight of our clones with that of wild-type-1, which contains hamster βAR. As might have been expected, all of the wild-type clones containing the hamster βAR yielded a coupling efficiency ratio of approximately 1, compared with wild-type-1, indicating similar coupling efficiency. The S261A mutant of the hamster βAR, which is expected to couple normally, also yielded a coupling efficiency ratio comparable to that of the other wild-type clones.

In addition, the Lβ2.5 and Lβ2.7 clones, which contained the human βAR, were also shown to yield a coupling efficiency ratio comparable to that of the clones containing hamster βAR. Both of these clones were expressed at much higher receptor levels than the clones transfected with hamster βAR. They

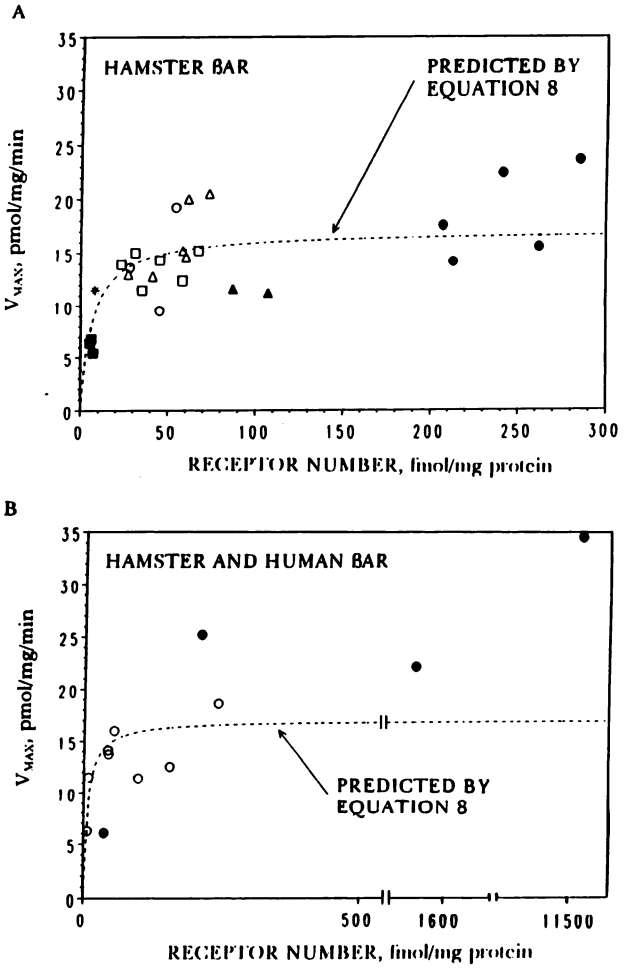


Fig. 2. Relationship of V_{max} to receptor levels. The change in V_{max} for epinephrine as βAR level varies in the membranes of L cells is shown. The V_{max} values and receptor numbers shown have been normalized as described in Materials and Methods. A, Plot of data for wild-type hamster βAR expressed at different levels listed in Table 1. □, Wild-type-1; △, wild-type-2; ■, wild-type-A; ○, wild-type-G; ●, wild-type-H; *, wild-type-I; ▲, wild-type-MB. B, Each *point* represents the average V_{max} and receptor level obtained for multiple membrane preparations of the 11 wild-type clones listed in Tables 1 and 2. The *n* values and standard deviations for measurements are shown in Tables 1 and 2. ○, Hamster βAR; ●, human βAR.

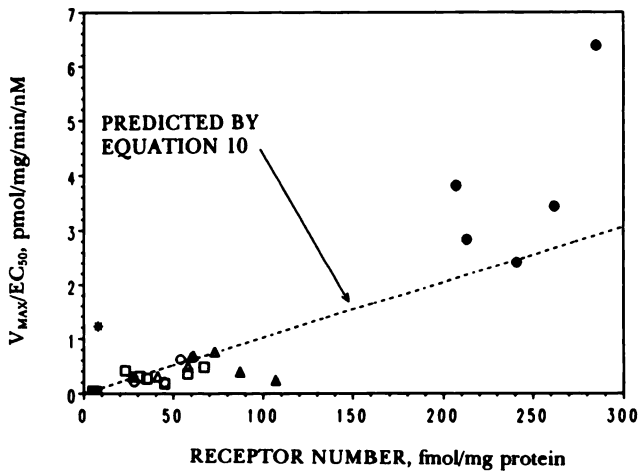


Fig. 3. Relationship of V_{max}/EC_{50} to receptor levels. The change in the ratio of V_{max} to EC_{50} as βAR level varies in the membranes of L cells is shown. *Points* were calculated from the data listed in Table 1. □, Wild-type-1; △, wild-type-2; ■, wild-type-A; ○, wild-type-G; ●, wild-type-H; *, wild-type-I; ▲, wild-type-MB.

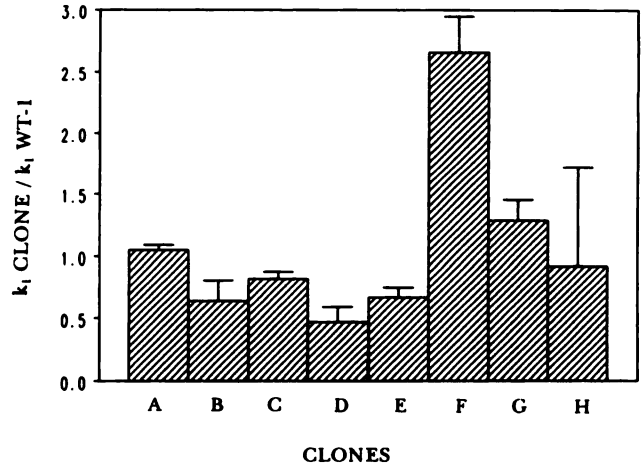


Fig. 4. Coupling efficiency ratios. Coupling efficiency ratios were calculated using eq. 7 for the eight clones described below, compared with wild-type-1. Of the resulting ratios of k_1 for the clone/k_1 for wild-type-1, the mean values obtained for each clone were plotted along with error bars representing the standard error obtained. The *n* values for each clone are the same as those listed in Tables 1 and 2. *A*, Wild-type-2; *B*, wild-type-A; *C*, wild-type-G; *D*, wild-type-MB; *E*, S261A; *F*, Lβ2.20; *G*, Lβ2.5; *H*, Lβ2.7. k_1/k_{-1} for wild-type-1 is 0.13 ± 0.070.

both yielded a much lower EC_{50} and a slightly higher V_{max} for adenylyl cyclase, compared with the wild-type-1 clone (see Tables 1 and 2). If only the kinetic parameters of adenylyl cyclase activation were taken into account and variation in receptor density were ignored, then these clones containing human βAR might be assessed to have very high coupling efficiencies, compared with the wild-type-1 clone, which contains hamster βAR. Using eq. 7 to calculate the k_1/k_{-1} we have been able to show that the low EC_{50} and the elevated V_{max} are not indicative of enhanced coupling to G_s in Lβ2.5 and Lβ2.7 but rather are a consequence of the high expression of βAR that occurs in these clones.

Lβ2.20 cells, which also contained human βAR, yielded a coupling efficiency ratio of >2, compared with wild-type-1. Although this result was unexpected, our inspection of measurements obtained in the four adenylyl cyclase assays performed on this clone indicates that these results are statistically significant (see Table 2). We have no explanation at this time as to why the receptor in Lβ2.20 should couple to the adenylyl cyclase system more efficiently than those in Lβ2.5 and Lβ2.7, because the clones all contain wild-type human β_2AR transfected into the same cell line. We do note, however, that the level of basal activity does seem to be unusually high in Lβ2.20 (see Table 2) and may be indicative of a more highly active βAR/adenylyl cyclase system.

These results clearly show that changes in EC_{50} and V_{max} that occur with varying expression levels for βAR must be accounted for when comparing the coupling efficiency of receptors from two different clones. Using the method presented here we were able to make a valid comparison between the S261A mutant and the other hamster wild-type clones, showing that coupling is not impaired or enhanced by this mutation. We were also able to compare the coupling efficiencies of hamster and human βAR despite great differences in the level of receptor expression. We acknowledge that these equations cannot factor out secondary consequences of the effects of

increasing receptor density, if they exist. For example, work by Samama *et al.* (17) and our own preliminary data indicate that increasing receptor density increases basal activity (see Tables 1 and 2). A higher basal activity of adenylyl cyclase may lead to greater activation of cAMP-dependent protein kinase, and all of the possible effects of this are difficult to predict. However, our preliminary study suggests that this equation may be useful in allowing a meaningful comparison of the EC_{50} values that are obtained for membrane preparations of two receptors expressed at different levels.

The study presented here indicates that direct comparison of V_{max} and EC_{50} to evaluate the effect of receptor modification on the ability of a receptor to stimulate adenylyl cyclase is valid only when receptor levels are identical. Although this is achieved on occasion, receptor densities quite often vary dramatically between two different clones. In addition, investigators in this and other laboratories have observed that receptor density for a single clone that is presumably stable may vary from one membrane preparation to the next. Our results suggest that V_{max} and EC_{50} values from different clones or from different membrane preparations of the same clone should not be compared without taking even seemingly small variations in receptor level into account.

Our findings also predict that the use of V_{max} and EC_{50} in assessing the desensitization of βAR must be coupled with a determination of receptor levels before an accurate evaluation of the various types of desensitization can be made. Down-regulation and sequestration may be compared with a decrease in receptor density. A decrease in coupling efficiency that occurs through receptor phosphorylation may be compared with a decrease in k_1. Examination of Figs. 1B and 2B and calculations with eqs. 8 and 9 predict that with high receptor levels (>200 fmol/mg) any decrease in $k_1 r$, due to either loss of r or change in k_1, will manifest itself as an increase in the EC_{50} with very little change in V_{max}. At intermediate to low receptor levels (20–200 fmol/mg) V_{max} will decrease with increases in EC_{50}. Therefore, the rate of change observed for both V_{max} and EC_{50} as a result of down-regulation, sequestration, or phosphorylation of βAR depends on the level of βAR expression found in the membrane preparation and should be considered when evaluating the ability of βAR to desensitize.

In testing our model with data published for other receptor systems, we found that it simulates quite well the results obtained from a study of the effect of differential expression of muscarinic receptors on the hydrolysis of phosphoinositide (26). This study involved a 10-fold change in receptor density for the human muscarinic receptor. The observed EC_{50} was shown to decrease from 30 to 3 μM and the maximum stimulation to increase 2-fold, in a manner predicted by eqs. 9 and 8, respectively (simulation not shown). This indicated that the model would be extended to at least some other systems in which receptors were coupled to G proteins. Other results from the same study included the monitoring of the effect of increased muscarinic receptor density on the inhibition of forskolin stimulation of adenylyl cyclase. The EC_{50} and the maximum stimulation of inhibition of cAMP formation by carbachol did not change with a 10-fold change in receptor level (26). These results could not be simulated by the equations presented here.

The model presented in this communication assumes that the βAR coupling to G_s is mobile. Therefore, eqs. 8 and 9 should not be expected to predict the effect of changes in receptor density on activation parameters for other receptor, G protein, or effector combinations that interact differently. This seems to be the case for the inhibition of adenylyl cyclase by the muscarinic receptor previously mentioned and for the 5-HT_{1A} receptor inhibition of adenylyl cyclase activation. The 5-HT_{1A} receptor has been studied after differential expression of the receptor in Chinese hamster ovary cells (27) and irreversible inhibition of postsynaptic 5-HT_{1A} receptors in the raphe nucleus (28). Both studies indicated that the EC_{50} for several agonists remained at levels not significantly different from the K_d, whereas the V_{max} decreased as receptor density decreased. Human 5-HT_{1A} receptors have been shown to inhibit adenylyl cyclase activation through coupling to inhibitory G proteins (29). G_i is known to co-purify with solubilized adenosine (30) and somatostatin (31) receptors, as opposed to G_s, which is separated from βAR upon purification. This evidence has been interpreted as an indication that G_i is precoupled to receptors in the membrane whereas G_s is not. A recent study showing a lack of cross-talk in agonist binding among G_i-coupled α_2-adrenergic, muscarinic, and opiate receptors in NG108-15 cells (43) also lent credence to the hypothesis that G_i interaction with receptors was quite different from that of G_s. Our model, based on the assumption that the receptor is mobile, should not apply in systems where the mechanism of G protein coupling to receptor is significantly different from that of βAR/G_s/adenylyl cyclase, but it is applicable to systems in which the receptor is coupled to a G protein in a manner similar to that of the system studied here.

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Send reprint requests to: Richard B. Clark, Graduate School of Biomedical Sciences, University of Texas, P.O. Box 20334, Houston, TX 77225.