The Use of Stimulus-Biased Assay Systems to Detect Agonist-Specific Receptor Active States: Implications for the Trafficking of Receptor Stimulus by Agonists

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A classic pharmacologic method of receptor and agonist classification is through the use of agonist potency ratios. Under null conditions, the relative potency of agonists is independent of the host cell for the receptor and depends only on the relative affinity and intrinsic efficacy of the agonists for the receptor type (see Appendix I). Deviations in agonist potency ratios therefore are used as presumptive evidence for differences in receptors. This technique is based on the tacit assumption that the mechanism of response production for the agonists involved is the same (i.e., that they produce the same active state of the receptor). Therefore, differences in agonist potency ratios, in contrast to furnishing evidence for differences in receptor types, may alternatively indicate lack of adherence to this tacit assumption. This article describes the induction of variation in agonist relative potency ratios in different tissue host cells for a single transfected receptor, the hCTR2. This raises the possibility that this effect indicates the production of agonist-specific receptor active states by the different agonists.

Materials and Methods

Molecular Biology and Generation of Stable Cell Lines

Full-length bovine Gas (short form) cDNA was kindly provided by Dr. Pat Casey at Duke University (Robishaw et al., 1986). The full-length cDNAs for mouse Gq (Strathmann and Simon, 1990), rat Gαq (Jones and Reed, 1987), and rat Gα1 (Jones and Reed, 1987) were provided by Steve Rees at GlaxoWellcome, UK. The NcoI/HindIII fragment of pTT-5/Gas, BamHI fragment of pSG5/Gaq, HincII fragment of pTT-5/Gao, and the HincII fragment of pTT-5/Gα1 were isolated and subcloned into pCIN expression vector (Rees et al., 1996). The full-length calcitonin receptor was isolated and subcloned...
into expression vector pcDNA3 as described previously (Chen et al., 1997).

The expression vectors containing different G proteins were then transfected into HEK 293 cells using calcium phosphate method (Promega, Madison, WI). On day 3 after transfection, the cells were selected using G418-supplemented media at a concentration of 600 µg/ml. After a 2-week selection, colonies were selected and expanded. Stable lines were checked for expression by immunoblotting.

**Gel Electrophoresis and Immunoblotting**

HEK 293 membrane preparations, made from stable clones containing various amounts of overexpressed Gα-proteins, were qualitatively evaluated using SDS-polyacrylamide gel electrophoresis according to the procedure of Laemmli (1970). Protein (20 µg) was dissolved in 30 µl of TBS-T buffer (20 mM Tris and 500 mM NaCl, pH 7.5, 0.1% Tween 20) and then incubated with 30 µl of 2× SDS loading buffer containing 5% 2-mercaptoethanol. This mixture was boiled for 5 min and cooled on ice. The denatured protein solution (30 µl [10 µg]) was then loaded into each well of the Novex 10-well, 10% 1.5-mm Tris-glycine gel and run at 120V for 90 to 100 min. The resolved proteins were transferred to Novex nitrocellulose membranes. The nitrocellulose membrane then was incubated in blocking buffer (Megga-Block 1:10 in TBS-T) for 1 h. After washing in TBS-T with 1:100 Megga-Block the membranes were incubated with various Gα-specific antibodies (Santa Cruz Biotech, Santa Cruz, CA) for 1 h. After a second washing, the membranes were incubated with a secondary horseradish peroxidase goat anti-rabbit antibody (1:5000) for 1 h. The blot then was developed using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ). Quantitative analysis of the Western blots was done with the BioRad GS-700 Imaging Densitometer (BioRad, Hercules, CA). Bands were measured and analyzed using the BioRad Molecular Analyst software package and data expressed in RDU. Clones were selected based on the increased density of G protein band compared with control.

**Transient Transfection of hCTR2**

HEK 293 cells enriched with α-subunits Gi, Gs, Go, and Gq were plated at a density of 10⁵ cells/225-mm² flask and grown overnight in DMEM plus 10% fetal calf serum supplemented with 1-glutamine (2 mM). Cells were transfected with 40 µg of pcDNA3 vector control (Invitrogen, Carlsbad, CA) or pcDNA3/hCTR2 using the calcium phosphate DNA transfection method (Davis et al., 1986). After a 6-h transfection, media were replaced and cells were grown an additional 48 h when they were collected for assay. HEK 293 cells (wild-type, Gαi-21, Gαo-24, Gαq-15, and Gαo-13 line) were plated to a concentration of 106 cells/100-mm dish. After 24 h, cells were cotransfected with clone 134/pMTR with pRSV/neo at a ratio of 10:1, respectively, according to the calcium phosphate method (Promega).

**Binding Studies**

**Membrane Preparation.** At confluency, cultured cells were harvested by manual scraping of the tissue culture flasks. Cells were pelleted by centrifugation at 2000 rpm for 15 min and then homogenized (three 15-s bursts) in ice-cold HEPES buffer (20 mM HEPES, pH adjusted to 7.4 with NaOH at 23°C). The homogenate was centrifuged at 48,000g for 15 min and washed twice through resuspension with new buffer. After a third centrifugation, the pellet was resuspended in fresh buffer containing 0.2 mM phenylmethylsulfonyl fluoride. Aliquots were frozen at −70°C.

**Saturation Binding Curves.** Membranes were equilibrated with either 125I-AC512 (2000 Ci/mmol; Amersham Pharmacia Biotech) or 125I-hCAL (2000 Ci/mmol; Amersham Pharmacia Biotech) in 20 mM HEPES buffer containing 0.5 mg/ml bacitracin, 0.5 mg/ml BSA, and 0.2 mM phenylmethylsulfonyl fluoride (all from Sigma Chemical, St. Louis, MO) for 60 min at 23°C (samples mixed on a Titer Plate Shaker; Lab Line Instruments, Melrose Park, IL). Non-specific binding was defined as the radioactivity remaining in the presence of 100 nM nonradioactive salmon calcitonin. Incubations were started by addition of membrane in triplicate tubes and binding was terminated by filtration through glass-fiber filters (presoaked 30 min in 0.5% polyethylenimine), with the Skatron semiautomatic cell harvester. Filters were placed in Sarstedt 68.752 51- × 12-mm polypropylene tubes and counted for 1 min in a gamma counter.

**Saturation binding data** were analyzed with the GlaxoWellcome statistical fitting package RADLIG (GlaxoWellcome Scientific Computing, Plan-les-Ouates, Geneva, Switzerland) to simultaneous equations describing total binding and a linear nonspecific binding curve (to yield a saturable binding curve). Saturation analysis yielded a nonlinear least-squares fit to the logistic equation with a half-maximal fitting parameter (the equilibrium dissociation constant of the ligand/receptor-complex under ideal conditions, denoted Kd) and a maximal asymptote (denoted Bmax, providing an estimate of the maximal number of binding sites in picomoles per milligram of protein). Complex displacement curves were fit to a two-population model, yielding two apparent affinities and relative quantities of the two apparent binding sites (or receptor states). Data with 125I-hCAL indicated complex two-phase binding with a low-capacity, high-affinity binding site and a high-capacity, low-affinity binding site, in keeping with standard agonist binding kinetics.

**Measurement of Calcium Transient Responses**

Stable HEK 293 clones were tested for their ability to mobilize calcium in the FLIPR system. Cells were harvested with 0.05% trypsin (Life Technologies, Gaithersburg, MD) and plated in black 96-well Viewplates (Polyfitronics, Rockland, ME) at a concentration of 10,000 cells/well in DMEM/F12 phenol red free medium (Life Technologies) containing 5% fetal bovine serum (Life Technologies). Approximately 30 h after cell plating, the media was removed by vacuum and replaced with DMEM/Ham's F12 phenol red-free medium without serum. Cells were kept in serum-free medium approximately 18 h before assay. At the time of assay, cells were washed with 100 µl FLIPR buffer (145 mM NaCl, 5 mM KCl, 0.5 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 10 mM glucose, pH 7.4). Dye was prepared as follows: 2 mM calcium green stock (C 3011; Molecular Probes, Eugene, OR) was prepared in dimethyl sulfoxide. The stock was mixed 50/50 with 20% pluronic acid (P 3000; Molecular Probes) and diluted to 4 µM final concentration in FLIPR buffer. Cells then were loaded with 50 µl of the 4 µM calcium green dye along with 2.5 mM probenecid in 2.5% NaOH and allowed to incubate for at least 1 to 2 h at 37°C. After incubation, the plates were allowed to come to room temperature. Plates were washed in FLIPR buffer twice and 50 µl left in each well. After loading plates into the FLIPR, basal intracellular calcium [Ca²⁺]i, conditions were monitored for 10 s before adding 50 µl of the agonist with an integrated 96-well pipettor. Fluorescence was measured from all 96 wells simultaneously using a charge-coupled device camera. Agonist activity was measured every second for the first 25 s then every 3 s for the next 15 s. Curves were calculated as percentage of 10 µM ionomycin control response.

**Results**

**Stimulus-Biased HEK 293 Cells.** Approximately 100 HEK 293 cell lines were transfected with Go-subunit cDNA for four different G proteins (Gai, Gαs, Gao, and Gqα) and made into stable clones. These were subjected to Western blot analysis for visualization of relative quantities of specific G proteins. As shown in Fig. 1, certain clones contained elevated levels of Gai, Gαs, Gao, and Gqα protein, respectively. Accordingly, cell lines 21 (for Gai-enriched cells), 24 (Gαs-enriched), 13 (Gao-enriched), and 15 (Gqα-enriched) were selected for further study. These stable cells lines then
were transiently transfected with hCTR2 cDNA for binding and functional studies.

Saturation Binding Studies. Saturation binding curves were obtained for the agonist $^{125}$I-hCAL and the calcitonin receptor antagonist $^{125}$I-AC512 (Chen et al., 1997) on membranes prepared from transiently transfected (expressing hCTR2) wild-type HEK 293 cells and Ga-subunit enriched HEK 293 cells. The maximal density of expressed hCTR2 sites as measured with the antagonist radioligand exceeded the number estimated with $^{125}$I-hCAL (Table 1) consistent with the idea that the G protein limited the production of high-affinity agonist binding state [i.e., the number of receptors exceeded the available G protein (Chen et al., 1997; Kenakin, 1997b)]. Transfection of the four cell lines led to varying levels of receptor expression being highest in the wild-type and Gaq-enriched and lower in Gao-, Gao-, and Gaq-enriched cell lines as measured with $^{125}$I-AC512 binding (Table 1). Little change in the amount of high-affinity binding complex with $^{125}$I-hCAL was obtained with Ga-subunit enrichment.

Effect of Receptor Density on Agonist Potency Ratios. As demonstrated by the different $B_{\text{max}}$ values for $^{125}$I-AC512 binding, receptor expression of hCTR2 after transient expression was not uniform. This precluded effective comparison among cell types and focused attention on the important aspect of these studies from the standpoint of receptor theory, namely the internal relative profiles of agonists within each cell type. According to the classic models of GPCRs, differences in receptor density should affect absolute potency but not relative potency; this is described more fully in Appendix I. The possible effect of calcitonin receptor density differences was examined in functional studies. The relative potency of agonists for hCTR2 under two different transfection conditions in HEK 293 cells was explored in a stable high expression cell line (30.05 ± 3 pmol/mg of protein hCTR2; Chen et al., 1997) and transient expression in wild-type HEK 293 cells. Figure 2A shows dose-response curves for rat calcitonin in the two cell lines. As shown in this figure, the maximal response to was greater in the stable HEK 293 cell type.

Figure 2B shows the correlation of the potencies of the agonists (quantified as pEC$_{50}$, the $\log$ of the EC$_{50}$, concentration producing half-maximal response) in the two cell lines. Linear regorsional analysis (Snedecor and Cochran, 1967; Armitage, 1971) indicated a highly significant regorsional coefficient ($T = 8.29$, d.f. = 6, $P < .001$). Fig. 2C shows a graphical representation of the pEC$_{50}$ values in the stable and the transient cell line. This representation shows the uniform phase shift in potency (slightly higher in the stable cell line) for the agonists; the pEC$_{50}$ values are given in Table 2. Although the absolute potencies differed, it is important to note that the relative potency, a parameter dependent only upon the agonists and receptor type (Appendix I), did not.

Stimulus-Biased Assay Systems: Effects of Agonists. The effects of standard agonists for endogenous receptors in wild-type and Ga-subunit enriched HEK 293 cells are shown in Fig. 3. As can be seen from this figure, carbachol produced 150% maximal ionomycin responses in Gao-enriched host cells and only 35% maximum response in Gao-enriched cells (Fig. 3A). Similarly, responses to ATP were enhanced in Gaq-enriched host cells and eliminated in Gao-enriched cells (Fig. 3B). These data indicated that, unlike binding for transfected calcitonin receptors, Gaq-, Gao-, and Gaq-protein enrichment produced differences in stimulus-response coupling. For both carbachol and ATP, little difference was seen with Gai-enriched cells compared with wild-type cells.

As shown in Fig. 4, the maximal responses, when compared with ionomycin, for amyllyn (Fig. 4A) and rat calcitonin (Fig. 4B) were enhanced by Gaq enrichment and slightly diminished by Gaq enrichment. Interestingly, the location parameters of the curves to rat calcitonin, but not amyllyn, also changed with Gaq-protein enrichment.

The relative potency ratios for eight agonists for hCTR2 were compared in wild-type, Gaq-enriched, and Gao-enriched HEK 293 cells. It should be noted while the maximal responses differed with respect to ionomycin in the different cell types, they did not differ within a given cell type for each of the agonists. Thus, all agonists produced a uniform maximal response (the maximal tissue response) and potency ratios were not complicated by differences in maximal asymptotc response.

Figure 5A shows the correlation between the pEC$_{50}$ values for the eight agonists in wild-type cells (abscissa) and Gao-enriched cells (ordinates). Linear regorsional analysis of the pEC$_{50}$ values indicated a highly significant regorsional coefficient ($T = 13.47$, d.f. = 6, $P < .001$). Fig. 5B shows the individual pEC$_{50}$ values graphically in wild-type and Gao-enriched cells. As can be seen from these figures, there was little change in the potencies of the agonists; the pEC$_{50}$ values are given in Table 3.

In contrast, Fig. 6A shows that Gaq enrichment produced some striking differences in the relative potencies of agonists. Linear regorsional analysis indicated a loss of correlation between wild-type and Gaq-enriched cell pEC$_{50}$ values ($T = 2.17$, d.f. = 6, n.s. at 5%). Amylyn lost potency in Gaq-enriched cells, some agonists did not change (rat and chicken CGRP, eel and salmon calcitonin) and some selec-
tively increased in potency (human, porcine and rat calcitonin). This resulted in a changed rank order of potency of the agonists in Gas-enriched cells (over wild-type)—see Fig. 6B. The pEC$_{50}$ values are shown in Table 3.

Figure 7 shows two examples of differing relative potencies in wild-type and Gas-enriched cells. Thus, although the relative potency of rat calcitonin and rat amylin was 1.2 in wild-type cells, it increased by a factor of 27 (to 33) in Gas-enriched host cells (Fig. 7A). Similarly, the potency ratio of porcine calcitonin and rat amylin changed by a factor of 46, from 3.0 to 138 (Fig. 7B).

**Discussion**

The observation of varying relative potencies of agonists for the same receptor in different host systems is highly unusual. If this were observed in different natural systems, it would be taken as presumptive evidence for differences in the receptors mediating the responses in those systems. The fact that this was observed for a single receptor in different host cells is particularly interesting in that such behavior cannot be accommodated by the tacit assumption made in classical receptor occupancy theory, namely that all agonists form a single active receptor state that then initiates cellular signaling.

In this study, the measurement of selective G protein enhancement was operational. Although the relative increase in the G protein content of the stimulus-biased cells was measured (relative to wild-type control), the resulting data are not relevant to the actual quantity of G protein accessible to the transfected receptor and available for receptor coupling and served only as a method of choosing cells for receptor transfection. The change in the responses to ligands for endogenous receptors (ATP and carbachol) and transfected hCTR2 was more relevant in that a bias, in terms of G protein coupling to these receptors, was demonstrated. This operational observation of G protein enrichment was used as the basis for further study of relative agonist potencies.

The fact that the amount of high-affinity agonist complex was so much lower than the total receptor number (as measured with the antagonist radioligand) indicated that the amount of G protein was limiting. Ga-subunit enrichment theoretically could have resulted in differences in the amount of high-affinity binding of an agonist radioligand. In keeping with these predictions, coexpression of secretin receptors with Gas has been shown to lead to an increase in the

| Table 1 | Saturation binding of $^{125}$I-hCAL and $^{125}$I-AC512 to membranes from HEK 293 cells expressing hCTR2 |
|-----------------|-----------------|-----------------|-----------------|
| **Cell Type**   | **$^{125}$I-hCAL** | **$^{125}$I-AC512** |
|                 | $pK_d$ $^a$ 95% c.i. | $B_{max}$ $^d$ 95% c.i. | $pK_d$ $^a$ 95% c.i. | $B_{max}$ $^d$ 95% c.i. |
| Wild-type       | 10.77 10.63 to 10.91 | 1.0 0.81 to 1.19 | 10.21 10.18 to 10.24 | 28.7 27.9 to 29.5 |
| Gai-enriched    | 10.76 10.6 to 10.93 | 1.02 0.8 to 1.25 | 10.13 10.08 to 10.16 | 28.8 27.8 to 29.8 |
| Gas-enriched    | 10.69 10.6 to 10.78 | 0.92 0.8 to 1.03 | 9.95 9.91 to 9.99 | 13.3 12.84 to 13.8 |
| Gao-enriched    | 10.03 9.83 to 10.23 | 1.96 1.2 to 2.72 | 10.31 10.22 to 10.4 | 10.5 9.27 to 10.81 |
| Gag-enriched    | 10.05 9.84 to 10.27 | 0.26 0.63 to 0.92 | 9.76 9.52 to 10.01 | 5.08 3.78 to 6.38 |

$^a$ Log equilibrium dissociation constant of the $^{125}$I-hCAL/hCTR2 complex for high-affinity binding.

$^b$ 95% confidence limits of the estimate. Estimates are the mean of three separate transient transfections.

$^c$ Number of high affinity binding sites (picomoles per milligram of protein) as estimated with the RADLIG program. Although an estimate was made of the number of low-affinity binding sites, there were insufficient datapoints to allow a meaningful maximal asymptote to be measured.

$^d$ Estimate of the maximal number of binding sites for the antagonist radioligand $^{125}$I-AC512.
number of high-affinity binding sites for $^{125}$I-secretin from 1.8% to 15% (Ishihara et al., 1991). However, for pleiotropic receptors that interact with more than a single G protein (such as hCTR2; Horne et al., 1994), the degree to which high-affinity binding would be enhanced depends upon the relative stoichiometries of the G proteins involved (i.e., if a relatively minor G protein is enhanced in the presence of a high concentration of the major interactant G protein for that receptor, then enrichment of a secondary protein may not be detectable as an increase in the number of high affinity binding sites). The relationship of the $B_{\text{max}}$ for an agonist observed in saturation binding and G protein is given from eq. 7 of Appendix II as $\alpha L (1 + \gamma_2 G_2)/\beta_2 K_2 [R_{\text{total}}]/(1 + \alpha L (1 + \gamma_1 G_1/\beta_1 K_1 + \gamma_2 G_2/\beta_2 K_2))$. It can be seen that enrichment of one of the G proteins would have little effect on $B_{\text{max}}$ if the

### TABLE 2

Relative potencies of calcitonin receptor agonists in transient wild-type and stably expressing HEK 293 wild-type cells

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Stable $^a$ HEK 293</th>
<th>Transient wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylin</td>
<td>8.0 ± 0.17</td>
<td>7.35 ± 0.2</td>
</tr>
<tr>
<td>Rat CAL</td>
<td>7.96 ± 0.21</td>
<td>7.43 ± 0.18</td>
</tr>
<tr>
<td>Human CAL</td>
<td>8.14 ± 0.2</td>
<td>7.45 ± 0.18</td>
</tr>
<tr>
<td>Porc CAL</td>
<td>8.29 ± 0.22</td>
<td>7.82 ± 0.2</td>
</tr>
<tr>
<td>Eel CAL</td>
<td>8.7 ± 0.24</td>
<td>8.38 ± 0.2</td>
</tr>
<tr>
<td>Salmon CAL</td>
<td>8.4 ± 0.17</td>
<td>8.07 ± 0.15</td>
</tr>
<tr>
<td>Rat CGRP</td>
<td>6.96 ± 0.15</td>
<td>6.92 ± 0.22</td>
</tr>
<tr>
<td>Chicken CGRP</td>
<td>7.92 ± 0.26</td>
<td>7.52 ± 0.2</td>
</tr>
</tbody>
</table>

$^a$ Stable HEK 293 cell line containing 30.05 pmol/mg of protein hCTR2.

### Table 3

Relative potencies of calcitonin receptor agonists in HEK 293 cells

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Wild-type</th>
<th>Goi-enriched</th>
<th>Gas-enriched</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylin</td>
<td>7.35 ± 0.2</td>
<td>7.45 ± 0.14</td>
<td>6.53 ± 0.11</td>
</tr>
<tr>
<td>Rat CAL</td>
<td>7.43 ± 0.18</td>
<td>7.14 ± 0.2</td>
<td>8.06 ± 0.16</td>
</tr>
<tr>
<td>Human CAL</td>
<td>7.45 ± 0.18</td>
<td>7.45 ± 0.18</td>
<td>8.12 ± 0.15</td>
</tr>
<tr>
<td>Porc CAL</td>
<td>7.82 ± 0.2</td>
<td>7.96 ± 0.2</td>
<td>8.68 ± 0.14</td>
</tr>
<tr>
<td>Eel CAL</td>
<td>8.38 ± 0.2</td>
<td>8.1 ± 0.1</td>
<td>8.0 ± 0.1</td>
</tr>
<tr>
<td>Salmon CAL</td>
<td>8.07 ± 0.15</td>
<td>8.1 ± 0.2</td>
<td>7.85 ± 0.16</td>
</tr>
<tr>
<td>Rat CGRP</td>
<td>6.92 ± 0.22</td>
<td>6.58 ± 0.17</td>
<td>7.02 ± 0.14</td>
</tr>
<tr>
<td>Chicken CGRP</td>
<td>7.52 ± 0.2</td>
<td>7.6 ± 0.2</td>
<td>7.52 ± 0.14</td>
</tr>
</tbody>
</table>

CAL, calcitonin.

### Fig. 3.

Responses to carbachol (A) and ATP (B) agonists for receptors endogenous to HEK 293 cells (■), Goi-enriched cells (○), Goi-enriched cells (□), and Gas-enriched cells (▲). No satisfactory dose-response relationships could be obtained with Gas-enriched cells. Data shown from one of three experiments.

### Fig. 4.

hCTR2-mediated responses to amylin (A) and rat calcitonin (B) in wild-type HEK 293 cells (■), Gas-enriched cells (○), and Goi-enriched (▲). Data shown from one of three transient transfections.

### Fig. 5.

The relative potency of hCTR2 agonists in wild-type transiently transfected HEK 293 cells and Goi-enriched cells. A, pEC$_{50}$ values for agonists in wild-type cells (abscissa) and Goi-enriched cells (ordinates). Values are for eel calcitonin (▲), salmon calcitonin (○), porcine calcitonin (□), chicken CGRP (●), human calcitonin (■), rat calcitonin (○), rat CGRP (●), and rat amylin (●). B, change in pEC$_{50}$ from stable to transient cells (ordinates = pEC$_{50}$). For A and B, bars represent S.E.M.

### Fig. 6.

The relative potency of hCTR2 agonists in wild-type transiently transfected HEK 293 cells and Gas-enriched cells. A, pEC$_{50}$ values for agonists in wild-type cells (abscissa) and Gas-enriched (ordinates). Values are for eel calcitonin (▲), salmon calcitonin (○), porcine calcitonin (□), chicken CGRP (●), human calcitonin (■), rat calcitonin (○), rat CGRP (●), and rat amylin (●). B, change in pEC$_{50}$ from stable to transient cells (ordinates = pEC$_{50}$). For A and B, bars represent S.E.M.
other G protein binding the receptor was in a relatively greater concentration or if the affinity of the receptor active state (denoted by \( \gamma \)) was lower for the enriched G protein. Thus there is a limitation of mass equivalence in the measurement of Gs-subunit protein in binding studies.

The limitation in binding studies of mass equivalence is not present in functional studies. When cell function is used as a measure of receptor stimulus, there can be disconnections between the amount of high-affinity complex and resulting amount of stimulus (and resulting response) produced by that complex. In general, stimulus-response mechanisms within cells greatly amplify the result of receptor/G protein interaction therefore G protein enrichment, insufficient to result in changes in high-affinity binding, may still produce observable effects on function. In light of the data with Gs-enrichment, this seems to be what occurred in these studies. The implications of this finding are worth considering.

Within a given cell type, if all agonists produce a uniform active receptor state, then the relative potency of these agonists will not vary (see Appendix I). The data obtained in Gs-enriched cells cannot be accommodated by this hypothesis, leading to the possibility that some of the agonists in this study produce at least two different receptor active states (see below). There are increasing data in the literature, from a range of experimental approaches, to suggest that this occurs with other receptors. Experimental data support the idea that agonist specific receptor activation for PACAP 1 receptor (pituitary adenylate cyclase activating polypeptide receptor type 1; Spengler et al., 1993), Drosophila tyramine receptors (Robb et al., 1994), and \( \beta \)-adrenoceptors (Chidiac et al., 1994). Agonist specific receptor activation has directly been proposed for dopamine D<sub>2a</sub> (Wiens et al., 1998), 5-HT<sub>2a</sub> (Berg et al., 1998), 5-HT<sub>1a</sub> (Van Hooft and Vijverberg, 1996), \( \beta \)-adrenoceptors (Krumins and Barber, 1997; Seifert et al., 1999), cannabinoid CB<sub>1</sub> receptors (Bonnaus et al., 1998; Glass and Northup, 1999) and \( \mu \)-opioid receptors (Keith et al., 1996; Blake et al., 1997; Yu et al., 1997).

Agonist-specific receptor active states have been hypothesized on the basis of a number of experimental approaches. These include observation of protean agonism [reversal of efficacy from positive to negative in quiescent versus constitutively active receptor systems (Kenakin, 1997c)], differences in agonist-induced receptor internalization, kinetic rates of activation, differences in rates of hydrolysis of G protein-induced nucleotide hydrolysis, and reversal of the relative potency of agonists for different stimulus-response pathways. Many of these approaches uncover agonist-selective receptor states, but it is still a theoretical hypothesis that these states are involved in signaling and will result in stimulus-trafficking. The present approach directly demonstrates that the agonists tested produce differing response patterns which may translate to different therapeutic profiles. On the other hand, complex differential G protein coupling would be expected to be dependent upon receptor/G protein stoichiometry and would thus be quite system dependent. Under these circumstances, it would not be expected that a given stimulus-biased assay such as this one would be universally useful for the detection of stimulus trafficking. With this in mind, it would be prudent to use as many techniques as possible to test ligand agonism because some may work better than others for given receptors and agonists.

The ternary complex model for GPCRs, either in the form of the extended ternary complex model (Samama et al., 1993), or the cubic ternary complex model (Weiss et al., 1996a,b,c) commonly are described as “two-state” models referring to the two spontaneously occurring active and inactive states of the receptor. The concept that agonists produce unique active receptor states may seem to be in conflict with these classic models. However, it should be noted that these models really are “multistate” models when describing activation of receptors by ligands. This is because the presence of the ligand opens the possibility of a modified affinity of the activated receptor for G proteins through thermodynamic constants \( \alpha \) (extended ternary complex model) or \( \gamma \) and \( \delta \) (cubic ternary complex model); see Table 4 and Fig. 8. Thus, the existing models of GPCR function accommodate agonist-specific receptor active conformations.

The formation of agonist-specific receptor active states becomes important from a cellular signaling point of view when multiple G proteins are involved. It is known that many G protein coupled receptors are pleiotropic with respect to the G proteins with which they interact (Kenakin, 1996). As discussed previously, human calcitonin receptors are among those known to activate Gs, Gi, and Gq (Horne et al., 1994). This could be important for signaling because studies have shown that different regions of the cytosolic loops of seven-transmembrane receptors activate different G proteins (Ikezu et al., 1992; Wade et al., 1999). Under these circumstances, it would not be expected that different overall receptor conformations would expose these different G protein-interacting sequences to signaling mechanisms in an identical manner. These ideas, taken in conjunction, open the

Fig. 7. Dose-response curves for amylin (○) and rat calcitonin (○) (A) and amylin (○) and porcine calcitonin (○) (B) in wild-type HEK 293 cells (left) and Gs-enriched HEK 293 cells (right). p.r., potency ratios for the agonists.
Theoretical possibilities that different active receptor conformations selectively activate different G proteins to direct stimulus to different biochemical pathways in cells. When this occurs, it would be predicted that the relative potencies of agonists producing different active states will vary according to the relative amounts of G protein available for stimulus-response coupling. This is discussed further in Appendix II.

Stimulus-biased assay systems, such as the \( \text{G}_\alpha \)-subunit-enriched HEK cells used in this study, are not meant to reflect natural physiology but rather are designed to furnish unique information about agonists. In drug discovery programs designed to find agonists, there usually are two possible targets: a complete mimic of the physiologically endogenous agonist or a mimic of a subset of agonism produced by the physiological agonist. Previously, the latter profile has been achieved solely by finding agonists for receptor subtypes or restriction of pharmacokinetics. The production of selective receptor active states theoretically offers another level of selective agonism. Specifically, if the spectrum of signal transduction initiated by a given agonist could be reduced, then a subset of physiological responses could be produced. If the pleiotropic nature of the endogenous receptor signaling is associated with a concomitant plethora of physiological responses (some of which are not desired in the therapeutic field), then reducing these may lead to a better mating of replacement agonist therapy for pathophysiological disorders. From this standpoint, agonist-selective receptor active states could represent the next effective level of agonist selectivity (Kenakin, 1997a).

Presently the relevance of agonist-specific cellular signaling to therapeutic targeting of synthetic agonists is not clear. The extent to which this idea can be capitalized upon therapeutically is unclear. At the least, however, it allows a method of classifying agonists by measures beyond those based simply on strength of agonism. This latter idea assumes that all agonists produce a uniform receptor active state that goes on to produce physiological response on the basis of stoichiometry and nothing more. The idea of agonist specific receptor active states extends that concept to include the "quality" of efficacy as well as the "quantity" of efficacy in describing the activity of agonists. The use of stimulus-biased host cells for surrogate expression of receptors may be a useful tool in this regard.

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### Appendix I

#### Effect of Receptor Density on Agonist Potency Ratios

**Extended Ternary Complex Model.** This model (Samama et al., 1993) defines agonism with the following equation:

\[
\rho = \frac{\beta L[Ri]K_a(1 + \gamma \alpha [A]/K_a)}{[A]/K_a(\alpha \gamma L[Ri]/K_a) + \beta L[Ri]/K_a + 1}
\]

where \( \rho \) is the fraction of signal-producing receptor species \([ARaG] + [RaG]\) as a fraction of the total G protein. The terms are described in Table 4. The observed potency for an agonist is given by:

\[
K_{obs} = \frac{K_a(1 + \beta L[Ri]/K_a)}{(\alpha \gamma L[Ri]/K_a)}
\]

Thus, the absolute potency of agonists depends upon terms relating affinity (\( K_a \)), efficacy (\( \alpha, \gamma \)), the receptor type (\( \beta, K_a, L \)), and the host system (\( [Ri], [G] \), and, for functional studies, the stimulus-response mechanism of the host system). It can be seen from eq. 2 that receptor density affects absolute potency.

For two agonists a and b, the relative potency is defined as:

\[
\text{Potency Ratio}_{a/b} = \frac{\alpha_\gamma \alpha b K_{obs}}{\alpha_\gamma a b K_{obs}}
\]

From eq. 3 it can be seen that the relative potency of the two agonists depends upon terms relating only to affinity (\( K_a \)) and efficacy (\( \alpha, \gamma \)).

**Cubic Ternary Complex Model.** This model (Weiss et al., 1996a,b,c) defines agonism with the following equation:

\[
\rho = \frac{\beta L[Ri]/K_a(1 + \delta \gamma [A]/K_a)}{[A]/K_a(\gamma [Ri]/K_a[1 + \delta a \beta L]) + 1 + [Ri]/K_a[1 + \delta a \beta L]}
\]

where \( \rho \) is the fraction of signal-producing receptor species \([ARaG] + [RaG]\). The terms are described in Table 4. The observed potency for an agonist is given by:

\[
K_{obs} = \frac{K_a(1 + [Ri]/K_a[1 + \delta a \beta L])}{(\gamma [Ri]/K_a[1 + \delta a \beta L])}
\]
The absolute potency of agonists depends upon terms relating affinity (K_A), efficacy (α, γ, δ), the receptor type (β, K_G), and the host system ([R], [G]), and for functional studies the stimulus-response mechanism of the host system. Thus, receptor density affects absolute potency.

For two agonists a and b, the relative potency is defined as:

\[
\text{Potency Ratio}_{a:b} = \frac{\gamma_0 K_{Aa} (1 + \delta_0 \alpha_a \beta_L)}{\gamma_0 K_{Ab} (1 + \delta_0 \alpha_b \beta_L)}
\]  
(6)

As with the extended ternary complex model, the terms relating relative potency involve only affinity and efficacy.

**Effect of Receptor Density on Relative Potency**

It can be seen from the two expressions for relative potency, in either the extended ternary complex model or the cubic ternary complex model, that the effects of receptor density cancel and molecular constants reflecting affinity and intrinsic efficacy control relative potency. Under these circumstances, the relative potency of two agonists (providing they both produce full agonist response) is a unique identifier of the agonist and receptor type. The guideline used in this process dictates that differences in the relative potency of full agonists denotes differences in the receptors’ mediating response. However, it can also be seen that differences in potency ratios can be brought about by differences in α or γ that reflect changes in the affinity of the ligand for the active (over the inactive) receptor state and differences in the affinity of the receptor for G protein when ligand is bound. This latter factor could be relevant if the ligand forms a different receptor state.

**Appendix II**

**Relative Potency Ratios for Promiscuous Receptor/G Protein Interactions**

The Ternary Complex Model [for this particular example, the extended ternary complex model by Samama et al. (1993) is used] can be expanded to include the interaction of the active State receptor (Ra) with two G proteins (denoted G1 and G2). Under these circumstances, the equations denoting response to an agonist (as defined by the production of a ternary complex with the agonist and active-state receptor with either G1 or G2 as:

\[
\rho = \frac{a L / [A] (1 + \gamma_0 [G_1] / \beta K_1 + [G_2] / \beta K_2)}{[A] / K_A (1 + \gamma_0 [G_1] / \beta K_1 + [G_2] / \beta K_2) + [L] / [G_1] / \beta K_1 + [G_2] / \beta K_2 + 1 + 1}
\]  
(7)

where ρ refers to the response-producing species (ARaG1, RaG1, ARaG2, RaG2); K_A to the equilibrium dissociation constant of the agonist for the receptor; α to the differential affinity of the agonist for the active receptor state; γ and δ to the differential affinity of the ligand-bound, active-state, agonist-receptor complexes for the G protein; and [G1]/β_K1, and [G2]/β_K2 to the relative quantities of the two G proteins interacting with the receptor as a fraction of the equilibrium dissociation constants of the active-state receptor/G protein complexes. From eq. 7, it can be seen that the observed affinity of an agonist in this type of system is given by:

\[
K_{obs} = \frac{K_{Aa} L / [G_1] / \beta K_1 + [G_2] / \beta K_2 + 1 + 1}{(1 + a L / [A] (1 + \gamma_0 [G_1] / \beta K_1 + [G_2] / \beta K_2))}
\]  
(8)

Therefore, for two agonists a and b, the potency ratio PRa/b is given by:

\[
\text{Potency Ratio}_{a:b} = \frac{K_{Aa} (1 + \delta_0 \alpha_a \beta_L)}{K_{Ab} (1 + \delta_0 \alpha_b \beta_L)}
\]  
(9)

The term γ refers to the change in the ability of the receptor to activate G proteins upon binding with ligand. Thus, different active states formed by different ligands would be represented by differing values of γ. An interesting feature of eq. 9 is that, if both agonists produce the same receptor active state (that is, if γ_a = γ and γ_b = γ_b), then the relative amounts of [G1] and [G2] will not change the relative potency of the two agonists and potency ratios would be equal in all cell types. Similarly, if one ligand has a different value for γ for one of the G proteins, then the relative amounts of the G proteins will change the relative potency ratios of the agonists.

**References**


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