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High-affinity Agonist Binding Is Not Sufficient for Agonist Efficacy at 5-Hydroxytryptamine$_{2A}$ Receptors: Evidence in Favor of a Modified Ternary Complex Model[1]

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ABSTRACT

In this study, the relationship between high-affinity agonist binding and second messenger production was examined at native and mutant 5-hydroxytryptamine$_{2A}$ receptors. At native 5-hydroxytryptamine$_{2A}$ receptors all agonists, with the exception of quipazine, discriminated between high- and low-affinity states of the receptor, as determined by analysis of competition binding assays. There was no correlation between the ability of selected agonists to label the high-affinity agonist state and to augment phosphoinositide hydrolysis. Quipazine, which did not discriminate between the affinity states of the receptor, behaved as a full agonist. Similar results were obtained when a point mutation (F340L) of a highly conserved phenylalanine located in transmembrane domain VI was examined. With the F340L mutant, most of the agonists tested labeled significantly fewer high-affinity sites, compared with the native receptor. There was no significant relationship between high-affinity agonist binding and second messenger production. Bufotenine and 4-iodo-3,5-dimethoxyphenylisopropylamine labeled similar percentages of high-affinity agonist binding sites (22% *vs.* 26%), but 4-iodo-3,5-dimethoxyphenylisopropylamine behaved as a full agonist, whereas bufotenine was devoid of detectable agonist activity. The inability of selected agonists to activate phosphoinositide hydrolysis was not due solely to lower agonist affinity for the mutant receptor, because the binding affinity of quipazine was unchanged by the F340L mutation but quipazine had no detectable agonist activity at the mutant receptor. Our results demonstrate that the ability of an agonist to promote the high-affinity state of the 5-hydroxytryptamine$_{2A}$ receptor is not correlated with its ability to augment second messenger production. These results are consistent with recent models of G protein-receptor functioning (*e.g.*, modified ternary complex model) that predict that additional transition states of the receptor-ligand complex are essential for agonist efficacy.

The molecular mechanisms by which agonists bind to and activate G protein-coupled serotonin (5-HT) receptors remain major enigmas for modern pharmacologists. Site-directed mutagenesis studies have highlighted the importance of highly conserved aspartic acid residues (Wang *et al.*, 1993; Sealfon *et al.*, 1995), serines (Johnson *et al.*, 1994) and phenylalanines (Choudhary *et al.*, 1993, 1995) for agonist binding and efficacy at various 5-HT receptors, including 5-HT$_{1A}$ and 5-HT$_{2A}$ subtypes. The aspartic acid group (from helix III) is thought to anchor the charged nitrogen from 5-HT, whereas serines (in helix V) and a phenylalanine (in helix VI) may anchor hydroxyl/amine residues and aromatic groups, respectively (Wang *et al.*, 1993; Choudhary *et al.*, 1993; Johnson *et al.*, 1994). These studies are in general accord with several computer-generated three-dimensional models of 5-HT-receptor interactions (Westkaemper and Glennon, 1993; Kristiansen and Dahl, 1996).

In some instances, point mutations of these highly conserved residues also diminish the ability of agonists to activate second messenger production. In the case of the 5-HT$_{2A}$ receptor, which activates PI hydrolysis (Conn and Sanders Bush, 1984; Roth *et al.*, 1984), a diminished ability to augment PI hydrolysis has been reported with mutations at aspartic acids found in helix II (Asp-120) (Wang *et al.*, 1993) and helix III (Asp-155) (Wang *et al.*, 1993) and a phenylalanine found in helix VI (Phe-340) (Choudhary *et al.*, 1993; Roth *et al.*, 1995). How these mutations, which alter ligand affinity, also affect agonist efficacy is unknown. An assumption of many prior studies has been that high-affinity agonist binding is essential for second messenger production.

In this paper, we directly test the hypothesis that high-affinity agonist binding is essential for second messenger production, using native and mutant 5-HT$_{2A}$ receptors. Our

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ABBREVIATIONS: DMEM, Dulbecco's modified Eagle medium; DMT, *N*,*N*′-dimethyltryptamine; DOB, (−)-4-bromo-3,5-dimethoxyphenylisopropylamine; DOI, 4-iodo-3,5-dimethoxyphenylisopropylamine; DOM, 4-methoxy-3,5-dimethoxyphenylisopropylamine; 5-HT, 5-hydroxytryptamine; 5-OMe-DMT, 5-methoxy-*N*,*N*′-dimethyltryptamine; PI, phosphoinositide.

findings suggest that the mere presence of the high-affinity agonist state of the receptor is not sufficient for receptor-effector coupling. Our findings are consistent with recent models that predict that additional transition states are essential for agonist-induced activation of second messenger production (*e.g.*, modified ternary complex model) (Lefkowitz *et al.*, 1993).

Experimental Procedures

Materials. Tissue culture reagents were from GIBCO/BRL (Gaithersburg, MD). [^{3}H]Ketanserin (89 Ci/mmol) was from New England Nuclear (Boston, MA). Molecular biology reagents were from Stratagene (Torry Pines, CA) or United States Biochemicals (Cleveland, OH). The rat 5-HT_{2A} receptor cDNA was a gift from D. Julius (University of California, San Francisco); the F340L mutation has been previously described (Choudhary *et al*., 1995). Other chemicals were of the highest grade commercially available. Quipazine, DMT, DOM, bufotenine, psilocybin and 5-OMe-DMT were supplied by the Mental Health Clinical Research Center, Case Western Reserve University Medical School; DOB and α-methyltryptamine were from Richard Glennon (Medical College of Virginia). 5-HT creatinine sulfate was from Sigma Chemical Co. (St. Louis, MO), whereas DOI was from Research Biochemicals Inc.

Cell lines. All stable cell lines were grown in DMEM containing 10% fetal calf serum and 600 μg/ml G418. An NIH/3T3 cell line expressing the 5-HT_{2A} receptor (GF-6) was from D. Julius (University of California, San Francisco) and was used to isolate a clonal cell line (GF-62) that expressed 5 to 7 pmol/mg protein of the 5-HT_{2A} receptor. For the F340L mutation, the F340L cDNA was subcloned into the eukaryotic expression vector pRc/CMV (Invitrogen) using *Not*I adaptors and was transfected into 3T3 and human embryonic kidney 293 cells using Lipofectin (GIBCO/BRL), exactly as described by the manufacturer. At 72 hr after transfection, the cells were split and grown in DMEM containing 600 μg/ml G418 and 10% fetal calf serum. Surviving clones were isolated and expanded for assessment of F340L receptor expression, as assessed by radioligand binding. One cell line expressing high levels of receptors in 3T3 cells (5–7 pmol/mg; M1C15) was used for subsequent experiments. Both cell lines (GF-62; M1C15) have been previously characterized (Roth *et al.*, 1995; Berry *et al.*, 1996).

Binding assays. Binding assays were performed in total volumes of 0.5 ml (for ^{3}H-radioligands) at 25°C for 90 min with 5 to 20 μg of membrane protein, as described previously (Roth *et al.*, 1987, 1995), in 50 mM Tris-HCl buffer containing 0.5 mM EDTA, 10 mM $MgCl_2$, 0.05% ascorbic acid and 10 μM pargyline (pH 7.40). Membranes were harvested with a Brandel SM-24 cell harvester, followed by three ice-cold washes onto polyethyleneimine-pretreated (0.1%) glass fiber filters. Filters were soaked for 18 hr in scintillation fluid before counting, with efficiency determined by the external standard method. Specific binding (determined with 10 μM mianserin) represented 90 to 97% of total binding in the experiments reported here; no more than 10% of total counts/assay tube was bound.

PI hydrolysis. Cells were harvested by trypsinization and split into 24-well plates with complete medium. Cells were washed with inositol-free DMEM 24 hr later and then incubated for an additional 18 hr with inositol-free DMEM containing 1 μCi/ml [^{3}H]inositol and 10% dialyzed fetal calf serum. Cells were then rinsed three times with a Krebs-bicarbonate buffer of the following composition: 118 mM NaCl, 4.7 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$ and 11 mM glucose. Before use the buffer was equilibrated with 95% O_2/5% CO_2. Cells were then incubated for 30 min with test agents together with 10 mM LiCl in Krebs-bicarbonate buffer. The reaction was then terminated by aspiration and the addition of 1.2 ml of methanol/water/HCl (25:25:0.1). Cells were then harvested into glass tubes and 0.6 ml of chloroform was added, followed by vigorous vortex-mixing. After phase separation, the upper aqueous phase was removed and [^{3}H]inositol monophosphate was isolated and quantified as previously detailed (Roth *et al.*, 1984). Typically a 20- to 40-fold activation of [^{3}H]inositol monophosphate accumulation was measured for both native and mutant receptors.

Data analysis. Binding data were analyzed using a weighted, nonlinear, least-squares program that determines binding to multiple sites using the law of mass action (LIGAND program), as previously detailed (Munson and Rodbard, 1980). For PI hydrolysis experiments, data were fit to a modified Michaelis-Menton equation (Roth *et al.*, 1995) using a nonlinear, least-squares, curve-fitting routine available in Sigma Plot. In all figures, lines represent the theoretical fits using the parameter estimates calculated by the curve-fitting programs. Protein was determined using a kit from Bio-Rad (Richmond, CA).

Results

A point mutation alters the affinity of selected agonists at 5-HT_{2A} receptors. In previous studies, we found that a mutation of a highly conserved phenylalanine (F340L), but not an adjacent conserved phenylalanine (F339L), drastically altered the affinities of several agonists and ergolines at the 5-HT_{2A} receptor (Choudhary *et al.*, 1993). We next determined whether other agonists were similarly affected by the F340L mutation. A complication encountered in investigating agonist efficacy and affinity at 5-HT_{2A} receptors, however, is the observation that high- and low-affinity states of the receptor exist. From our initial data (Choudhary *et al.*, 1993), it was unclear whether the high- or low-affinity agonist state(s) might be altered by the F340L mutation. Thus, we first needed to characterize agonist affinity at the high- and low-affinity states of the 5-HT_{2A} receptor.

Competition binding assays disclosed high- and low-affinity states of the 5-HT_{2A} receptor. For all of the agonists except for quipazine, a two-site model fit the data significantly better than a one-site model (figs. 1 and 2; table 1). The affinities of agonists at the high- and low-affinity sites are in general agreement with our previous results, as well as those of others (Roth *et al.*, 1987; Segal *et al.*, 1990; Teitler *et al.*, 1990).

With the F340L mutant receptor, for most agonists tested, a two-site model fit the data significantly better than a one-site model (figs. 1 and 2; table 1). For DOM, DMT and quipazine binding, a two-site model did not improve the fit (F test; $P > .05$). With the exception of quipazine, all tested agonists showed significantly lower affinity for both the high- and low-affinity sites at the F340L mutant receptor, compared with the native receptor (F test; $P < .01$). For the phenylisopropylamines, the shifts in affinity were generally of a lower magnitude for the high-affinity site (average, 4.4-fold), compared with the tryptamines (average, 62.2-fold). For both groups of compounds, there were fewer receptors in the high-affinity state in cells expressing the mutant receptor, compared with cells expressing the native receptor (mean, 26.6 ± 5% *vs.* 44.5 ± 8%; $P < .01$; F test). We next examined the effect of the F340L mutation on agonist efficacy.

A point mutation alters the ability of selected agonists to augment PI hydrolysis. As can be seen in figure 3 and table 2, all tested compounds behaved as agonists at the cloned 5-HT_{2A} receptor. Table 2 lists the K_{act} and V_{max} values (relative to 5-HT for the native receptor or DOI for the F340L mutant) for these compounds. As can be seen from table 2, a

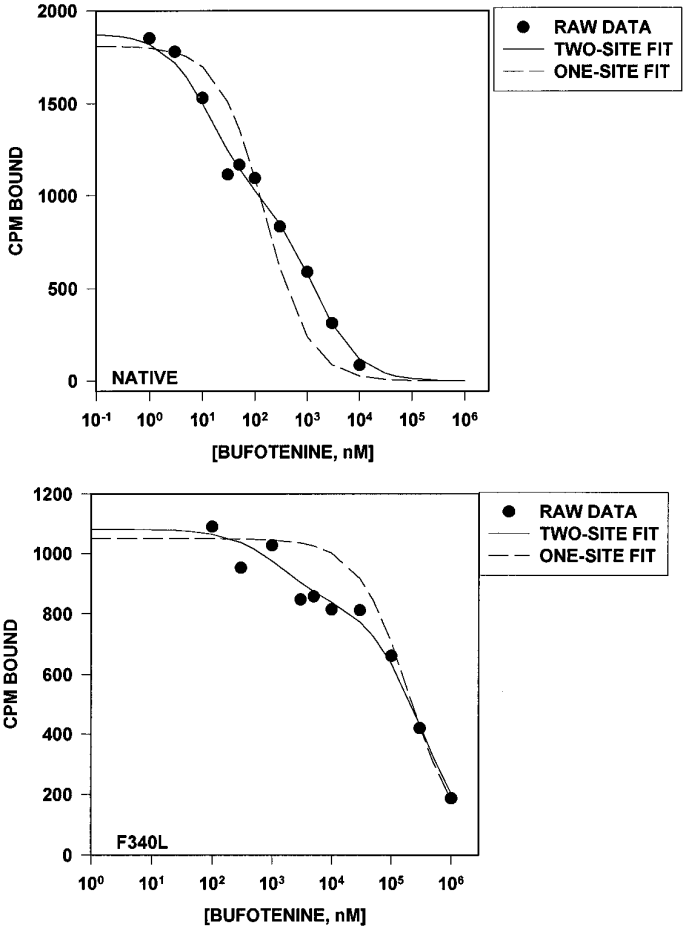

Fig. 1. Competition binding isotherms for bufotenine at native and F340L mutant receptors. Inhibition by bufotenine of [^{3}H]ketanserin-labeled native (top) and F340L mutant (bottom) receptors was measured and then sequentially fit to one-site (dashed line) and two-site (solid line) models. A two-site model significantly improved the fit ($P < .01$; *F* test) for both native and F340L mutant receptors.

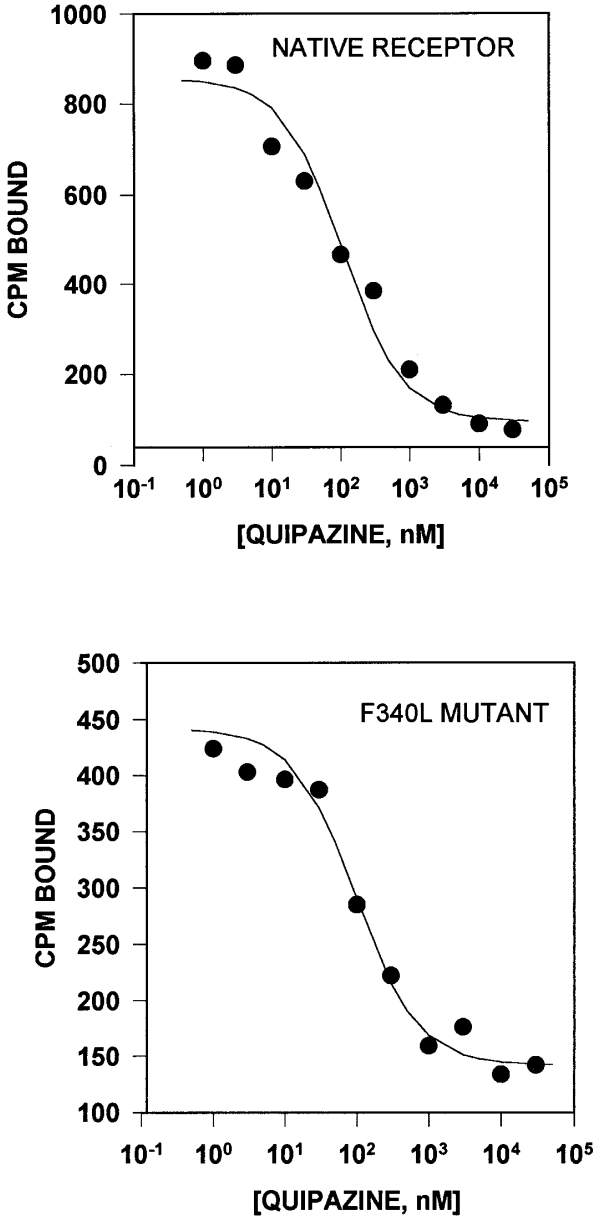

Fig. 2. Competition binding isotherms for quipazine at native and F340L mutant receptors. Inhibition by quipazine of [^{3}H]ketanserin-labeled native (top) and F340L mutant (bottom) receptors was measured and then fit to a one-site model. No improvement of fit was seen with a two-site model ($P > .05$; *F* test).

range of intrinsic activities was seen, with some compounds behaving as full agonists (DOB, DOI, DOM, bufotenine, α-methyltryptamine, quipazine and 5-HT) and others behaving as partial agonists (DMT and 5-OMe-DMT).

The F340L mutation had varying effects on the efficacies of agonists (fig. 3; table 2). In agreement with our previous results, the F340L mutation caused 5-HT to behave as a partial agonist. Interestingly, DOB, (±)-DOI and DOM all had significantly greater efficacies than did 5-HT at the F340L receptor, whereas most of the tested tryptamine analogs had lower efficacies than did 5-HT (table 2). DOI behaved as a full agonist at both native and mutant receptors.

High- and low-affinity binding is not correlated with second messenger production. We next investigated whether a correlation might exist between the high-affinity agonist state of the receptor and second messenger production. Figures 4 and 5 show the results of these studies. The percentage of high-affinity sites varied, depending on the agonist used, from 25% (5-OMe-DMT) to 80% (DOI). There was no correlation between the percentage of high-affinity sites and the ability of selected agonists to activate PI hydrolysis with the native receptors (fig. 4).

A similar lack of correlation was seen in cells expressing the F340L mutation. Thus, for instance, bufotenine and 5-OMe-DMT had 29% and 42% high-affinity sites, respectively, but produced a minuscule activation of PI hydrolysis in cells expressing the F340L mutation (table 2). DOB and (±)-DOI, on the other hand, had fewer receptors (15% and 26%, respectively) in the high-affinity state but produced a robust activation of PI hydrolysis (14.6- and 18.8-fold activation of PI hydrolysis, respectively). DOM bound to only one state of the receptor but was able to greatly augment PI hydrolysis. DOI had a significantly greater efficacy than 5-HT; the efficacy of DOI was similar to that produced in cells expressing the native 5-HT$_{2A}$ receptor (average 22-fold acti-

TABLE 1

Binding affinities of various agonists for high- and low-affinity states of 5-HT_{2A} receptors at native and F340L mutant receptors

Data represent mean ± SD of computer-derived estimates for inhibition of [^{3}H]ketanserin binding at native and F340L mutant receptors.

Drug	Bufo[a]	DMT	5-OMe	5-HT	α-Me-5-HT	DOM	(−)-DOB	(±)-DOI	QUIP
Native									
K_H (nM)	2.7 ± 1.3	94 ± 37	22 ± 5	7 ± 4	15 ± 3	1.9 ± 1.5	3.7 ± 3	6.2 ± 3.5	919 ± 128
K_L (nM)	337 ± 91	1,195 ± 598	676 ± 149	2,622 ± 420	3,299 ± 693	208 ± 58	38 ± 14	28 ± 20	
% R_H	45 ± 5	34 ± 23	25 ± 6	25 ± 1	25 ± 5	40 ± 7	44 ± 6	80 ± 8	100
% R_L	55 ± 4	66 ± 26	75 ± 10	75 ± 5	75 ± 5	60 ± 6	56 ± 10	20 ± 6	
F340L									
K_H (nM)	203 ± 172	8,960 ± 1,882	2,320 ± 417	12.4 ± 6	99,000 ± 27,000	2,806 ± 416	12.8 ± 10	33 ± 17	256 ± 35
K_L (nM)	85,941 ± 8,500		75,105 ± 55,000	158,000 ± 47,000			1,355 ± 162	508 ± 50	
% R_H	22 ± 8	100	42 ± 4	29 ± 8	100	100	15 ± 2	26 ± 8	100
% R_L	78 ± 7		58 ± 6	71 ± 14			85 ± 8	76 ± 6	

[a] Bufo, bufotenine; 5-OMe, 5-methoxytryptamine; α-Me-5-HT, α-methyl-5-HT; QUIP, quipazine.

vation of PI hydrolysis), despite the fact that fewer receptors were in the high-affinity state.

We also compared the relative affinities of selected agonists at the high- and low-affinity sites with their K_{act} values for PI hydrolysis (fig. 5). There was no correlation between the K_i values for the high-affinity ($r^2 = 0.08$) or low-affinity ($r^2 = 0.05$) sites and the K_{act} values for activating PI hydrolysis in cells expressing the native receptor. Finally, no correlation was noted between K_H and percentage of efficacy (data not shown).

Discussion

The major finding of this paper is that the ability of an agonist to promote high-affinity agonist binding is not necessarily correlated with agonist efficacy. As demonstrated below, our findings are consistent with a modified ternary complex model of ligand-receptor-G protein interactions (Lefkowitz *et al.*, 1993). We arrived at our conclusions by three mutually supportive lines of evidence, 1) the lack of correlation between the number of high-affinity agonist sites and the efficacy of an agonist in activating PI hydrolysis in cells expressing native and mutant receptors, 2) the preferential loss of agonist efficacy for tryptamine analogs without large alterations in the number of high-affinity agonist sites with the F340L mutation and 3) the maintenance of phenylisopropylamine efficacy despite the reduction in the affinity and number of high-affinity agonist sites with the F340L mutation. Each of these points is discussed, and our results are then interpreted with the aid of a modified ternary complex model of receptor-G protein activation (Lefkowitz *et al.*, 1993).

Our first observation was that there was no clear correlation between the number of high-affinity sites and the ability of an agonist to activate PI hydrolysis (K_{act}). In cells expressing native receptors, agonists had variable efficacies ranging from 77% to 100% of that of the full agonist 5-HT and differed in the formation of the high-affinity state of the 5-HT_{2A} receptor from a low of 25% to a high of 80%. A prediction of the simple ternary complex model (fig. 6) is that there should be some direct correlation between agonist efficacy and the relative number of receptors in the high-affinity state. There was no correlation between these two parameters.

With mutant receptors, the lack of a correlation between the number of high-affinity agonist sites and K_{act} was even more striking. 5-HT, DOI and DOB were all able to promote similar degrees of high-affinity binding but showed quite variable degrees of second messenger production. There was

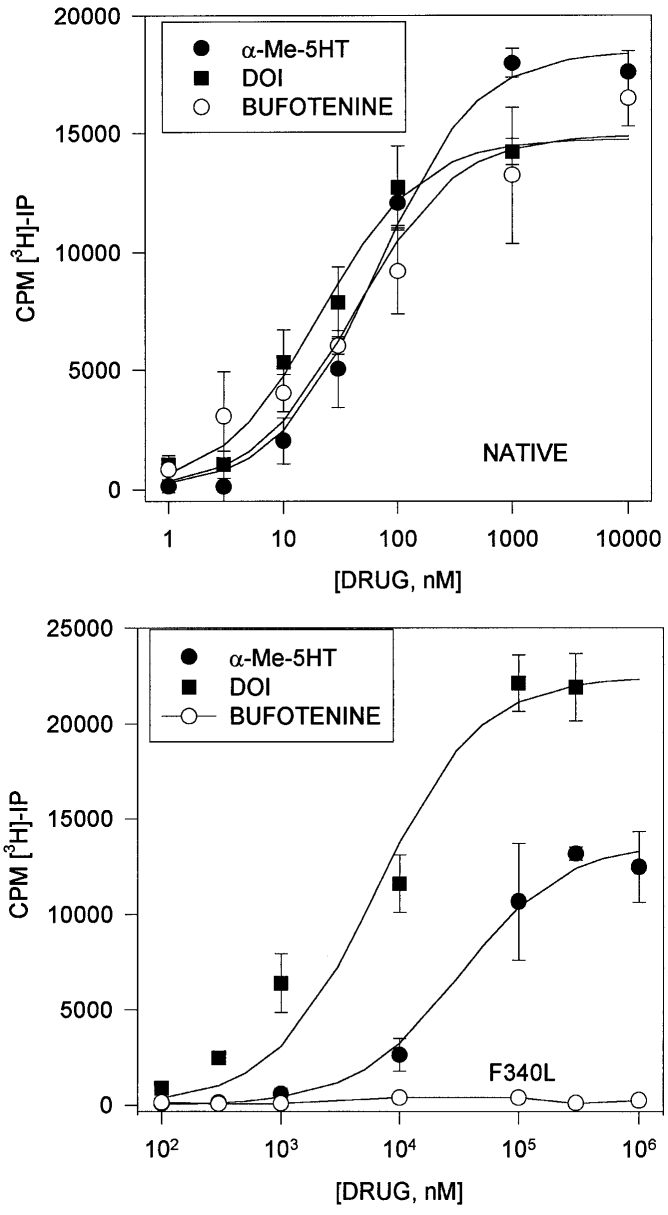

Fig. 3. Effects of F340L mutation on agonist efficacy. The ability of α-methyl-5-HT (●), DOI (■) and bufotenine (○) to activate PI hydrolysis was evaluated with native (top) and F340L mutant (bottom) receptors stably expressed in NIH/3T3 cells. Data represent mean ± S.E.M. of a typical experiment, which has been replicated at least three times for each compound. IP, inositol phosphates.

TABLE 2
Differential effects of the F340L mutation on the ability of agonists to activate PI hydrolysis

Drug	Native Receptor K_{act}	Native Receptor V_{max}	F340L Mutant K_{act}	F340L Mutant V_{max}
	nM	*%*	*μM*	*%*
(−)-DOB	59 ± 15	100 ± 3.4	32 ± 16	50 ± 19
(±)-DOI	11.6 ± 3	93 ± 3	26 ± 12	100
DOM	171 ± 32	93 ± 4	28 ± 14	50 ± 4
Bufotenine	127 ± 24	90 ± 14	1.9 ± 1.9	10.5 ± 1.4
DMT	731 ± 239	77 ± 5	ND[a]	<10
5-OMe-DMT	988 ± 169	86 ± 2.6	ND	<10
α-Methyltryptamine	1331 ± 529	99 ± 12	ND	<10
5-HT	62 ± 20	100	7.2 ± 2	18 ± 3
α-Methyl-5-HT	65 ± 9.3	100	6.3 ± 2	65 ± 13
Quipazine	395 ± 36	100	ND	0

[a] ND, not detectable (<20% increase of PI hydrolysis over basal). For a typical experiment with stably transfected cells expressing the 5-HT_{2A} receptor, the basal activity was 2,000 dpm, with the maximum stimulation with saturating concentrations of (−)-DOB being 46,180 dpm (23-fold stimulation). For the M1C15 cell line, the basal level was 600 dpm, with the maximum stimulation with saturating concentrations of (±)-DOI being 27,250 dpm (45-fold stimulation). V_{max} is expressed as the percentage of maximum stimulation of 5-HT.

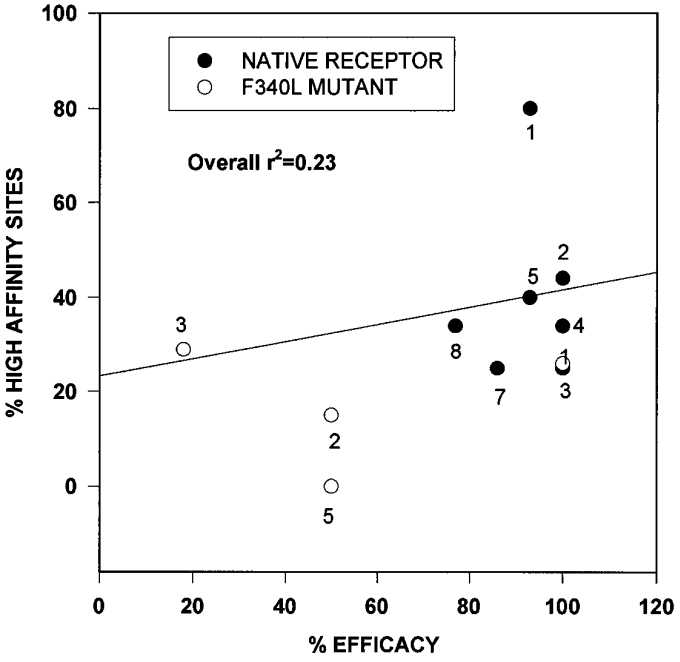

Fig. 4. Relationship between the relative number of high-affinity sites and efficacy at native and F340L mutant receptors. Shown is the relationship between the mean percentage of high-affinity sites and the percentage relative efficacy for native (●) and F340L mutant (○) receptors. For the native receptors, 5-HT was used as 100% relative efficacy; for the F340L mutant, DOI was used as 100% relative efficacy. The relationship was statistically significant ($P > .05$). 1, DOI; 2, DOB; 3, 5-HT; 4, bufotenine; 5, DOM; 6, quipazine; 7, DMT; 8, 5-OMe-DMT; 9, 5-methoxy-5-HT.

no correlation between the number of high-affinity sites and the ability of drugs to activate PI hydrolysis in mutant receptors.

Our second observation was that tryptamine analogs showed a preferential loss of efficacy without a corresponding loss of the number of high-affinity agonist sites at the F340L mutation. Thus, for instance, 5-HT displayed 18% efficacy despite the fact that the relative proportions of high- and low-affinity sites were only marginally altered. Other tryptamine analogs showed even more dramatically diminished efficacies with the F340L mutation. Decreased affinity at the high-affinity site was not, however, associated with diminished efficacy. Thus, for instance, bufotenine, DMT and 5-OMe-DMT displayed nearly 100-fold lower K_i values for the high-affinity site with the F340L mutation, and all had efficacies diminished by at least 90%. On the other hand, 5-HT had only a 6-fold lower affinity for the F340L high-affinity state but displayed an 82% decrease in efficacy, relative to the native receptor.

Our third observation was that phenylisopropylamines showed diminished affinities and numbers of high-affinity sites without corresponding losses of agonist efficacy. Interestingly, the shift in K_i values for the high-affinity state (8–20-fold) was much less than the change in K_{act} (7,200–36,000-fold). If high-affinity binding was directly correlated with agonist efficacy, we would have expected similar shifts in affinity. Additionally, DOI had many fewer sites in the high-affinity state (80% *vs.* 26%) with the mutant receptor but displayed equivalent agonist efficacy, relative to the native receptor. Taken together, these three types of observations demonstrate that high-affinity agonist binding cannot be directly connected with agonist efficacy.

A similar lack of correlation was seen with quipazine. Quipazine labeled only a single site at both native and mutant receptors and had no change in apparent binding affinity with the F340L mutation, in agreement with our previous study (Roth *et al.*, 1995). Despite the fact that no apparent change in the K_i of quipazine occurred with the F340L mutation, quipazine was inactive at the F340L mutant and fully active at the native receptor. The simple ternary complex model directly predicts that a loss of agonist efficacy would be accompanied by lower binding affinity.

Earlier studies with other receptors have implied that the high-affinity agonist "state" of the receptor is the state that is coupled to G proteins in the absence of bound GTP or GDP (fig. 6). The receptor cycles from this state to a low-affinity state depending on its coupling to G proteins and the presence of GTP or GDP. This model of receptor coupling is based on a number of mutually supportive lines of evidence (see discussion in Lefkowitz *et al.*, 1993), including point mutations of highly conserved aspartic acid residues that abolish high-affinity agonist binding and that abolish G protein coupling as well. Studies by Hausdorff *et al.* (1991), however, demonstrated that a deletion mutant of the *beta* adrenergic receptor was able to bind agonists with high affinity but not be coupled to adenylate cyclase. Those authors concluded

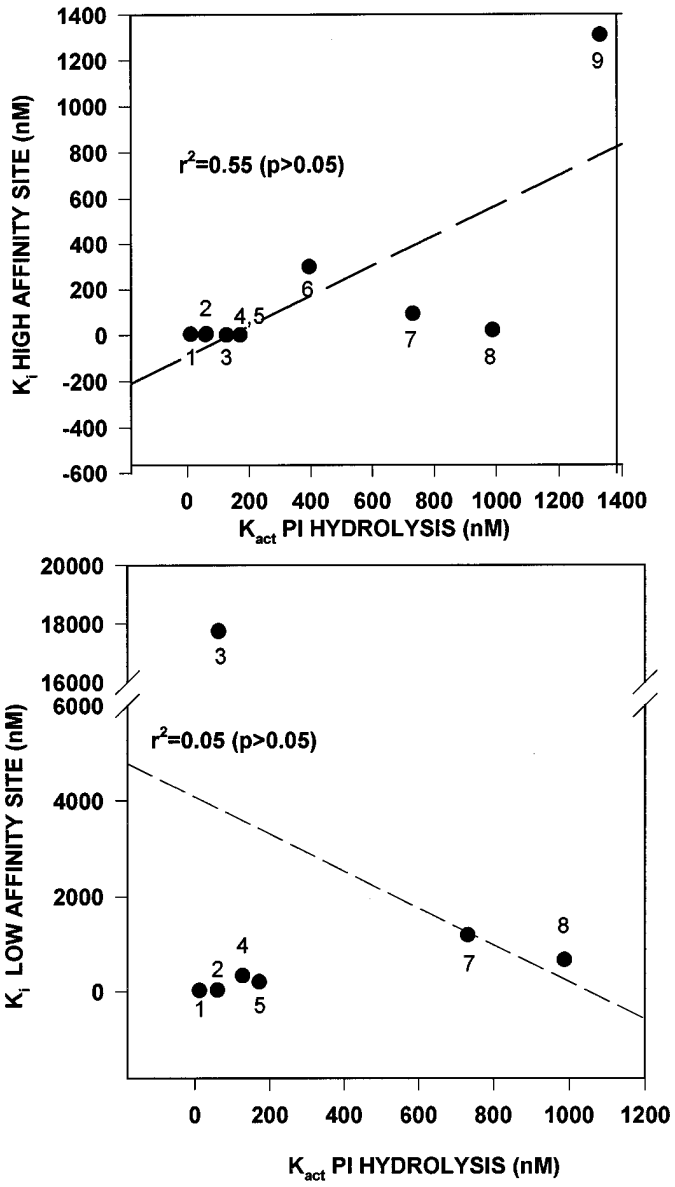

Fig. 5. Lack of correlation between binding affinities and potencies for agonists at 5-HT_{2A} receptors. Top, relationship between mean K_i values for the high-affinity site and K_{act} values for PI hydrolysis at native receptors. Bottom, relationship between mean K_i values for the low-affinity site and K_{act} values for PI hydrolysis at native receptors. Neither relationship was statistically significant ($P > .05$ for both). 1, DOI; 2, DOB; 3, 5-HT; 4, bufotenine; 5, DOM; 6, quipazine; 7, DMT; 8, 5-OMe-DMT; 9, 5-methoxy-5-HT.

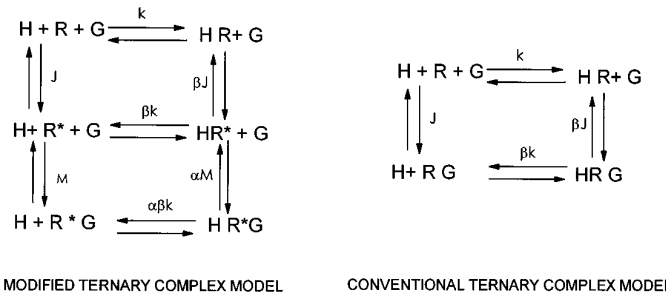

Fig. 6. Comparison of the modified ternary complex model and the conventional ternary complex model (adapted from Lefkowitz *et al.*, 1993). The modified ternary complex model proposes the existence of an intermediate state of the receptor (R*) that is essential for G protein (G) binding and activation. Ligands (H) may interact directly with either the R, R* or R*G form of the receptor, whereas the receptor may fluctuate between R and R* conformations. The model differs from the conventional ternary complex model by the introduction of an intermediate state of the receptor (R*).

that, for *beta* adrenergic receptors, the processes of high-affinity agonist binding and G protein coupling are likely to be distinct processes and that at least one additional state of the receptor is essential for agonist efficacy. That group subsequently proposed a slightly more complicated scheme of ligand binding and G protein coupling, which has been called a modified ternary complex model (fig. 6).

Using this modified model as a template, then, how can one describe situations in which complex binding kinetics (*e.g.*, multiple affinity states) can be disconnected from activation of G proteins? From the model it is clear that agonists (H) can interact with three separate receptor species, *i.e.*, R, R* and R*G. Furthermore, each binding reaction is described by distinct kinetic parameters, *i.e.*, k, βK and $\alpha\beta k$, respectively; only one kinetic parameter (M parameter) describes the process leading to activated G proteins. Additionally, each of these states of the ligand-receptor-G protein complex exists in multiple equilibria, of which six possible combinations are described in the most simple situation. G protein activation, according to this model, depends solely on the final entities (R*G or HR*G), which themselves are dependent on many equilibrium reactions, according to this simplified scheme. It is clear, then, that multiple equilibrium binding sites corresponding to different affinity states of the receptor (HR, HR* and potentially others) can exist separately from the activated state of the receptor, HR*G.

This model also predicts that receptors can directly interact with G proteins but that an activated receptor state (R*) is necessary for this to occur. We have recently found that 5-HT_{2A} receptors can interact with $G_{\alpha q}$ in the absence of agonists but in the presence of magnesium, presumably *via* a transitional state of the receptor (E. A. Hyde and B. L. Roth, manuscript in preparation). Additionally, Casey *et al.* (1996) have provided preliminary findings that constitutively active 5-HT_{2A} receptors (R*) have higher agonist affinity than native receptors. It is likely, of course, that this simplified model will not ultimately be suitable for describing 5-HT_{2A} receptor-ligand-G protein interactions and that more complex kinetic schemes will be discovered that more accurately describe ligand-receptor interactions. For rhodopsin, the model G protein-coupled receptor, several transition states before receptor activation have been identified (Stewart *et al.*, 1975; Hamdorf and Kirschfeld, 1980), so it will not be surprising if analogous processes occur with 5-HT_{2A} receptors and other G protein-coupled receptors.

For 5-HT_{2A} receptors, only a few other studies have examined the effects of mutations on high- and low-affinity states and signal transduction processes. Wang *et al.* (1993) demonstrated that a mutation at Asp-120 (in the rat 5-HT_{2A} receptor) abolished both signal transduction and the ability of the 5-HT_{2A} receptor to be regulated by guanine nucleotides. Similar results have been obtained for the *beta* adrenergic receptor (Strader *et al.*, 1988).

Johnson *et al.* (1994) investigated the effects of several mutations in transmembrane region V that altered the structure-activity relationships of N^1-substituted ergolines and tryptamines. They reported that one mutation (A242V) apparently abolished the high-affinity state but did not alter the ability of 5-HT or 5-methoxytryptamine to augment PI hydrolysis. These results are in accord with our findings that mutations may alter the number of high- and low-affinity sites without affecting second messenger production.

How can this model be used to directly clarify our findings obtained with mutant receptors? According to this model, mutations may be constructed that have discrete effects on ligand affinity for the R and R* forms of the receptor (*e.g.*, K_H and K_L, which correspond to k and βk in the model), the equilibrium between these two states (*e.g.*, percentages of high- and low-affinity states; J parameter) and/or the ability of G proteins to directly bind to the receptor (M parameter). Thus, for several tryptamines, second messenger production is abolished (R*→R*G transition, governed by the M parameter) by the F340L mutation but the R→R* transition is relatively unaffected (*e.g.*, percentages of high- and low-affinity states present; J parameter). In the same manner, the data of Wang *et al.* (1993) can be explained by suggesting that the D120N mutation selectively abolishes the R*→R*G transition (M parameter).

Likewise, for compounds like quipazine, for which only one state of the receptor is measured, interaction (according to this model) can only be with the R* form at the native receptor. An alternative explanation is that the R→R* transition for quipazine is rapid, relative to ligand binding, and only one affinity state is measured. The model also predicts that DOM binds to the R* form of the F340L mutant, because only one affinity state was seen and the receptor was still coupled to PI hydrolysis. Additionally, the model predicts that quipazine interacts only with the R* form of the receptor with the F340L mutant but that the F340L mutant interferes with G protein coupling. Our assumption is that the single affinity state of quipazine corresponds to its high intrinsic efficacy, an assumption that will need to be tested in further experiments.

Testing additional predictions of this model is quite difficult, because we have no way of measuring conformational changes of receptor proteins. Kobilka and co-workers, however, were able to demonstrate such transition states with purified *beta* adrenergic receptors (Gether *et al.*, 1995). Experiments currently underway in which large quantities of 5-HT_{2A} receptors are being purified may help to clarify these issues; we have obtained preliminary evidence that purified 5-HT_{2A} receptors can directly interact with purified $G_{\alpha q}$ proteins but that this interaction depends on the presence of magnesium (E. A. Hyde and B. L. Roth, manuscript in preparation).

The results with the phenylisopropylamines are somewhat more difficult to explain, because each compound was differentially affected by the F340L mutation. For DOM and DOB, the F340L mutation appears to alter the R*→R*G interaction, whereas for DOI the effect is primarily on ligand binding affinity (k constant), because efficacy was not affected. These results suggest that a single point mutation can have multiple effects on ligand binding and efficacy, depending on the ligand used and the assay conditions selected.

In conclusion, we demonstrate that the relationship between high-affinity agonist binding states and second messenger production is more complicated than previously suggested for 5-HT_{2A} receptors. Our data are in accord with models that suggest that intermediate state(s) of the receptor (R*) are formed before agonist-induced activation of PI hydrolysis and that single point mutations may independently alter multiple steps of receptor-ligand activation.

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