

Use of Constitutive G Protein-Coupled Receptor Activity for Drug Discovery

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ABSTRACT

This article describes the behavior of transiently transfected human receptors into melanophores and the potential use of constitutive receptor activity to screen for new drug entities. Specifically, transient transfection of melanophores with different concentrations of receptor cDNA presumably leads to increased levels of receptor expression. This leads to an increased response to agonists (both maxima and potency) and, in some cases, an agonist-independent constitutive receptor activity. Transfections with increasing concentrations of the G_s protein-coupled human calcitonin receptor type 2 (hCTR2) cDNA produced sufficient levels of constitutively activated receptor to cause elevated basal cellular responses. This was observed as a decrease in the transmittance of light through melanophores (consistent with G_s protein activation) and increased response to human calcitonin. The receptor-mediated nature of this response was confirmed by its reversal with the hCTR2 peptide inverse agonist AC512. A collection of ligands for hCTR2 either increased or decreased constitutive hCTR2

activity, suggesting that the constitutive system was a sensitive discriminator of positive and negative ligand efficacy. Similar results were obtained with G_i -protein-coupled receptors. Transient transfection of NPY1, NPY2, NPY4, CXCR4, and CCR5 cDNA produced increased light transmittance through melanophores (consistent with G_i -protein activation). NPY1 cDNA produced little constitutive response on transfection, whereas maximal levels of constitutive activity ranging from 30 to 45% were observed for the other G_i -protein-coupled receptors. Responses to agonists for these receptors increased (both maxima and potency) with increasing cDNA transfection. The receptor/ G_i -protein nature of both the constitutive and agonist-mediated responses was confirmed by elimination with pertussis toxin pretreatment. These data are discussed in terms of the theoretical aspects of constitutive receptor activity and the applicability of this approach for the general screening of G protein-coupled orphan receptors.

High-throughput screening with combinatorial chemical libraries is an effective method of discovery of new ligands that interact with receptor targets. The interference of receptor signals mediated by the interaction of the receptor with known ligands is the common method of detection of new entities. In the case of orphan receptors, for which there are still no known ligands, this system is capable only of discovering excitatory (agonist) ligands that cause receptor activation and thus a measurable signal.

G protein-coupled receptors (GPCRs) can spontaneously form active states that subsequently can activate G proteins and thus produce a measurable pharmacologic response (Costa and Herz, 1989; Samama et al., 1993). Unless ligands have identical affinities for the different activation states of seven transmembrane receptors present in constitutive receptor systems, their binding will redistribute the species, and this will be detected as either an increase or a decrease in the constitutive receptor activity (see predictions of ter-

nary complex models describing this effect in *Appendix I*). There are data to show that a measurable constitutive GPCR activity can be obtained by receptor overexpression in recombinant systems (*Appendix II*; Kenakin, 1996). Therefore, one approach to the screening of GPCRs is to overexpress the receptor to the point of observing constitutive activity and then allowing ligands with affinity to redistribute the receptor species. The following are requirements for such assays: 1) the receptor must have some proclivity to spontaneously form an active state, 2) there must be suitable G proteins available in the cell to interact with the active state, and 3) there must be a means to monitor the amount of activated G protein (Kenakin, 1997).

This article describes the study of constitutive receptor activity in *Xenopus laevis* melanophores, cells that fulfill the second and third prerequisites of the assay. Specifically, these cells contain a wide range of G_α proteins (Jayawickreme et al., 1994); therefore, the functional expression of

ABBREVIATIONS: hCTR2, human calcitonin receptor type 2; GPCR, G protein-coupled receptor; PTX, pertussis toxin; CFM, conditioned fibroblast medium; hCAL, human calcitonin.

numerous foreign GPCRs can be facilitated (Potenza et al., 1992, 1994; Karne et al., 1993; Graminski et al., 1993; McClintock et al., 1993; Graminski and Lerner, 1994; Jayawickreme et al., 1994a,b; Lerner, 1994). This system can be monitored in real time by the dispersion and aggregation of melanin. Melanosome dispersion can be affected via activation of adenylyl cyclase (Potenza et al., 1992; McClintock et al., 1993) or phospholipase C (Graminski et al., 1993), whereas melanosome aggregation results from the inhibition of adenylyl cyclase (Potenza et al., 1992; McClintock et al., 1993). Because both states (dispersion or aggregation) of intracellular melanosome distribution are easily detectable, GPCRs can be studied by monitoring ligand-mediated melanosome translocation by either measuring the change in light transmittance through the cells or by imaging the cell response (Potenza et al., 1992, 1994; Karne et al., 1993; McClintock et al., 1993; Graminski et al., 1993; Graminski and Lerner, 1994; Jayawickreme et al., 1994a,b; Lerner, 1994).

Experimental Procedures

Materials. Leibovitz (L-15) medium came from Sigma Chemical Co. (St. Louis, MO), BSA from Boehringer Mannheim (Indianapolis, IN), and pertussis toxin (PTX) from Calbiochem (516560; La Jolla, CA).

Construction of Expression Vectors. The full-length cDNA for human calcitonin receptor type 2 (hCTR2) (Chen et al., 1997), NPY1, NPY2, NPY4 (Matthews et al., 1997), CX chemokine receptor type 4 (CXCR4), and chemokine C receptor 5 (CCR5) (Chen et al., 1998) were amplified by a reverse-transcriptase polymerase chain reaction strategy as described. DNA fragments containing coding sequences were isolated and subcloned into the melanophore expression vector pJG3.6 (Graminski et al., 1993). Plasmid DNA used for melanophore transfections was prepared by a modification of the triton-lysozyme method and double banded in CsCl/ethidium bromide equilibrium gradients as described (Davis et al., 1994).

Functional Bioassay. Melanophores were maintained in cell cultures as previously described (Jayawickreme et al., 1994a,b). Transient expression of GPCR plasmid DNA in melanophores was achieved after electroporation (Graminski et al., 1993; Jayawickreme et al., 1994). After electroporation, cells were seeded into flat-bottom 96-well tissue culture plates (Falcon Labware, Oxnard, CA) to a density of 20,000 cells/well in conditioned fibroblast medium (CFM) and incubated at 27°C for 24 to 48 h. Nontransfected cells did not respond to the agonists used in this study.

The ligand-mediated responses in recombinant melanophores were monitored by measuring the change in transmittance with an SLT Spectra plate reader (Hillsborough, NC). For an experiment, media was removed from the plates and replaced with $0.7 \times$ L-15/0.1% BSA containing test drugs. Soon after the addition of reagents, the zero time reading (T_i) was obtained. The plates were then placed in the dark and read at appropriate time intervals (T_p). The extent of the response was quantified (Jayawickreme et al., 1994a; Potenza et al., 1994) as $(1 - T_p/T_i)$.

In the studies with PTX, the transfected cells were incubated overnight (16–18 h) in CFM with 1 μ g/ml of PTX. Just before the assay, the media was removed and replaced with $0.7 \times$ L-15/0.1% BSA containing various reagents and/or drugs.

Results

G_s Protein-Coupled Receptor (hCTR2). Transient transfection of melanophores with cDNA for hCTR2 resulted in a cDNA-dependent decreased light transmittance and the acquisition of responses to human calcitonin (hCAL). Figure 1I shows the effect of transfection of melanophores with 32 μ g of cDNA for hCTR2. Also shown are the effects of increasing concentrations of the inverse agonist AC512. The reduction in the melanin dispersion by AC512 indicates that it was caused by constitutive activation of G_s protein by the transiently expressed receptor.

Figure 2A shows the positive temporal response to hCAL;

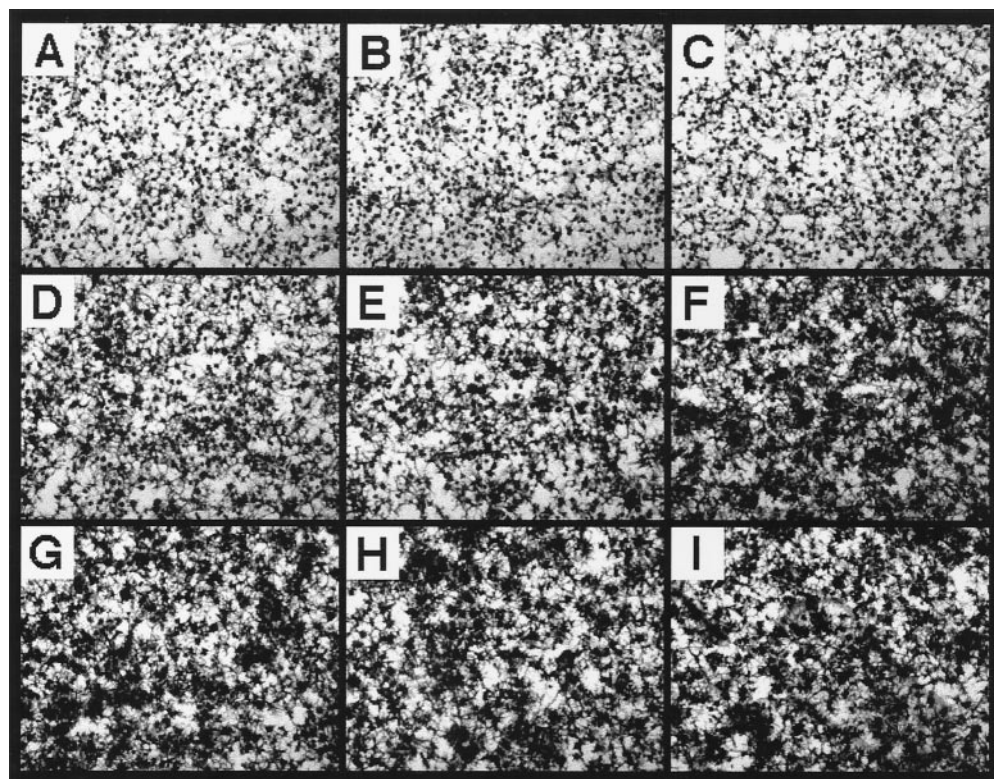


Fig. 1. Constitutive activity in melanophores expressing hCTR2 receptor. Melanophores were transfected with 32 μ g of hCTR2 cDNA. A, effect of 1 μ M AC512. B, effect of 100 nM AC512. C, effect of 10 nM AC512. D, effect of 1 nM AC512. E, effect of 100 pM AC512. F, effect of 10 pM AC512. G, effect of 1 pM AC512. H, effect of 0.1 pM AC512. I, effect of no AC512.

equilibrium is attained within 60 to 90 min. The temporal effects of the inverse agonist AC66 are shown in Fig. 2B. The dose-response relationships for these effects are shown in Fig. 2C.

Figure 3 shows the relationship of dose-response curves to hCAL and the inverse agonist AC512 with different levels of constitutive receptor activity. The elevated baseline and dose-response curve to hCAL after transfection of cells with 16 μg of cDNA are shown in Fig. 3A. Also shown in this figure is the inverse agonism by the inverse agonist peptide AC512. Figure 3B shows the effects of hCAL and AC512 in cells transfected with 32 μg of cDNA; the constitutive basal response is greater in these cells.

The effects of a wider range of cDNA is shown in Fig. 4A. It can be seen from this figure that increasing cDNA levels cause increasing constitutive activity, whereas the maximal responses to hCAL increase to the level shown for 16 μg of cDNA and progress no higher at 32 μg of cDNA. The location parameters of the dose-response curves shift to the left as predicted by the ternary complex model for GPCRs (see *Appendix III*). Figure 4B shows the corresponding effects of increasing cDNA on the inverse agonism with AC512. In this case, as predicted by theory, the dose-response curves shift to the right with increasing constitutive activity (*Appendix III*). The constitutive receptor activity of hCTR2, as a function of receptor level, is shown in Fig. 5, along with the corresponding maximal responses to hCAL. These data show that the maximal constitutive activity was approximately 60% of the maximal possible response to the full agonist hCAL.

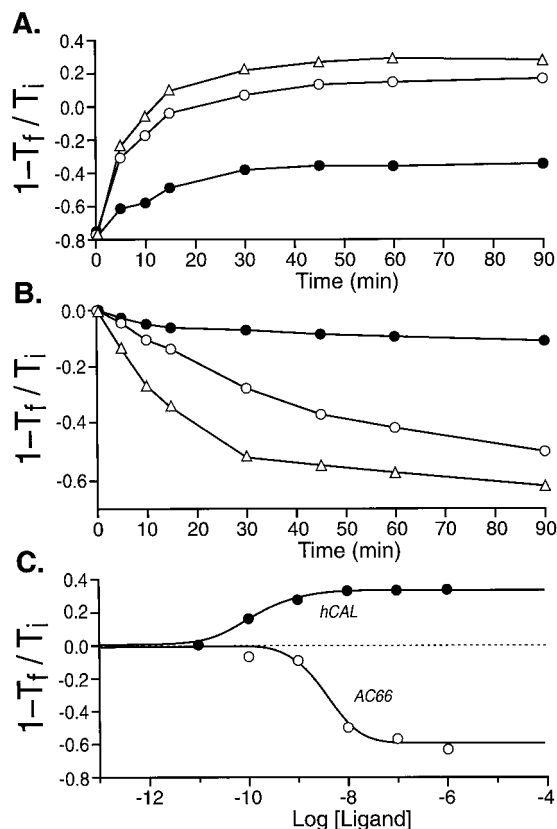


Fig. 2. Temporal effects of agonists and inverse agonists on melanophores transfected with 8 μg of hCTR2 cDNA. A, effects of 0.1 nM hCAL (●), 1 nM (○), and 10 nM (△) with time. B, effect of the inverse agonist AC66 at 1 nM (●), 10 nM (○), and 100 nM (△) with time. C, dose-response curves for hCAL and AC66.

The effects of several agonists and antagonists for hCTR2 were tested in a constitutive system resulting from transfection of melanophores with 8 μg of cDNA for hCTR2 (structures of ligands shown in Table 1). As can be seen in Fig. 6, all the ligands tested produced either positive or inverse agonism. A higher level of constitutive activity (16 μg of cDNA) showed a similar profile for these agonists, except that the maximal range for increases to the positive agonists was closer to the basal level, leading to a diminished maximal delta response to these agonists. In contrast, the maximal delta range for the inverse agonists was increased (data not shown).

G_i Protein-Coupled Receptors. Corresponding data were collected for G_i-coupled receptors. Note that activation of G_i protein causes increased light transmittance, which, in turn, produces more negative values for $1 - (T_f/T_i)$. Thus,

Human Calcitonin Receptor Type 2

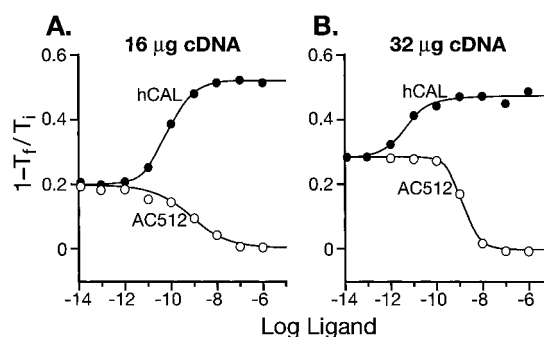


Fig. 3. Effect of transfection with hCTR2 cDNA on basal activity and response to hCAL (●) and the inverse agonist AC512 (○). Cells transfected with 16 μg (A) and 32 μg (B) of hCTR2 cDNA.

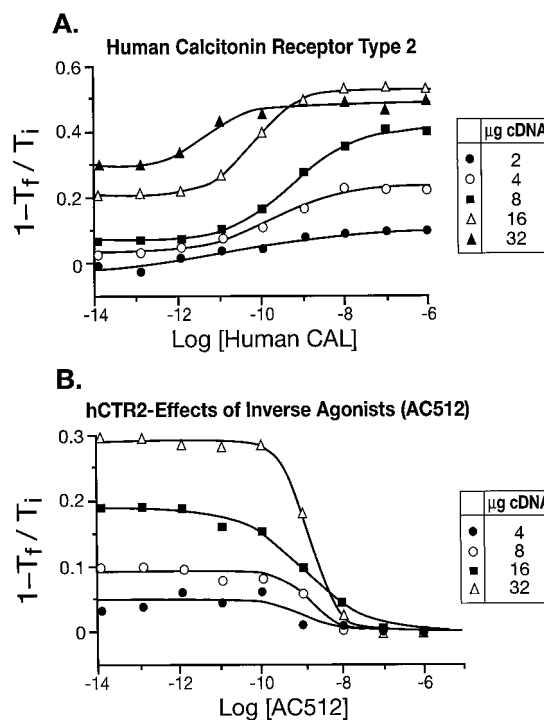
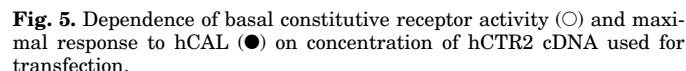


Fig. 4. Effects of different levels of hCTR2 transfection. Dose-response curves to hCAL (A) and AC512 (B) in melanophores transfected with hCTR2 cDNA in concentrations shown in legends to the right of the graphs.

Similar data were obtained for human CCR5. As seen in Figure 9A, transfection with increasing concentrations of



Ligand	Amino Acid Sequence
Rat amylin ^a	NNLGP S V S L
Porcine CAL ^a	RKCNTATCATQRLANFLV PPTNVGSNTY _{NH₂}
Rat CGRP ^a	CSNLSTCVLSAYWRNLNNFHRFSGMGFGPETP _{NH₂} GVVKD G N S F
hCAL ^a	SCNTATCVTHRLLAGLLSR VPTNVGSEAF _{NH₂}
Rat CAL ^a	CGNLSTCMLGTYTQDFNKFHTFPQTAIGVGAP _{NH₂}
Eel CAL ^a	CGNLSTCMLGTYTQNKNFHTFPQTSIGVGAP _{NH₂}
AC66 ^b	CSNLSTCVLGKLSQELHKLQTYPRTDVGAGTP _{NH₂}
AC512 ^b	VLGKLSQELHKLQTYPRNTGSGTP _{NH₂}
AC25 ^b	(Ac)LGXLSQELHRLQTYPRNTGSENTY _{NH₂}
PYY ^a	VTHRLAGLLSRSGGVKKNNFVPTNVGSKAF _{NH₂} YPAKPEAPGEDASPEELSRYYASLRHYLNLVTRQRY _{NH₂}

A similar pattern was observed for NPY4 (Fig. 12). Thus, transfection with increasing concentrations of NPY4 cDNA leads to increased response and responsiveness to PYY and constitutive activity with a small threshold for constitutive activity. PTX pretreatment prevented these effects.

The discovery of constitutive GPCR activity presented a theoretical approach to the identification of ligands for orphan receptors. The basic premise for this idea is that different tertiary conformations of the receptor protein will display different binding domains for ligands. Unless the affinities of

the ligands for these different domains were identical, they would bind to each conformation according to mass action kinetics (the concentration of ligand and the respective equilibrium dissociation constants for the ligand-receptor conformation complex). The differential binding to these conformations necessarily would change their relative abundance (see Appendix D), and this redistribution necessarily would alter the concentration of the signaling species, namely, R_aG (see Fig. 13). The magnitude of the observed response depends on the amount of R_aG species formed and the sensitivity of the stimulus-response machinery of the cell. Theoretically, any sensitive functional assay (e.g., reporters, yeast) could be used for constitutive screening. Melanophores were chosen in this study because of the high sensitivity of the stimulus-

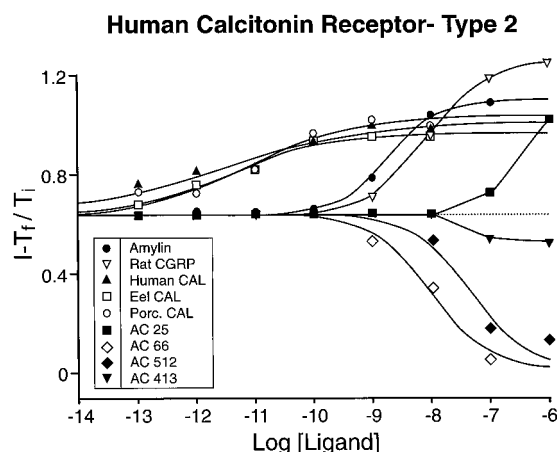


Fig. 6. Effects of peptide calcitonin ligands for hCTR2 in melanophores transfected with 8 µg of hCTR2 cDNA.

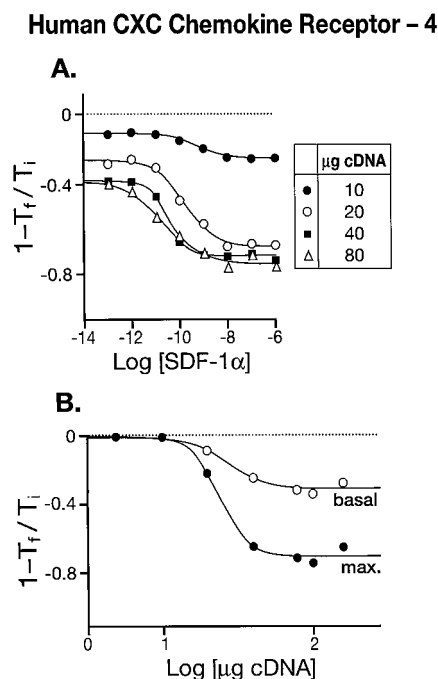


Fig. 7. Effects of different levels of CXCR4 transfection on responses to SDF-1α (A) and basal and maximal agonist response (B). A, dose-response curves to SDF-1α in melanophores transfected with CXCR4 cDNA at 10 µg (●), 20 µg (○), 40 µg (■), and 80 µg (△). B, dependence of basal constitutive receptor activity (○) and maximal response to SDF-1α (●) on concentration of CXCR4 cDNA used for transfection.

Human CXC-Chemokine Receptor – 4

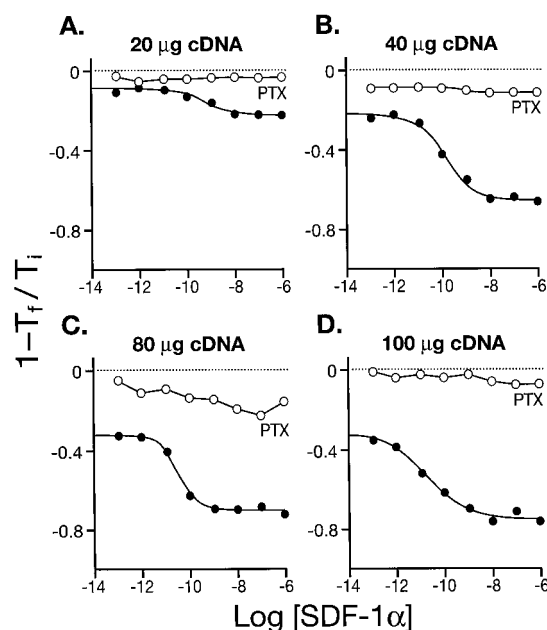


Fig. 8. Effects of PTX treatment on constitutive and agonist-induced responses of melanophores transfected with CXCR4. Responses to SDF-1α in melanophores not treated (●) and treated with PTX (○). Melanophores transfected with 20 µg (A), 40 µg (B), 80 µg (C), and (D) 100 µg of CXCR4 cDNA.

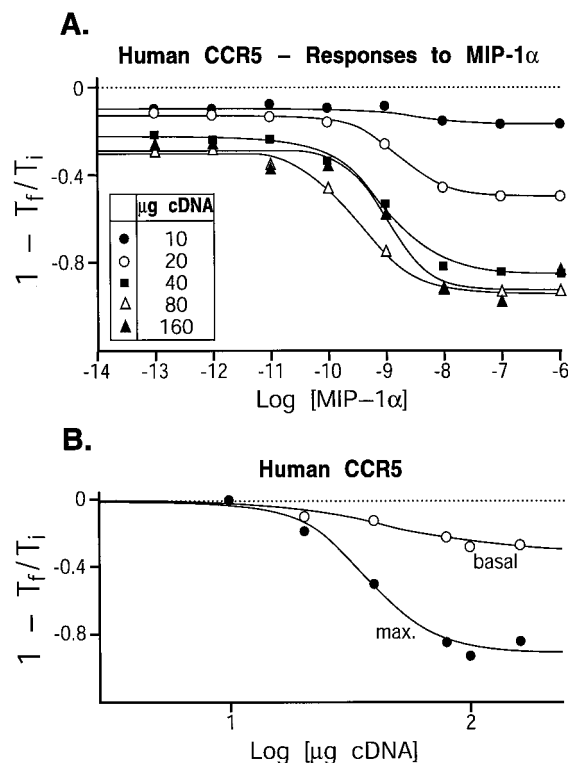


Fig. 9. Effects of different levels of CCR5 transfection on responses to MIP-1α (A) and basal and maximal agonist response (B). A, dose-response curves to MIP-1α in melanophores transfected with CCR5 cDNA at 10 µg (●), 20 µg (○), 40 µg (■), 80 µg (△), and 160 µg (▲). B, dependence of basal constitutive receptor activity (○) and maximal response to MIP-1α (●) on concentration of CCR5 cDNA used for transfection.

response mechanisms for G_s , G_i , and G_q in this cell line. Many GPCRs have been successfully transiently expressed in these cells with concomitant observation of G_i , G_s , and G_q activation. Finally, note that responses in melanophores can be viewed in real time (see Fig. 2), thereby allowing the direct observation of steady states.

The propensity of a given GPCR to form the active state is an inherent property of the receptor. In terms of the ternary complex model, this is defined by the allosteric constant (L in Fig. 13) as $[R_a]/[R_i]$. Although there are instances where this can be altered biochemically (i.e., removal of Na^+ ; Costa and Herz, 1989; Tian et al., 1994) for some receptors, the usual method of creating a constitutively active receptor system is to manipulate the stoichiometry of receptors and G proteins. For example, constitutive activity has been observed with increasing receptor expression (i.e., β_2 -adrenoceptors; Samama et al., 1993; thyroid-stimulating hormone receptors; Van Sande et al., 1995) and enrichment of G proteins (Senogles et al., 1990). The rationale for this approach is that, because the allosteric constant controls the fraction of receptors in the activated state, a critical concentration of active receptors, for the creation of observable response, can be obtained by simply increasing the number of receptors expressed. The data obtained with the receptors in this study are consistent with this idea but also highlight the uniqueness of different types of receptors. Relatively high levels of constitutive activity were attained with the G_i protein-coupled receptors NPY2, NPY4, CXCR4, and CCR5; however,

NPY1 was uniquely quiescent and produced little observed constitutive activity at cDNA levels that clearly allowed cell surface receptor expression (as evidenced by the responses to the agonist PYY).

Note that the application of the models to the observed data tacitly assumes that exposing the cells to increasing levels of receptor cDNA leads to increasing receptor expression. However, this assumption is not limiting to the use of constitutive activity for screening purposes, because the transient transfection is titrated to a given level of response regardless of the actual receptor density present on the membrane.

If the host cell sensitivity to active-state receptor is low, then, conceivably, high receptor transfection levels would be required before sufficiently high levels of active receptor could spontaneously be generated to produce visible response. In contrast, there would be much less limitation on agonist-induced response, because saturating concentrations of full agonist would lead to conversion of all existing receptors into the active state. Under these circumstances, a threshold phenomenon would be predicted in which receptor transfection would take place (and response to agonist would be observed), but no constitutive receptor activity would be observed until considerably greater levels of receptor expression. Surprisingly, whereas a slight threshold effect was observed in these studies for NPY2 and NPY4, essentially constitutive receptor activity was observed with almost all levels of receptor expression. This suggests that melanophores have high responsiveness characteristic to GPCR activity, an idea supported by the high sensitivity of agonists in this system.

Another interesting outcome of this study was the results

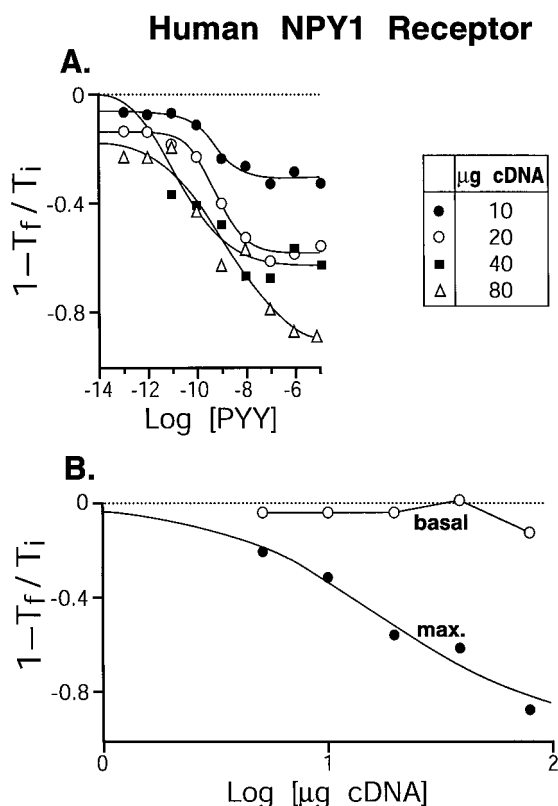


Fig. 10. Effects of different levels of NPY1 receptor transfection on responses to PYY (A) and basal and maximal agonist response (B). A, dose-response curves to PYY in melanophores transfected with NPY1 cDNA at 10 μ g (●), 20 μ g (○), 40 μ g (■), and 80 μ g (△). B, dependence of basal constitutive receptor activity (○) and maximal response to PYY (●) on concentration of NPY1 cDNA used for transfection.

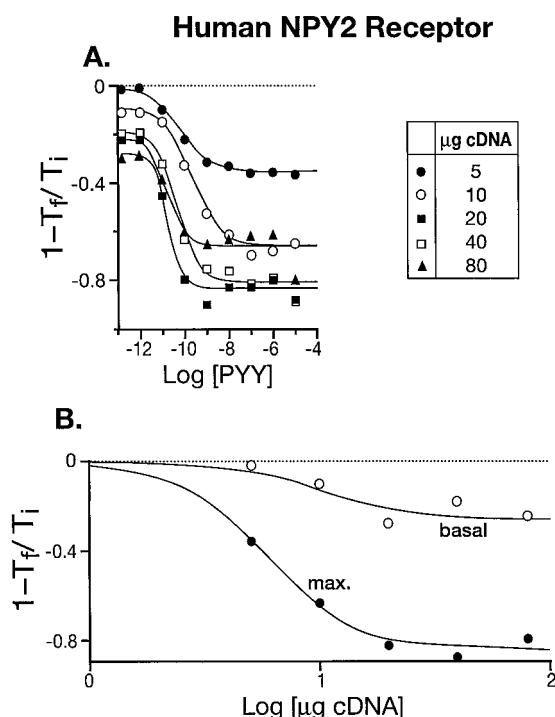


Fig. 11. Effects of different levels of NPY2 receptor transfection on responses to PYY (A) and basal and maximal agonist response (B). A, dose-response curves to PYY in melanophores transfected with NPY2 cDNA at 5 μ g (●), 10 μ g (○), 20 μ g (■), 40 μ g (□), and 80 μ g (▲). B, dependence of basal constitutive receptor activity (○) and maximal response to PYY (●) on concentration of NPY2 cDNA used for transfection.

of the limited test of the notion that most ligands with affinity for any given GPCR will have differential affinity for the various states of the receptor and thus show a detectable response in the constitutively active receptor system. As shown in Fig. 6, ligands known to bind to hCTR2 either produced positive agonism or negative agonism; there were no "neutral" antagonists in this collection of ligands. Although this clearly is a limited sample, it is interesting that the data were consistent with the thermodynamic prediction.

Two versions of the ternary complex model predict different degrees of maximal constitutive receptor activity from GPCR systems (*Appendix II*). The extended ternary complex model predicts that the same maximal response that is produced by an agonist should be observed constitutively with high receptor expression levels (if the amount of receptor is not limiting). A different prediction is consistent with the

cubic ternary complex model, which allows for the inactive state of the receptor to interact with G proteins. Interestingly, antagonist-induced receptor/G protein interactions leading to ternary complexes that do not signal (AR_iG complex in Fig. 13) have been reported for MOR-1 (Brown and Pasternak, 1998). Also, nonsignaling ternary complexes with the inverse agonist SR 144528 have been reported for cannabinoid receptors (Bouaboula et al., 1997, 1999). If such nonsignaling complexes are formed, then this model predicts that a limit can be reached where the maximal constitutive response will be lower than the agonist-induced maximal response according to system parameters describing the ability of the receptor to form the active state (allosteric factor L) and the differential affinity of the activated receptor (over the inactive state) for G proteins (β in Fig. 13). As seen in Figures 5, 7, and 9 through 12, the maximal constitutive activity observed was substantially lower than the agonist-induced maximal response. Whereas this ostensibly indicates that the cubic model better describes GPCR systems, note that there is no way of knowing whether the amount of receptor transfected into the melanophores did not limit the maximal interaction between receptor and G protein. Therefore, the submaximal constitutive receptor activity does not furnish definitive evidence for nonsignaling receptor/G protein complexes.

The data presented with these receptors indicate that a constitutive GPCR assay is a viable alternative for screening orphan receptors. The advantage of such an approach lies in the expanded window of detection. Not only will agonists be found but also inverse agonists. This option is not available in nonconstitutively active screens in which only positive agonists will be detected. Another advantage of constitutive screens is the fact that they can be more sensitive to agonists than quiescent assays (*Appendix III* and Fig. 4). Theoretically, this also applies to inverse agonists, although for these ligands (where α and $\gamma < 1$), the enhancing effects of constitutive activity will be severely dampened so as to become nearly insignificant. Interestingly, whereas an increased sensitivity to the positive agonist human calcitonin was observed in this study (Fig. 4A), little change in potency for the inverse agonist AC66 was seen (Fig. 4B), agreeing with this prediction.

The possible disadvantage of this assay is the added variability of screening with transient transfections. The level of constitutive activity varies with the efficiency of receptor transfection, which, in turn, affects the sensitivity of the assay to positive and negative agonism (see *Appendix III*). It is difficult to predict, in general terms, whether this variabil-

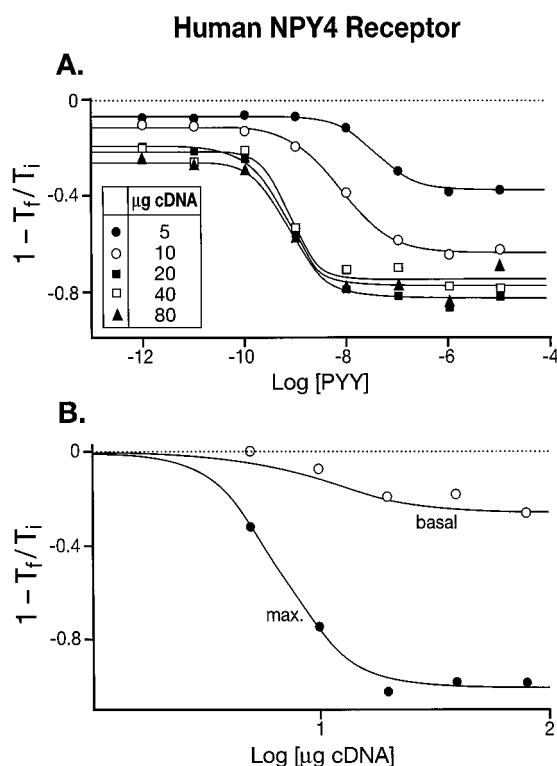


Fig. 12. Effects of different levels of NPY4 receptor transfection on responses to PYY (A) and basal and maximal agonist response (B). A, dose-response curves to PYY in melanophores transfected with NPY4 cDNA at 5 μ g (●), 10 μ g (○), 20 μ g (■), 40 μ g (□), and 80 μ g (▲). B, dependence of basal constitutive receptor activity (○) and maximal response to PYY (●) on concentration of NPY4 cDNA used for transfection.

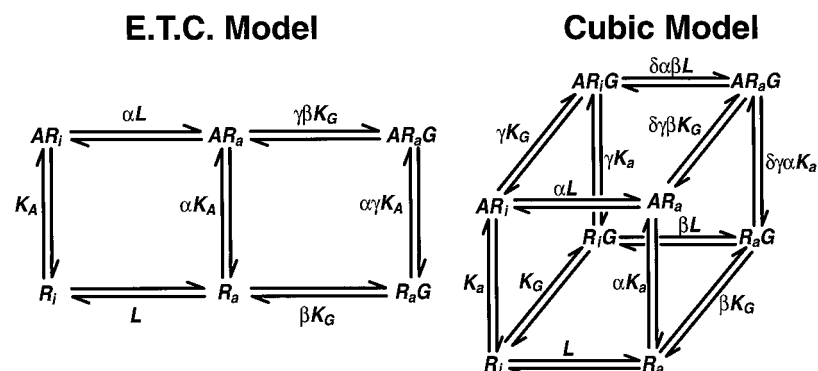


Fig. 13. Two models of GPCR systems. A, extended ternary complex model describing two receptor states (R_i and R_a) whereby the active state R_a interacts with G protein (G) (Samama et al., 1993). B, the cubic ternary complex whereby both inactive and active states of the receptor are allowed to interact with the G protein, but only AR_aG mediates response (Weiss et al., 1996a,b).

ity is too high a cost for constitutive screening. The key probably lies in the nature of the receptor, the efficiency of receptor expression, the magnitude of the allosteric constant L , and the host cell system (i.e., stoichiometry of the G proteins available for interaction with the active-state receptor). In general, the main drawback to constitutive systems would be the possibility of decreasing the positive scale for potential agonism (i.e., the constitutive activity approaches the endogenous agonist maximal response). With appropriate controls (i.e., the measurement of the maximal detectable levels of increased G_s or G_i/G_q activation with standard agonists), the control of constitutive receptor activity below these levels would be sufficient to ensure the possibility of detection of positive agonists.

These data are consistent with the idea that constitutive GPCR systems can be made sufficiently sensitive and stable to be used in screening for ligands. The fact that all but one of the receptors we tested provided substantial constitutive activity suggests that this approach would be especially useful for the screening of orphan receptors.

Appendices

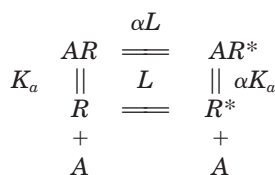
Certain predictions can be made from the extended ternary complex (ETC) model as presented by Samama et al. (1993; Fig. 13A) and the cubic ternary complex (CTC) model (Weiss et al., 1996a,b; Fig. 13B) about the relationship among receptor density, constitutive response, sensitivity to ligands, and the ability to discern receptor conformations. Note that the models described below are binding models that do not take into account GTP activation of the G protein, and, as such, they may not adequately describe functional systems.

Appendix I: Enrichment of Receptor Conformation by Conformational Selection

Differential affinities for different receptor conformations (as found in constitutively active receptor systems) leads to enrichment of the species for which the ligand has the highest affinity. This can be illustrated with a system containing two receptor conformations R and R^* that coexist in the system according to an allosteric constant denoted L'



Assume that the ligand A binds to R with an equilibrium association constant K_a and R^* by an equilibrium association constant αK_a . The factor α denotes the differential affinity of the agonist for R^* , i.e., if $\alpha = 10$, then the agonist has a 10-fold greater affinity for the R^* form. The complete scheme with ligand involved is then



This question then, how can selective affinity of A for R^* (i.e., $\alpha > 1$) enrich the R^* species? This can be calculated by

examining the amount of R^* species (both as R^* and AR^*) present in the system in the absence of ligand and in the presence of ligand. The equilibrium expression for $[R^*] + [AR^*]/[R_{tot}]$, where $[R_{tot}]$ is the total receptor concentration given by the conservation equation $[R_{tot}] = [R] + [AR] + [R^*] + [AR^*]$ is

$$\rho = \frac{L(1 + \alpha[A]/K_A)}{[A]/K_A(1 + \alpha L) + 1 + L} \quad (2)$$

where the concentration of ligand is $[A]$, L is the allosteric constant, K_A is the equilibrium dissociation constant of the agonist-receptor complex ($K_A = 1/K_a$), and α is the differential affinity of the ligand for the R^* state. It can be seen that, in the absence of agonist ($[A] = 0$), $\rho_0 = L/(1 + L)$ and in the presence of a maximal concentration of ligand (saturating the receptors; $[A] \rightarrow \infty$) $\rho_\infty = [\alpha(1 + L)]/(1 + \alpha L)$. Therefore, the effect of a ligand on enriching the R^* state is given by the ratio ρ_∞/ρ_0 ; when this ratio is >1 , then the presence of the ligand enriches the R^* state. This ratio is given by

$$\frac{\rho_\infty}{\rho_0} = \frac{\alpha(1 + L)}{1 + \alpha L} \quad (3)$$

It can be seen from eq. 3 that, if the ligand has an equal affinity for both the R and R^* states ($\alpha = 1$), then ρ_∞/ρ_0 will equal unity, and no enrichment of the R^* will result from maximal ligand binding. However, if $\alpha > 1$, then the presence of the conformationally selective ligand will cause the ratio ρ_∞/ρ_0 to be >1 . For example, if the affinity of the ligand is 10-fold greater for the R^* state, then in a system where 10% of the receptors are spontaneously in this state ($L = 0.1$), the saturation of the receptors with this agonist will increase the amount of R^* by a factor of 1.8 (10–18%), and positive agonism (if R^* mediates constitutive response) will result. Similarly, if $\alpha < 1$, then a ligand will diminish the amount of R^* , and inverse agonism will result.

Appendix II: Dependence of Basal Constitutive Receptor Activity on Receptor Density

ETC Model. The equilibrium equations for the G protein species are

$$[G] = \frac{[AR_aG]}{\alpha\gamma\beta L[R_i][A]K_g} \quad (4)$$

and

$$[R_aG] = \frac{[AR_aG]}{\alpha\gamma[A]K_a} \quad (5)$$

$$\text{response} = \rho = \frac{[AR_aG] + [R_aG]}{[G_{tot}]} \quad (6)$$

The conservation equation is

$$[G_{tot}] = [G] + [R_aG] + [AR_aG] \quad (7)$$

The equation for the production of response either constitutively or through an agonist ligand $[A]$ is

$$\rho = \frac{\beta L[R_i]/K_G(1 + \alpha\gamma[A]/K_A)}{[A]/K_A(\alpha\gamma\beta L[R_i]/K_G) + [1 + (\beta L[R_i]/K_G)]}, \quad (8)$$

where $K_G = 1/K_g$, and $K_A = 1/K_a$.

The basal constitutive receptor activity is given by eq. 8 when $[A] = 0$:

$$\text{basal} = \frac{\beta L[R_i]/K_G}{1 + \beta L[R_i]/K_G} \quad (9)$$

The maximal response to a full agonist is obtained as $[A] \rightarrow \infty$. This equals unity; thus, eq. 9 also yields the expression for constitutive activity as a function of receptor density expressed as a fraction of the maximal response to a full agonist. It can be seen from eq. 9 that, if receptor density is not limiting (i.e., as $[R_i] \rightarrow \infty$), then the maximal constitutive activity predicted by the **ETC** model is unity (i.e., the maximal response produced by a full agonist).

CTC Model. The equilibrium equations for the G protein species are

$$[G] = \frac{[AR_aG]}{\delta\gamma\alpha\beta L[R_i]K_g[A]K_a} \quad (11)$$

$$[R_iG] = \frac{[AR_aG]}{\delta\gamma\alpha\beta L[A]K_a} \quad (12)$$

$$[R_aG] = \frac{[AR_aG]}{\delta\gamma\alpha[A]K_a} \quad (13)$$

$$[AR_iG] = \frac{[AR_aG]}{\delta\alpha\beta L} \quad (14)$$

Basal and agonist-induced response is given as

$$\text{response} = \rho = \frac{[R_aG] + [AR_aG]}{[G_{\text{tot}}]} \quad (15)$$

The conservation equation is

$$[G_{\text{tot}}] = [G] + [R_iG] + [R_aG] + [AR_iG] + [AR_aG] \quad (16)$$

Thus, response, as a function of receptor density and ligand concentration, is

$$\rho = \frac{\beta L[R_i]/K_G(1 + \delta\gamma\alpha[A]/K_a)}{[A]/K_A[\gamma[R_i]/K_G(1 + \delta\alpha\beta L)] + 1 + [R_i]/K_G(1 + \beta L)} \quad (17)$$

where $K_G = 1/K_g$, and $K_A = 1/K_a$.

The basal constitutive receptor activity is given by eq. 17 when $[A] = 0$

$$\text{basal} = \frac{\beta L[R_i]/K_G}{1 + [R_i]/K_G(1 + \beta L)} \quad (18)$$

The maximal response to a full agonist is given as $[A] \rightarrow \infty$

$$\text{maximal} = \frac{\delta\alpha\beta L}{1 + \delta\alpha\beta L} \quad (19)$$

Therefore, the expression for constitutive activity as a function of receptor density expressed as a fraction of the maximal response to a full agonist is

$$\frac{\text{basal}}{\text{maximal}} = \frac{[R_i]/K_G(1 + \delta\alpha\beta L)}{\delta\alpha[1 + [R_i]/K_G(1 + \beta L)]} \quad (20)$$

If receptor concentration is not limiting (i.e., as $[R_i] \rightarrow \infty$), then the constitutive activity, as a function of the maximal agonist-stimulated activity, will reach an asymptotic value of

$$\frac{\text{basal}}{\text{maximal}} = \frac{1 + \delta\alpha\beta L}{\delta\alpha(1 + \beta L)} \quad (21)$$

This equation reduces to

$$\frac{\text{basal}}{\text{maximal}} = \frac{1/\delta\alpha + \beta L}{1 + \beta L} \quad (22)$$

For a high-efficacy agonist ($\delta\alpha \gg 1$), then, $1/\delta\alpha \rightarrow 0$, and the expression reduces to

$$\frac{\text{basal}}{\text{maximal}} = \frac{\beta L}{1 + \beta L} \quad (23)$$

Thus, the maximal amount of constitutive activity produced by the receptor depends on the allosteric constant L (the natural proclivity of the receptor to form the active state) and the differential factor defining increased affinity of interaction of the active-state receptor with G protein over the inactive state ($[R_i]$). It can be seen from eq. 23 that, unlike the prediction of the **ETC** model, the maximal basal constitutive activity need not reach the maximal response produced by a full agonist.

Appendix III: Effect of Constitutive Activity on Observed Potency of Agonists

ETC Model. From the equation describing response (eq. 8), the observed affinity of an agonist is given by

$$K_{\text{obs}} = \frac{K_A(1 + \beta L[R_i]/K_G)}{\alpha\gamma\beta L[R_i]/K_G} \quad (23)$$

Isolating the $[R_i]/K_G$ term from eq. 23 and from the equation describing constitutive activity (eq. 9) and equating the $[R_i]/K_G$ terms leads to the following equation relating the observed affinity to an agonist and the level of constitutive activity

$$K_{\text{obs}} = \frac{K_A}{\alpha\gamma\text{basal}} \quad (24)$$

From eq. 24, it can be seen that, as the constitutive activity of the system increases (increasing levels of basal), the observed equilibrium dissociation constant of the ligand-receptor complex will decrease (increased potency for ligands). The effect will be very much less with inverse agonists ($\alpha, \gamma < 1$), if observed at all.

CTC Model. From the equation describing response (eq. 17), the observed affinity of an agonist is given by

$$K_{\text{obs}} = \frac{K_A[\gamma[R_i]/K_G(1 + \delta\alpha\beta L)]}{1 + [R_i]/K_G(1 + \beta L)} \quad (25)$$

Isolating $[R_i]/K_G$ from eq. 25 and from the equation describing constitutive activity (eq. 17) and equating the $[R_i]/K_G$ terms leads to the following equation relating the ob-

served affinity to an agonist and the level of constitutive activity

$$K_{\text{obs}} = \frac{\beta L K_A}{\gamma(1 + \delta \alpha \beta L) \text{basal}} \quad (26)$$

As with the extended ternary complex model, it can be seen that, as the constitutive activity of the system increases (increasing levels of basal), the observed equilibrium dissociation constant of the ligand-receptor complex will decrease (increased potency for ligands).

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