Evidence for a Model of Agonist-induced Activation of 5-Hydroxytryptamine 2A Serotonin Receptors That Involves the Disruption of a Strong Ionic Interaction between Helices 3 and 6

5-Hydroxytryptamine 2A (5-HT2A) receptors are essential for the actions of serotonin (5-hydroxytryptamine; 5-HT) on physiological processes such as vascular smooth muscle contraction, platelet aggregation, perception, and emotion. In this study, we investigated the molecular mechanism(s) by which 5-HT activates 5-HT2A receptors using a combination of approaches including site-directed mutagenesis, molecular modeling, and pharmacological analysis using the sensitive, cell-based functional assay R-SAT. Allosteric-scanning mutagenesis of residues close to the intracellular end of H6 of the 5-HT2A receptor implicated glutamate Glu-318(6.30) in receptor activation, as also predicted by a newly constructed molecular model of the 5-HT2A receptor, which was based on the x-ray structure of bovine rhodopsin. Close examination of the molecular model suggested that Glu-318(6.30) could form a strong ionic interaction with Arg-173(3.50), localized in the interface between the third transmembrane segment and the second intracellular loop (2). A direct prediction of this hypothesis, that disrupting this ionic interaction by an E318(6.30)R mutation would lead to a highly constitutively active receptor with enhanced affinity for agonist, was confirmed using R-SAT. Taken together, these results predict that the disruption of a strong ionic interaction between transmembrane helices 3 and 6 of 5-HT2A receptors is essential for agonist-induced receptor activation and, as recently predicted by ourselves (B. L. Roth and D. A. Shapiro (2001) Expert Opin. Ther. Targets 5, 685–695) and others, that this may represent a general mechanism of activation for many, but not all, G-protein-coupled receptors.

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David A. Shapiro‡, Kurt Kristiansen‡, David M. Weiner§, Wesley K. Kroese‡, and Bryan L. Roth‡

From the ‡Department of Biochemistry, Case Western Reserve University Medical School, Cleveland, Ohio 44106-4935, §ACADIA Pharmaceuticals, Inc., San Diego, California 92121 and the Departments of Neurosciences and Psychiatry, University of California at San Diego, La Jolla, California, and the Department of Pharmacology, Institute of Pharmacy, University of Tromsø, Tromsø N-9037, Norway

5-Hydroxytryptamine 2A (5-HT2A)1 receptors are essential for the actions of serotonin (5-hydroxytryptamine; 5-HT) on a number of key physiological processes including platelet aggregation, vascular and nonvascular smooth muscle contraction, perception, and emotion (1). Additionally, 5-HT2A receptors represent a major site of action of hallucinogens such as lysergic acid diethylamide, which are agonists, and atypical antipsychotic drugs such as clozapine, which are antagonists (2). 5-HT2A receptors are unique in that both agonists and antagonists induce receptor internalization (3) that is dynamin-dependent and arrestin-independent (4). Despite considerable study (5–9), the molecular and atomic mechanisms by which 5-HT induces activation of the 5-HT2A receptor or of the other 15 cloned 5-HT receptors are currently unknown.

Prior studies have suggested several potential models of agonist-induced activation of 5-HT receptors in particular and other G-protein-coupled receptors (GPCRs) in general. Initial site-directed mutagenesis studies of the 5-HT2A (10) and dopamine-adrenergic receptors (11, 12) implicated a negatively charged residue in transmembrane helix 3 (H3), which could form a strong interaction with positively charged/polar residues in H7 ("H3-H7 interaction model"). Another model ("H2-H7 proximity model") (7) suggested that hydrogen bonding interactions between H2 and H7 were most important for the activation of 5-HT2A receptors and gonadotrophic hormone receptors (14). Predictions based on the H2-H7 proximity model suggest that this could be a general model for GPCR activation (14, 15). More recent studies have predicted that agonist-induced activation of rhodopsin and β-adrenergic receptors (16, 17) and muscarinic m5 receptors (18, 19) occurs via agonist-induced rotations of H6 and H3 ("H3-H6 rotation model"). This model was quite recently modified based on examination of the crystal structure of rhodopsin (20) and extended to include disruption of a strong ionic interaction between residues in H6 and H3 ("H6-H3 interaction model") (21, 41). Indeed, the H6-H3 interaction model has recently been supported by mutagenesis and modeling studies of the β2-adrenergic receptor (21).

In recent studies (6, 9), we demonstrated that the H3-H7 interaction model does not adequately describe agonist-induced 5-HT2A receptor activation. Instead, we have proposed (6, 22) that agonists interact with H6 via aromatic residues and that this interaction facilitates H6 motion and subsequent receptor activation. We have also predicted (41) that agonist binding to

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1 The abbreviations used are: 5-HT2A, 5-hydroxytryptamine 2A; 5-HT, 5-hydroxytryptamine; TM, transmembrane; PI, phosphoinositide; GPCR, G-protein-coupled receptor; WT, wild type; DOI, 2,5-di-methoxy-4-iodophenyl-2-aminopropane.
residues in H6 leads to the disruption of a strong ionic interaction between H3 and H6. In the current study, we test this “H3-H6 interaction model” via a combination of site-directed mutagenesis and molecular modeling studies. Functional expression via R-SAT (receptor selection and amplification technology) was used to characterize the effects of receptor mutations on basal, agonist-independent receptor activity. Taken together, these results are consistent with a model of 5-HT2A receptor activation in which agonists induce a disruption of a strong ionic interaction between H3 and H6, which is facilitated by agonist-induced movements of H6. We also suggest, based on an examination of aligned amino acids, that this is likely to be a widely used, although not universal, mechanism for agonist-mediated activation of G-protein coupled 5-HT receptors.

MATERIALS AND METHODS

Receptor Numbering Schemes—Where appropriate, amino acid residues have been labeled both by standard amino terminus-based numbering and parenthetically by a numbering scheme introduced in 1995 by Ballesteros and Weinstein (23) in which relative amino acid positions are highlighted. This scheme facilitates the efficient comparison of residues within different GPCRs. With this method, every amino acid identifier starts with the transmembrane helix number and is followed by the position relative to a reference residue (arbitrarily assigned the number 50) among the most conserved amino acids in that transmembrane helix. In the seven-transmembrane (TM) GPCRs, this generalized numbering scheme utilizes Asn-1.50, Asp-2.50, Arg-3.50, Tyr-4.50, Pro-5.50, Pro-6.50, and Pro-7.50 as seven reference positions, corresponding in rat 5-HT2A receptors to Asn-246, Asp-338, Arg-377, respectively. Phe-340, for example, lies corresponding in rat 5-HT2A receptors to Asn-92, Asp-120, Arg-173, Trp-200, respectively. The extreme N- and C-terminal parts of the long third intracellular loop segments.

Methods for inactive models of the bovine rhodopsin (20) as a template. By using the WHATIF program (28), amino acid residues in these parts of the bovine rhodopsin structure (chain A) were substituted to their rat 5HT2A receptor counterparts. According to the mutation data in Ref. 6, the rotational orientation of helix 5 was modified such that the Phe-340(5.44) side chain pointed away from the binding site and the Phe-243(5.47) and Phe-5.48 side chains pointed into the binding site. A model of the rat 5-HT2A receptor was constructed by using computer graphics visualizations, for Biased Probability Monte Carlo simulations (27), and for calculation of accessible surface area. A model of the rat 5-HT2A receptor was constructed by using computer graphics and molecular mechanics energy calculations. The model included all parts of the amino acid sequence (residue ranges as follows: Leu-711(1.29)–Thr-266(5.70) and Met-312(6.24)–Gln-398(7.71)) with the exception of the amino-terminal segment and most of the carboxyl-terminal and third intracellular loop segments.

An initial model comprising the seven transmembrane helices, the short loop between H1 and H2, a putative cytoplasmic helix after H7, and part of the second extracellular loop was built by using the x-ray structures of GPCRs. The N-terminal and third intracellular loop segments.

Energy Gradient Difference between Successive Minimization Steps. A model of the rat 5-HT2A receptor was constructed by using computer graphics and molecular mechanics energy calculations. The model included all parts of the amino acid sequence (residue ranges as follows: Leu-711(1.29)–Thr-266(5.70) and Met-312(6.24)–Gln-398(7.71)) with the exception of the amino-terminal segment and most of the carboxyl-terminal and third intracellular loop segments.

For radioligand binding assays, cells were switched 24 h after transfection to Dulbecco’s modified Eagle’s medium containing 5% dialyzed fetal calf serum, and 24 h later they were switched to serum-free Dulbecco’s modified Eagle’s medium. Cells were then harvested 24 h later for radioligand binding assays, which were performed as previously described (4, 6). P1 hydrolysis experiments were performed exactly as previously detailed (4, 6). Receptor expression was verified concurrently with binding experiments by immunohistochemistry using an antibody specific for the 5-HT2A receptor.

RESULTS

Alanine-scanning Mutagenesis Identifies Residues in H6 That Alter Agonist-induced 5-HT2A Receptor Activation—In initial studies, we performed alanine-scanning mutagenesis of 12 residues (Ser-316(6.28)–Val-328(6.40)) at the i3/i6 inter-
All calculations were obtained using the software program Ligand for tors. Values represent the means of three or more experiments from HEK 293 cells transiently expressing WT or mutant receptors. Values represent the means of three or more experiments ± S.E. All calculations were obtained using the software program Ligand for tors. Values represent the means of three or more experiments ± S.E.

Table I

| Mutant | Kd (nM) | Bmax (fmol/mg) | Kd (μM) |
|--------|---------|----------------|---------|
| WT 2A  | 0.47 ± 0.09 | 172 ± 14 | 0.90 ± 0.17 |
| S316A | 0.46 ± 0.05 | 412 ± 21 | 1.2 ± 0.06 |
| N317A | 0.43 ± 0.07 | 357 ± 21 | 2.1 ± 0.59 |
| E318A | 0.28 ± 0.08 | 1137 ± 136 | 0.29 ± 0.05 |
| Q319A | 0.67 ± 0.10 | 491 ± 34 | 1.1 ± 0.49 |
| K320A | 3.17 ± 0.89 | 105 ± 10 | 1.5 ± 0.32 |
| G322A | 0.57 ± 0.14 | 285 ± 20 | 0.38 ± 0.11 |
| K323A | 0.74 ± 0.18 | 180 ± 16 | 0.54 ± 0.17 |
| V324A | 0.89 ± 0.17 | 306 ± 24 | 0.25 ± 0.10 |
| L325A | 0.80 ± 0.09 | 324 ± 9.7 | 0.10 ± 0.01 |
| G326A | 0.52 ± 0.08 | 272 ± 16 | 0.29 ± 0.07 |
| I327A | 0.62 ± 0.10 | 219 ± 15 | 0.26 ± 0.13 |
| V328A | 0.28 ± 0.02 | 728 ± 36 | 0.62 ± 0.37 |

*Significantly different from WT (p ≤ 0.05).

The residues at positions 6.33–6.37 are assumed to be localized at a mixed hydrophobic-hydrophilic environment at the lipid/water interface as proposed from spin labeling experiments with rhodopsin (30). As shown in Table I, all of these mutant receptors were expressed as functional receptors as measured by [125I]-DOI binding, although some variations in receptor expression were measured. Immunochemical studies also verified receptor expression (not shown). Table I also shows that all mutants retained low micromolar or submicromolar affinities for 5-HT, although most mutants had slightly increased (E318(6.30)A, C322(6.34)A, V324(6.36)A, L325(6.37)A, G326(6.38)A, and I327(6.39)A) or decreased (N317(6.29)A, Q319(6.31)A, K320(6.32)A) or decreased (E318(6.30)A, C322(6.34)A, V324(6.36)A, V328(6.40)A); 2) mutations that diminished the potency of 5-HT-stimulated PI hydrolysis (N317(6.29)A, Q319(6.31)A, K320(6.32)A); 3) mutations that reduced the efficacy of 5-HT-stimulated PI hydrolysis (L325(6.37)A); and 4) mutations that had no effect on 5-HT-stimulated PI hydrolysis (S316(6.28)A, K320(6.35)A, G326(6.38)A, I327(6.39)A). Visualization of the locations of these residues in a 5-HT2A receptor model (model 1, Fig. 2a) suggests that where mutation to alanine enhances agonist potency, it probably does so via distinct mechanisms. Among these, Glu-318(6.30)A appeared to form an ionic interhelical interaction with Arg-173(5.50), whereas the hydrophobic residues had their side chains directed toward hydrophobic residues in H5 (Cys-322(6.34)A) or H7 (Val-324(6.36), Val-328(6.40)).

The side chains of all residues where mutation to alanine decreased potency are predicted to be involved in intrahelical hydrogen bonding interactions in model 1 (Fig. 2b, pre-MD simulation). The side chain CO moiety of Glu-318(6.31)A interacted with the NH2 moiety of Lys-320(6.32), whereas the NH2 moiety of Asn-317(6.29)A interacted with one carboxylic oxygen atom of Glu-318(6.30). In model 2 of the wild type receptor, the Asn-317(6.29)A side chain interacted with residues at the junction between H7 and the cytoplasmic helix (OH moiety of Thr-386(7.59) and main chain NH moiety of Lys-385(7.58)), whereas the Lys-320(6.32)A side chain interacted with residues in H5 (main chain CO moiety of Ala-265(5.69) and the side CO moiety of Glu-318(6.30)).

All residues where mutation to alanine did not affect 5-HT-stimulated receptor activation (Ser-316(6.28), Lys-320(6.35), Ile-327(6.39)A) were localized on the membrane-facing surface of H6 (Fig. 2b). The side chain of Leu-325(6.37)A, where mutation to alanine decreased efficacy of 5-HT-stimulated PI hydrolysis, was predicted to be localized in the cavity formed between the transmembrane helices 2, 3, 6, and 7.

Molecular Modeling Predicts a Strong Ionic Interaction between H3 and H6 of the 5-HT2A Receptor—The localizations in model 1 of the various residues of the 5-HT2A receptor that

Table II

| Mutant | Kmax (nM) | Vmax (% of WT) |
|--------|----------|---------------|
| WT 2A  | 18.1 ± 1.2 | 101.2 ± 4.1 |
| S316A | 36.8 ± 9.0 | 92.0 ± 5.1 |
| N317A | 91.5 ± 20.6* | 91.6 ± 13.0 |
| E318A | 4.0 ± 1.5* | 88.5 ± 14.1 |
| Q319A | 47.0 ± 17.2* | 71.3 ± 6.7 |
| K320A | 13.4 ± 17.5* | 67.8 ± 9.3 |
| C322A | 7.9 ± 0.20 | 87.0 ± 3.6 |
| K323A | 10.4 ± 2.4 | 70.2 ± 16.6 |
| V324A | 6.7 ± 1.2* | 94.3 ± 19.3 |
| L325A | 39.0 ± 4.3 | 81.9 ± 8.2* |
| G326A | 7.7 ± 1.7 | 90.1 ± 11.4 |
| I327A | 22.6 ± 5.1 | 77.1 ± 6.8 |
| V328A | 0.54 ± 0.04* | 92.3 ± 11.1 |

*Significantly different from WT (p ≤ 0.05).
were targeted for alanine-scanning mutagenesis are shown in Fig. 2, a and b. In this model, the carboxylate moiety of Glu-318(6.30) was involved in 1) salt bridge/hydrogen bonding interactions with the two NH2 moieties of Arg-173(3.50) and 2) a hydrogen bonding interaction with the NH2 of Asn-317(6.29), respectively (Fig. 2c). In model 2, the interaction between Arg-173(3.50) and Glu-318(6.30) side chains was purely electrostatic and did not involve any direct hydrogen bonding interactions (Fig. 2d). During the molecular dynamics simulation, the side chains of Arg-173(3.50) and Glu-318(6.30) took part in a network of hydrogen bonding interactions involving polar side chains in H2, H6, and H7. The OH moiety of Thr-109(2.39) interacted through hydrogen bonding interactions involving polar residues having hydrogen bonding interactions (Fig. 2), and V328(6.40) respectively (Fig. 2e). During the molecular dynamics simulation, the side chains of Arg-173(3.50) and Glu-318(6.30) took part in a network of hydrogen bonding interactions involving polar side chains in H2, H6, and H7. The OH moiety of Thr-109(2.39) interacted with the COO− moiety of Glu-318(6.30) and the side chain of Arg-173(3.50) as a hydrogen bond donor and acceptor, respectively. In addition, the side chain of Arg-173(3.50) interacted through hydrogen bonding interactions with the backbone CO group of Glu-318(6.30), whereas the COO− moiety of Glu-318(6.30) interacted through hydrogen bonding interactions with the NH2 moiety of Asn-107(2.37), the OH moiety of Thr-386(7.59), and the NH2 moiety of Asn-384(7.57).

**Molecular Modeling of Wild Type and Mutant 5-HT2A Receptors Reveals Differences in Hydrogen Bonding Interactions**—Comparison of models 1 of wild type and the R173E(3.50)/E318R(6.30) double mutant receptors revealed that the arginine side chain adopted different conformations at positions 3.50 and 6.30 (Fig. 2c). In the wild type receptor, the R173(3.50) side chain adopted a conformation that allows both of its NH2 moieties to form hydrogen bonds to the carboxylate moiety of Glu-318(6.30). In contrast, the Arg-318(6.30) side chain adopted a conformation that allows its NH/NH2 face to interact with E173(3.50) in the double mutant. This hydrogen bonding/salt bridge interaction was also present in model 2 of the double mutant, the side chain of Arg-318(6.30) had hydrogen bonding interactions with the backbone CO and side chain OH moieties of Ser-314(6.26) (Fig. 2f).

In the energy-minimized average conformation of V328(6.40)A, the OE1 atom of the Glu-318(6.30) side chain had hydrogen bonding interactions with Arg-173(3.50) and Thr-109(2.39), whereas the OE2 atom of Glu-318(6.30) had hydrogen bonding interactions with Asn-317(6.29) and Lys-385(7.58) (Fig. 2g). Comparison of the highly constitutively active mutants V328(6.40)E and E318(6.30)R with the other constructs (models 2) revealed important differences in hydrogen bonding interactions in the polar pocket between helices 1, 2, and 7. The Asp-120(2.50) side chain attained conformations that allowed it to interact with the NH2 moiety of both Asn-92(1.50) and Asn-376(7.49) in the models of V328(6.40)A and E318(6.30)R and with the NH2 moiety of Asn-376(7.49) and the backbone moiety of Thr-88(1.46) and Ser-373(7.46) in the model of the double mutant (R173(3.50)/E318(6.30)R).

**Hydrophobic Type Interactions between Transmembrane Helices**—It is possible that interhelical interactions between hydrophobic residues may also contribute to constraining the wild type receptor to its inactive state conformation. The side chains of three hydrophobic residues in H6 where alanine substitution induces activation formed strong Van der Waals interactions with residues in adjacent helices in model 1 (Cys-322(6.34): Arg-173(3.50), Ile-177(3.54), Tyr-254(5.58), and Ile-258(5.62); Val-324(6.36): Val-379(7.52), Phe-383(7.56), and Asn-384(7.57); Val-328(6.40): Leu-113(2.43), Leu-166(3.43), Asn-376(7.49), and Val-379(7.52)). Comparison of the different models obtained by energy minimization of the average over the last 500 ps in molecular dynamics trajectories (models 2) revealed that the surface exposure of the Val-324(6.36) side chain differed markedly (ratio of accessible surface of Val-324(6.36) relative to standard exposed surface for the residue type as follows: R173(3.50)/E318(6.30)R: 0.06; WT: 0.23; V328(6.40)A: 0.27, E318(6.30)R: 0.49). In model 2 of the double mutant, the Val-324(6.36) side chain was buried among Lys-323(6.35), Leu-325(6.37), Val-379(7.52), Tyr-380(7.53), Asn-384(7.57), and Phe-383(7.56) close to the intracellular end of H6 and H7.

**Mutation Analysis of Residues Predicted to Form a Strong Ionic Interaction**—To test the prediction of the modeling studies that Arg-173 and Glu-318 formed a strong ionic interaction, R173(3.50)/E and E318(6.30)R as well as a double mutant con-
Data are plotted as a function of from three separate experiments each with triplicate determinations. The double mutant E318(6.30)R/R173(3.50)E reversely, the E318(6.30)R mutation produced a 23-fold decrease severely inhibited by the R173(3.50)E mutation (Table III), Receptor activation, as measured by PI hydrolysis, was most was detectable to below 0.5 hydrolysis activity). Basal, agonist-independent PI hydrolysis E318(6.50)R mutant produced a detectable concentration-de- (tiated) level of PI hydrolysis was measured (Fig. 3). Only the transfect HEK cells was performed, and the basal (unstimu-
ated) level of PI hydrolysis was measured (Fig. 3). Only the E318(6.50)R mutant produced a detectable concentration-de-pendent increase in basal PI hydrolysis (i.e. constitutive PI 
dependent increase in basal PI hydrolysis activity). Basal, agonist-independent PI hydrolysis increased with increasing DNA transfection concentration and was detectable to below 0.5 μg of DNA transfected per well of a six-well culture plate. Untransfected HEK 293 cells displayed less than 225 cpm (background) under the assay conditions used (data not shown).

R-SAT Assays Confirm PI Activation Data and Accurately Predict Constitutive Activity of Native and Mutant 5-HT2A Receptors—R-SAT assays were then used to further define the functional effects of the various 5-HT2A receptor mutants. Seven selected receptor constructs representing the interface between H3 and the i2 loop (R173(3.50)E), H6 (N317(6.29)A, E318(6.30)A, E18(6.30)R, K320(6.32)A, and V328(6.40)A) and the double mutant (R173E/E318R) were chosen for R-SAT analysis, and their respective potencies for the full agonist serotonin, the full inverse agonist ritanserin, and their corre-
sponding degrees of constitutive signaling were determined. A titration of the cDNAs used to transfect NIH3T3 cells as part of the R-SAT assay was performed. As depicted in Fig. 4a, each receptor mutant displayed a distinct and quantifiable degree of basal signaling, which increased in a DNA concentration-de-
pendent manner. As depicted in Fig. 4 and Table IV, the WT 5-HT2A receptor displayed a moderate degree of basal signaling (18 ± 2%) in this assay. Mutagenesis of R173(3.50)E created a significantly impaired receptor characterized by a complete loss of basal signaling and a 60-fold decrease in potency for serotonin (Fig. 4b and Table IV). The opposite phenotype was observed for the E318(6.30)R mutant, which displayed profoundly enhanced biological activity. This receptor was nearly fully activated in the absence of 5-HT (89 ± 11% basal activity), precluding accurate determinations of functional po-
tency for serotonin. Interestingly, this mutant receptor displayed a 6.4-fold decrease in functional potency for the inverse agonist ritanserin (Table IV). The double mutant R173(3.50)E/E318(6.30)R displayed an intermediate phenotype, with a functional potency for serotonin 7.6-fold greater than the R173(6.30)E mutant yet 9.3-fold less than wild type and a measurable but minimal degree of basal activity (3.8 ± 2%). As depicted in Fig. 4 and Table IV, the four remaining TM6 mu-
tants displayed a range of constitutive activity phenotypes. The N317(6.29)A and K320(6.32)A mutants displayed a slight loss of function with 5.0- and 2.2-fold decreased potency for sero-
tonin, respectively, coupled with a mild decrease in basal sig-
naling. The E318(6.30)A and V328(6.40)A mutants displayed substantial and graded gain of function phenotypes character-
ized by increased potencies for serotonin (1.7- and 33-fold, respectively) and decreased potencies for ritanserin (1.5- and 9.5-fold, respectively). These receptor mutants displayed sig-
nificant increases in basal signaling from the 18% seen with the wild type receptor to 45 and 76%, respectively. In summary, the rank order of constitutive activity of these 5HT2A receptors as measured in this assay was R173(3.50)E < R173E/E318R double mutant < N317(6.29)A < K320(6.32)A < WT ≪ E318(6.30)A ≪ V328(6.40)A = E318(6.30)R.

DISCUSSION

The main findings of this study are that 1) a strong ionic interaction (i.e. “salt bridge”) between Arg-173(3.50) in H3 and Glu-318(6.30) in H6 of the 5-HT2A receptor stabilizes the inactive state of the receptor, 2) disruption of this interaction leads to constitutive activation of the 5-HT2A receptor, 3) hydrophobic interactions also appear to stabilize the inactive state of the 5-HT2A receptor, and 4) functional expression of directed H3 and H6 mutants that disrupt these normal inter-
actions can affect receptor expression as well as dramatically affect potency for receptor agonists and inverse agonists.

In this study, we examined the effect of mutation of the residues in the N-terminal part of H6 on receptor activation in an attempt to determine the extent to which the residues in this region play a role in receptor activation. It has been pre-
viously suggested that residues in the third intracellular loop of the 5-HT2A receptor may be responsible for interactions with G-proteins (G_i) and the transmission of subsequent effector function (1), although direct tests of this hypothesis have not been made previously.

The data from Table II suggest that a number of residues in this region (Asn-317(6.29), Glu-318(6.30), Gln-319(6.31), Lys-
320(6.32), Cys-322(6.34), Val-324(6.36), Leu-325(6.37), and Val-328(6.40)) all participate in the regulation of receptor ac-
tivity. Our current working model hypothesizes that the recep-
tor is stabilized in a “ground” or inactive state by interaction of a number of charged and noncharged residues. Our model also predicts that agonist binding is accompanied by a rotation of TM6, leading to a subsequent rotational movement of H6 and disruption of key stabilizing ionic interactions between resi-
dues of H6 and residues at the i2/H3 interface, although the current study does not provide biochemical confirmation of this prediction. Of the residues mutated in this study, those that decreased potency after mutation to alanine are all predicted to
be involved in intra- and interhelical hydrogen bonding interactions. Such intra- and interhelical hydrogen bonding interactions might play an important structural role in stabilizing active state conformations. We cannot exclude the possibility that the N317(6.29)A, Q319(6.31)A, and K320(6.32)A mutations specifically disrupt hydrogen bonding interactions with the Gq protein. Studies in progress predict that portions of the i3 loop may directly interact with Gq.

The (E/D)RY motif near the cytoplasmic end of TM3 is highly conserved among the seven-transmembrane GPCRs. Arg-3.50 is almost fully conserved in family 1 GPCRs (Arg in 98% of the sequences in the tGRAP10 family 1 alignment). There is now a growing body of evidence suggesting that Arg-173(3.50) plays an important role in activation of several GPCRs (15, 21, 31–33), although without a defined mechanism. In support of our current hypothesis, and in direct agreement with our findings, R135(3.50)W and R135(3.50)L mutant rhodopsin receptors were found to be spectrally normal (each bound 11-cis-retinal and was converted to the alternate spectral form with \( \lambda_{\text{max}} = 380 \text{ nm} \) upon illumination) but were defective in the signal transduction pathway (32). Related findings were reported for the α2-adrenergic receptor (31), in which D142(3.49)A caused significant constitutive activity. The effect of direct alanine mutation of Arg-3.50 on the activity of

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**FIG. 4.** Constitutive activity of 5-HT2A receptor mutants revealed by R-SAT. Reciprocal mutations of the H3 and H6 drastically affect 5HT2A receptor basal signaling properties. a, constitutive activity as a function of DNA concentration. NIH-3T3 cells were transiently transfected with 0.5–50 ng/well receptor DNA as part of the R-SAT assay. Constitutive activity was defined as (basal response — full inverse agonist response) (full agonist response — full inverse agonist response) shown to reach maximal levels at ~25 ng/well receptor DNA. Data are derived from 3–5 separate experiments. S.E. bars have been omitted for clarity but were all less than 15%. b, representative concentration response curves are shown for the full agonist serotonin (filled squares) and the inverse agonist ritanserin (filled diamonds) for each of the receptor mutants assessed at 25 ng/well of plasmid DNA. Data are plotted as the percentage response observed in the R-SAT assay, defined as full agonist response/full inverse agonist response. The data are from nine-point concentration-response curves performed in duplicate. Control (no drug treatment) values are depicted on the left of each graph.

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2 W. K. Kroeze and B. L. Roth, manuscript in preparation.
3 A detailed alignment of GPCRs can be found on the World Wide Web at tGRAP.uit.no/fam1w.html.
the α1µ-adrenergic receptor was, however, undetermined. More convincingly, direct mutation of the homologous residue in gonadotropin-releasing hormone receptor to Gln, His, or Lys severely inhibited agonist-induced inositol phosphate generation (15). Finally, in a most recent analysis of homologous residues in the β2-adrenergic receptor, the authors propose direct interaction of Arg-131(3.50) with Glu-268(6.30) and the neighboring Asp-130(3.49) (21). Together, these findings suggest an important role for Arg-173(3.50) in GPCR activation that appears to be conserved across all receptor families. E318(6.30)A showed considerable constitutive receptor activity by R-SAT (45% of the maximum stimulable activity), eclipsed only by V328(6.40)A (76%) and E318(6.30)R (89%). Both of the tested E318(6.30) mutants had 1) enhanced affinity for agonist binding, 2) enhanced potency for activation by 5-HT (as measured by PI hydrolysis and R-SAT), and 3) increased basal activity in both assay systems, all hallmarks of constitutive activity. Interestingly, only mutations in the 5HT2A receptor that induced profound constitutive activity (>70%, E318R and V328A) displayed decreased functional potencies for the full inverse agonist ritanserin. R-SAT confirmed the basal signaling properties of the mutant receptors and proved its utility for the measurement of inverse agonist responses in those receptors for which routine measurements of constitutive activity are unobtainable. The sensitivity of the R-SAT technology has already proven useful in identifying novel psychotherapeutic agents (e.g. antipsychotics) that differ more in their inverse agonist than antagonist properties (34).

Interestingly, the E318(6.30)R mutation elicited greater constitutive activity than E318(6.30)A, suggesting that a direct polar repulsion between this residue and Arg-173(3.50) is at least partially responsible for the constitutive phenotype. Results obtained with the E318(6.30)R mutant further suggest that, within the guidelines of allowable motion of H6, additional rotation of this segment away from the ground state conformation leads directly to increased receptor activity. The double (reciprocal) mutant E318(6.30)R/R173(3.50)E had a much reduced potency of 5-HT-stimulated PI hydrolysis and minimal constitutive activity (3%) compared with the wild type receptor. This could be explained by differences in the hydrogen bonding interactions observed between the WT and the reciprocal mutant during molecular dynamics simulations (Fig. 2, d and e) or after Monte Carlo simulations (Fig. 2c). During molecular dynamics simulations, stronger salt bridge/hydrogen bonding interactions between H3 and H6 were observed in the double mutant than in the wild type receptor. The Arg-318(6.30) side chain of the double mutant interacted with the Asp-173(3.49) and Glu-173(3.50) side chains (Fig. 2e), whereas the Arg-173(3.50) side chain of the wild type receptor interacted with Glu-318(6.30) (Fig. 2d). The R173(3.50)E mutant had also the characteristics of an inactive state conformation. Interhelical interactions between E3.50 and other residues may lock this mutant in the inactive state.

According to the present mutagenesis and modeling data, it is likely that interactions between hydrophobic residues in helix 6 (Cys-322(6.34), Val-324(6.36), Val-328(6.40)) and helices 5 and 7 also contribute to the stabilization of inactive state conformations. Amino acid substitutions at position 6.34 in rat 5-HT2A (35), rat 5-HT2C (36), and other family A receptors (e.g. Refs. 37–39) have been shown to lead to constitutive activity. Site-directed mutagenesis experiments with the Gαs-coupled A1A1 angiotensin receptor have identified I245(6.40)L as one of 16 single point substitutions that induce constitutive activity (40). Finally, random saturation mutagenesis of TM6 in the muscarinic m5 receptor revealed a periodicity of mutants that induce constitutive receptor signaling, leading the authors to conclude that TM6 is involved in stabilizing the off state of the receptor and that rotational changes in this portion of the molecule were critical to receptor activation (18, 19, 25). Our systematic mutagenesis and modeling studies confirm that minor changes in the side chain volumes at the interfaces between helices 6 and 7 and between helices 6 and 5 promote constitutive receptor activation.

The molecular dynamics simulations of wild type and mutant receptors also suggested that the transition from inactive to active state conformations may involve a slight increase in distance between the intracellular halves of H6 and H7. Interestingly, the side chain of the highly conserved Asp-120(2.50) changed conformation in the simulations of the constitutively active mutants (V328(6.40)A and E318(6.30)R), leading to rearrangement in the hydrogen bonding interactions at the polar pocket between helices 1, 2, and 7. This observation supports previously incompletely explained findings from site-directed mutagenesis studies, which suggested that both the Asp-120 and Asn-376 side chains are essential for 5-HT2A receptor activation (7).

In conclusion, these studies imply that the ground (inactive) state of the 5-HT2A receptor is stabilized by both ionic and novel hydrophobic interactions involving residues in the intracellular half of H6. A strong ionic interaction between Glu-318(6.30) and Arg-173(3.50) was confirmed to be a major factor in stabilizing the ground state of the 5-HT2A receptor. Finally, R-SAT technology was used to precisely quantify the constitutive activity of native and mutant GPCRs, providing insight into the structural requirements for receptor activation.

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REFERENCES
1. Roth, B. L., Willins, D. L., Kristiansen, K., and Kroeze, W. K. (1998) Pharmacol. Ther. 78, 231–257
2. Roth, B. L., Lopez, E., Patel, S., and Kroeze, W. K. (2000) Neuron 25, 252–262
3. Roth, B. L., and Willins, D. L. (1999) Neuron 25, 629–631
4. Bhatnagar, A., Willins, D. L., Gray, J. A., Woods, J., Benovic, J. L., and Roth, B. L. (2001) J. Biol. Chem. 276, 8269–8277
5. Almala, N., Ebersole, B. J., Zhang, D., Weinstein, H., and Sealfon, S. C. (1996) J. Biol. Chem. 271, 14672–14675
6. Shapiro, D. A., Kristiansen, K., Kroeze, W. K., and Roth, B. L. (2000) Mol. Pharmacol. 58, 877–886
7. Sealfon, S. C., Chi, L., Ebersole, B. J., Rodie, V., Zhang, D., Ballesteras, J. A., and Weinstein, H. (1995) J. Biol. Chem. 270, 16683–16688
8. Choudhary, M. S., Craigo, S., and Roth, B. L. (1993) Mol. Pharmacol. 43, 755–761
9. Kristiansen, K. R., Kroeze, W. K., Willins, D. L., Gelber, E. I., Savage, J. E.,
10. Wang, C. D., Gallaher, T. K., and Shih, J. C. (1993) *Mol. Pharmacol.* **43**, 931–940

11. Porter, J. E., and Perez, D. M. (1999) *J. Biol. Chem.* **274**, 34535–34538

12. Porter, J. E., Hwa, J., and Perez, D. M. (1996) *J. Biol. Chem.* **271**, 28318–28323

13. Robinson, P. R., Cohen, G. B., Zhukovsky, E. A., and Oprian, D. D. (1992) *Neuron* **9**, 719–725

14. Zhou, W., Flanagan, C., Ballesteros, J. A., Konvicka, K., Davidson, J. S., Weinstein, H., Millar, R. P., and Sealfon, S. C. (1993) *Mol. Pharmacol.* **43**, 931–940

15. Ballesteros, J. A., Kitanovic, S., Guarnieri, F., Davies, P., Fromme, B. J., Konvicka, K., Chi, L., Millar, R. P., Davidson, J. S., Weinstein, H., and Sealfon, S. C. (1998) *J. Biol. Chem.* **273**, 10445–10453

16. Gether, U., Lin, S., Ghanouni, P., Ballesteros, J. A., Weinstein, H., and Kobilka, B. K. (1997) *EMBO J.* **16**, 6737–6747

17. Kobilka, B. K., and Gether, U. (1998) *Adv. Pharmacol.* **42**, 470–473

18. Spalding, T. A., Burstein, E. S., Wells, J. W., and Brann, M. R. (1997) *Biochemistry*** 36**, 10109–10116

19. Burstein, E. S., Spalding, T. A., and Brann, M. R. (1998) *J. Biol. Chem.* **273**, 24322–24327

20. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, R. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) *Science*** 289**, 739–745

21. Ballesteros, J. A., Jensen, A. D., Liapakis, G., Rasmussen, S. G., Shi, L., Gether, U., and Javitch, J. A. (2001) *J. Biol. Chem.* **276**, 29171–29177

22. Roth, B. L., Shoham, M., Choudhary, M., and Khan, N. (1997) *Mol. Pharmacol.* **52**, 259–266

23. Balint, C. J. A., and Weinstein, H. (1995) *Methods Neurosci.* **25**, 366–428

24. Price, R. D., Weiner, D. M., Chang, M. S. S., and Sanders-Bush, E. (2001) *J. Biol. Chem.* **276**, 44663–44668

25. Spalding, T. A., Burstein, E. S., Henderson, S. C., Ducote, K. R., and Brann, M. R. (1998) *J. Biol. Chem.* **273**, 21563–21568

26. Cornwell, W. D., Ciapik, P., Bayly, C. I., Gould, I. R., Merz, K. M. J., Ferguson, D. M., Spellmeyer, D. C., Fox, T., Caldwell, J. W., and Kollman, P. A. (1995) *J. Am. Chem. Soc.* **117**, 5179–5179

27. Abagyan, R., and Totrov, M. (1994) *J. Mol. Biol.* **235**, 983–1002

28. Friend, G. (1980) *J. Mol. Graph.* **8**, 52–56

29. Guex, N., and Peitsch, M. C. (1997) *Electrophoresis*** 18**, 2714–2723

30. Cornell, W. D., Ciaplak, P., Bayly, C. I., Gould, I. R., Merz, K. M. J., Ferguson, D. M., Spellmeyer, D. C., Fox, T., Caldwell, J. W., and Kollman, P. A. (1995) *J. Am. Chem. Soc.* **117**, 5179–5179

31. Porter, J. E., and Perez, D. M. (1999) *J. Biol. Chem.* **274**, 34535–34538

32. Porter, J. E., Hwa, J., and Perez, D. M. (1996) *J. Biol. Chem.* **271**, 28318–28323

33. Robinson, P. R., Cohen, G. B., Zhukovsky, E. A., and Oprian, D. D. (1992) *Neuron* **9**, 719–725

34. Zhou, W., Flanagan, C., Ballesteros, J. A., Konvicka, K., Davidson, J. S., Weinstein, H., Millar, R. P., and Sealfon, S. C. (1993) *Mol. Pharmacol.* **43**, 931–940

35. Ballesteros, J. A., Jensen, A. D., Liapakis, G., Rasmussen, S. G., Shi, L., Gether, U., and Javitch, J. A. (2001) *J. Biol. Chem.* **276**, 29171–29177

36. Roth, B. L., Choudhary, M., and Khan, N. (1997) *Mol. Pharmacol.* **52**, 259–266

37. Price, R. D., Weiner, D. M., Chang, M. S. S., and Sanders-Bush, E. (2001) *J. Biol. Chem.* **276**, 29171–29177

38. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, R. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) *Science*** 289**, 739–745

39. Ballesteros, J. A., Jensen, A. D., Liapakis, G., Rasmussen, S. G., Shi, L., Gether, U., and Javitch, J. A. (2001) *J. Biol. Chem.* **276**, 29171–29177

40. Lattion, A., Abuin, L., Nenniger-Tosato, M., and Cotecchia, S. (1999) *FEBS Lett.* **457**, 302–306

41. Paron, C., Bardin, S., Miserey-Lenkei, S., Guedin, D., Corvol, P., and Clauser, E. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 7615–7620

42. B. L. Roth and D. A. Shapiro (2001) *Expert Opin. Ther. Targets* **5**, 685–695