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Structural Basis for Ligand Binding and Specificity in Adrenergic Receptors: Implications for GPCR-Targeted Drug Discovery†

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ABSTRACT: Crystal structures of engineered human β_2-adrenergic receptors (ARs) in complex with an inverse agonist ligand, carazolol, provide three-dimensional snapshots of the disposition of seven transmembrane helices and the ligand-binding site of an important G protein-coupled receptor (GPCR). As expected, β_2-AR shares substantial structural similarities with rhodopsin, the dim-light photoreceptor of the rod cell. However, although carazolol and the 11-*cis*-retinylidene moiety of rhodopsin are situated in the same general binding pocket, the second extracellular (E2) loop structures are quite distinct. E2 in rhodopsin shows β-sheet structure and forms part of the chromophore-binding site. In the β_2-AR, E2 is α-helical and seems to be distinct from the receptor's active site, allowing a potential entry pathway for diffusible ligands. The structures, together with extensive structure−activity relationship (SAR) data from earlier studies, provide insight about possible structural determinants of ligand specificity and how the binding of agonist ligands might alter receptor conformation. We review key features of the new β_2-AR structures in the context of recent complementary work on the conformational dynamics of GPCRs. We also report 600 ns molecular dynamics simulations that quantified β_2-AR receptor mobility in a membrane bilayer environment and show how the binding of an agonist ligand, adrenaline (epinephrine), causes conformational changes to the ligand-binding pocket and neighboring helices.

HISTORICAL CONTEXT OF RESEARCH ON G PROTEIN-COUPLED RECEPTORS

The cAMP second messenger system was discovered and reported almost exactly 50 years ago by E. W. Sutherland and T. W. Rall (*1, 2*). Their work provided a biochemical underpinning explaining the physiological effects of hormones and led to the hypothesis that a specific cell-surface receptor might mediate the effect they observed. Adrenergic receptors were subsequently isolated, characterized, and cloned, and precise biochemical signaling pathways were elucidated (*3*). Almost entirely in parallel, the biochemistry and molecular biology of the vertebrate visual system were elucidated. In the case of the visual system, the photoreceptor, rhodopsin, was understood in considerable detail even before the second messenger in the rod cell, cGMP, was identified in 1985 (*4, 5*). In a landmark report in 2000, a high-resolution crystal structure for rhodopsin, from bovine rod cells, was reported (*6*). And now, finally, high-resolution crystal structures of engineered monoaminergic receptors, the human β_2-adrenergic receptor (AR),[1] have been reported (*7*−*9*) as

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well, which provide a unique opportunity to suggest how ligand binding modulates receptor activity.

Although perhaps now almost taken for granted, the existence of a superfamily of so-called seven-transmembrane (7-TM) or heptahelical receptors that couple to heterotrimeric G proteins [G protein-coupled receptors (GPCRs)] became clear only during a frenzy of cloning and bioinformatics analysis over a 15-year period culminating in the sequencing of the human genome. The importance of heptahelical receptors in "druggable" target space[2] is not in dispute. According to Overington et al. (*10*), of the 266 human targets for approved drugs, a remarkable 27% correspond to rhodopsin-like, or family A, GPCRs. And since, to paraphrase Sir James W. Black, the most likely path to a new drug is to start with an existing drug, GPCRs will remain hot as drug targets in the foreseeable future, especially since more than 100 of the 726 heptahelicals encoded by the human genome remain "orphan" receptors[3] and most of these are expressed in the central nervous system.

THE PROBLEM AT HAND

GPCR signaling complexes are allosteric machines. Agonist receptor ligands outside of the cell induce guanine nucleotide exchange on a G protein inside the cell where the ligand and nucleotide binding sites are ≥8−10 nm apart. To describe the pharmacology of a drug (ligand), the concepts of affinity and efficacy are used. Affinity refers to the ability of the drug to bind to its molecular target, whereas efficacy refers to the ability of a drug to induce a biological response in its molecular target. Partial agonists exhibit less efficacy compared to (full) agonists. Neutral antagonists do not change the basal activity of the receptor (zero efficacy), and inverse agonists are able to stabilize the inactive state of the receptor; i.e., they exhibit inversion of efficacy. Although it has been studied in great detail by a number of approaches, it could be argued that the structural (and thermodynamic) basis of ligand recognition by monoaminergic receptors has remained a largely unsolved problem.

Initially, in classical work, medicinal chemistry and pharmacology generated a vast inventory of structure−activity relationship data. Receptor subtypes were defined by specific ligands (mostly antagonists) and validated in tissue physiology models, if available. With the cloning and heterologous expression of receptors (and chimeric/mutant receptors), a second generation of experiments led to mapping of ligand-binding sites on receptors representing members of the main pharmacological targets (muscarinic, α- and β-adrenergic, dopamine, and serotonin receptors). However, only relatively few subtypes were analyzed in this way.

The third generation of experiments was focused on mutagenesis-based structure determination for mapping the interactions between the highly conserved residues on TM helices, which together with the bacteriorhodopsin structure and the available three-dimensional low-resolution cryo-electron microscopy projection structures of rhodopsin, resulted in the first models of the TM domains of a larger series of homologous receptors. The fourth generation of experiments might be defined as those focused on the dynamics of receptor activation, culminating in the "helix movement model of receptor activation" and some level of understanding of the structural basis for constitutive receptor activity and constitutively activating mutations (CAMs) (*11*−*15*).

Today, with the dawning of a new generation of structural studies, we are finally able to compare and contrast the high-resolution structure of rhodopsin with that of engineered β_2-ARs on the background of deduced primary structures of a substantial number of related receptors from various genomes. The human genome alone contains more than 40 monoaminergic GPCRs, including the new class of trace amine-associated receptors (TAARs) (*16*). All of them bind endogenous ligands with a β-aryl-ethylamine as the common structure (one can argue that the acetyl group of acetylcholine is a minimal "aryl"). The "monoamine" histamine is probably acting as a "diamine" dication in the binding pocket, stabilized by a second counterion in TM helix 5 (*17*). The residues in the binding pocket of these receptors are not conserved between the families, and their phylogenetic relationships (*16*) probably do not help much in attempting to understand their ligand specificities. In fact, as discussed below, many drugs originally believed to be receptor-specific have been found to be promiscuous.

PHARMACOLOGICAL IDENTITY CRISIS

Screening for exquisitely selective drugs (*18*) may lead to new drugs with fewer side effects. However, highly receptor selective drugs may not be the most effective therapeutic choices, especially for complex disorders with complex and largely obscure molecular pathophysiology, like CNS-mediated mood disorders. Therefore, first-choice drugs are often nonselective ["dirty" (*19*), promiscuous (*18*)] drugs with less stringent receptor selectivity, but with more peripheral (and often adverse) effects. Newer "chimeric" drugs with two or more simultaneous targets, like risperidone and clozapine in the central nervous system or carvedilol and labetalol in the peripheral vascular system, are bridging the gap between specific and nonselective drugs. While the therapeutic significance of non-β-blocking properties of carvedilol and labetalol has been controversial, at least with atypical antipsychotics, the mix of metabotropic acetylcholine/dopamine D2/dopamine D4/serotonin 2A receptor affinities led to some slow progress (*20*). One could speculate that advances in the biophysics and molecular biology of GPCRs might facilitate rational development of "selectively nonselective drugs" (*18, 19*).

One central question is why there are fewer agonist than antagonist drugs. Are the screening methods insufficient for identifying and optimizing new agonist leads, or is it simply too difficult to estimate the relevant in vivo activity? Or perhaps it is because we do not know enough about the G protein taxonomy in the target tissue and, therefore, do not know the extent to which the "high-affinity" form of the ternary ligand−receptor−G protein complex can be formed (*21*). And the situation becomes even more complicated as

[1] Abbreviations: AR, adrenergic receptor; CAMs, constitutively activating mutations; DHA, dihydroalprenolol; E2, second extracellular loop; FRET, fluorescence resonance energy transfer; GPCR, G protein-coupled receptor; IpBABC, *p*-(bromoacetamido)benzyl-1-[^{125}I]iodocarazolol; LCP, lipidic cubic phase; MD, molecular dynamics; PWR, plasmon-waveguide resonance studies; SAR, structure−activity relationship; TAARs, trace amine-associated receptors; TEV, tobacco etch virus; 7-TM, seven-transmembrane.

[2] Druggable target space can be defined as the potential total universe of biological targets for which drugs might be developed.

[3] Orphan receptors are expressed but have no known exogenous ligand, although such a ligand or ligands are presumed to exist.

former antagonists turn out to be inverse agonists, or sometimes even agonists (case in point, chlorpromazine is an agonist for TAAR-1) (*22*).

Drugs thought to act on a single receptor sometimes turn out actually to act on multiple targets. For example, dopamine and amphetamines both activate TAAR-1, which in turn leads to phosphorylation of the dopamine transporter, resulting in efflux of stored dopamine from the cytosol (*23*). Therefore, this $G\alpha_s$-coupled receptor functionally antagonizes the action of the $G\alpha_i$-coupled presynaptic dopamine autoreceptor D_2-short (*24*). In this case, agonism on TAAR-1 and antagonism on D_2-short would provide a synergistic mechanism for chlorpromazine!

The therapeutic effects of drugs that seemingly act on a single target may actually be due to them acting synergistically on several targets. The obvious consequence of this dilemma of multiple receptors responding to a single ligand, and of a single receptor responding to multiple ligands, is that the definition of a pharmacological receptor can be difficult (*25*). Moreover, the pharmacological differentiation of homologous receptor molecules can be difficult or impossible with the set of ligands currently available, at least to the academic community. The question is how can new GPCR structures, which enable structure-based (biophysical) computational methods, contribute to the drug discovery process. More specifically, can basic principles of ligand recognition be developed and used not only to discover and optimize receptor selective drugs but also to design a new generation of multitarget or promiscuous drugs? However, we first need the structures.

CRYSTAL STRUCTURES OF HUMAN β_2-AR

Rasmussen et al. (*9*) reported the crystal structure of an engineered human β_2-AR that consists of a construct modified at the N-terminus by addition of an HA signal sequence followed by a FLAG epitope. The receptor is truncated after position 365 so that it lacks 48 amino acid residues at its C-terminus. A glycosylation site was also removed by site-directed mutagenesis (N187E). A second receptor construct was also crystallized that contained a tobacco etch virus (TEV) protease cleavage site, so that the receptor lacked 24 amino acids from the N-terminus after proteolysis. The receptor clones were expressed in Sf9 insect cells, solubilized in dodecyl maltoside detergent, and purified by successive antibody affinity and ligand (alprenolol) affinity chromatography. N-Linked glycosylation was removed by glycosidase treatment, and the FLAG epitope was removed where possible with the AcTEV protease, an enhanced form of TEV protease. The purified protein was relatively stable, but only approximately one-half of the purified receptors appeared to be functional and capable of ligand binding.

In parallel work, a monoclonal antibody was developed that binds to the C3 loop of the native receptor, but not to denatured receptor protein. Fab fragments were prepared and reacted with the purified receptor, which was further stabilized by carazolol, an inverse agonist ligand with picomolar affinity. The resulting complex was purified by size exclusion chromatography and mixed with bicelles composed of the phospholipid DMPC and the detergent CHAPSO suitable for crystallization trials. Long thin plate-like crystals were cryoprotected, and an entire data set was collected from a single crystal. Although the initial resolution was ~3.0 Å, the diffraction was somewhat anisotropic and the resolution of the final model was 3.4 Å, in the plane of the putative membrane, and 3.7 Å, perpendicular to the plane of the membrane. The final structures (one for each of the two constructs; PDB entries 2R4R and 2R4S) were determined using molecular replacement using the immunoglobulin domain search models for the Fab.

Several regions of the receptor are either unresolved in the crystal structure or obscured by the Fab fragment. In particular, the C-terminal tail region is missing from the engineered receptor construct and the C3 loop is bound to the Fab fragment. However, the binding of the Fab apparently did not affect agonist or antagonist binding and did not prevent the conformational changes concomitant with receptor activation. The ligand-binding site can be identified as an extended flat structure near the extracellular receptor surface; however, the carazolol itself is not resolved, and the active site of the receptor is not seen in detail. The Fab itself and the TM helices near the cytoplasmic surface of the receptor are the best resolved regions of the structure.

Cherezov et al. (*7*) engineered a β_2-AR with the same modifications in the N- and C-terminal tails and the same N187E substitution. However, instead of using an Fab fragment to stabilize the receptor, they created a fusion protein by inserting a synthetic gene encoding a slightly modified version of the enzyme T4 lysozyme between Ile233(5.72) and Arg260(6.22). The β_2-AR−T4 lysozyme fusion construct was also expressed in Sf9 insect cells and purified by a method very similar to that of Rasmussen et al. The engineered receptor construct crystallized in bicelles, and as observed by Rasmussen et al., the system could not be optimized to provide resolution beyond ~3.4 Å. Therefore, lipidic cubic phase (LCP) crystallization trials were performed using in meso crystallization robotics (*26*). Final diffraction data from more than 40 crystals were considered in gathering a full data set at complete 2.4 Å resolution. Initial phases of the construct were obtained by molecular replacement using both T4 lysozyme and a polyamine model of rhodopsin as search models. Most likely due to the use of the LCP method, the quality of the structure reported by Cherezov et al. (PDB entry 2RH1) is superior to that reported by Rasmussen et al.

Excellent quality electron density is observed for residues 29−342 of the 365-amino acid full-length construct. Two disulfide bonds, Cys106(3.25)−Cys191(5.30) and Cys184(4.76)−Cys190(5.29), are observed, and a palmitic acid linked to Cys341 is clearly visible in the $F_o - F_c$ omit maps. Importantly, the active site contains a well-resolved carazolol ligand. The borders of all of the TM helices and loops can be defined from the structure, except, of course, where the T4 lysozyme was introduced into the C3 loop. Interestingly, a short helical segment near the middle of the E2 loop is seen, which is not present in rhodopsin and was also not predicted by automated secondary structure prediction algorithms. Interactions between residues of the T4 lysozyme and receptor components are discussed in some detail (*7*). Basically, there are minimal intermolecular interactions between the T4 lysozyme component of one molecule and the receptor component of a neighboring molecule in the LCP crystal lattice, and it is argued that these interactions cause no particular structural perturbation.

It is informative to compare and contrast the structures of

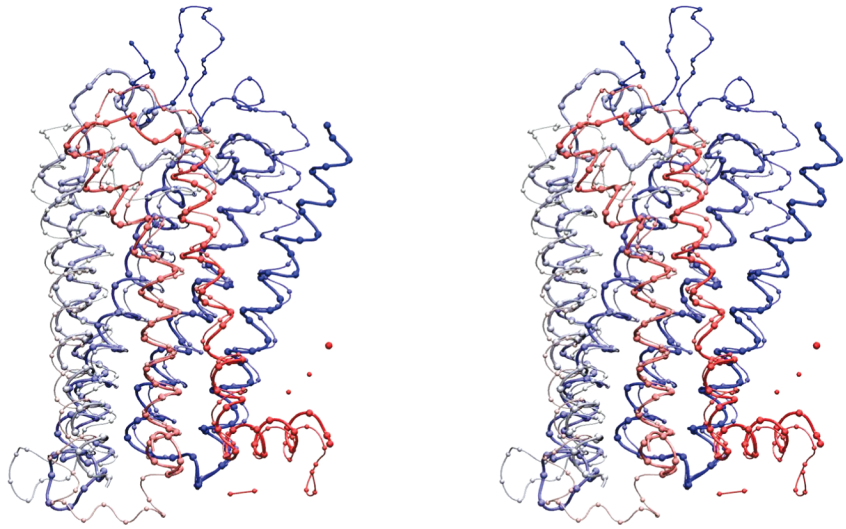

FIGURE 1: Structural alignment of the β_2-AR (*7, 8*) and rhodopsin (*78*) (PDB entries 2RH1 and 1GZM). The DaliLite algorithm (*79*) results in a well-defined solution of the three-dimensional structure-based sequence alignment problem. The protein backbones are shown as spline curves (thick for β_2-AR and thin for rhodopsin), color-coded from the N-terminus (blue) to the C-terminus (red). The Cα atoms are represented as spheres. Cross-eyed stereo presentation generated with VMD1.8.6 (*80*). The T4 lysozyme (T4L) structure that replaces part of cytoplasmic loop C3 has been omitted for the sake of clarity. Note the striking similarity of TM helices 1−7 and amphipathic helix 8.

rhodopsin and the β_2-AR. The N-terminus of the β_2-AR is disordered, whereas that of rhodopsin is resolved clearly, interacts extensively with extracellular domains, and forms a small four-strand β-sheet in concert with the E2 loop, which essentially blocks access to the retinal-binding site (*27*). In the β_2-AR, a short helical segment on the E2 loop, which includes a disulfide-bonded Cys, sits well above the carazolol binding site. Carazolol and retinal are situated in similar binding sites in the core of the helical bundle, but near the extracellular surface. We observe that the TM helical segments of rhodopsin and the β_2-AR superimpose very precisely, except for TM helix 1 (Figure 1). TM helix 1 of the β_2-AR, despite containing a Pro not found in rhodopsin, is straight and angled away from the axis of the receptor by ~18°.

The functional properties of the expressed β_2-AR−T4 lysozyme fusion construct were studied in detail (*8*). In summary, the construct bound the antagonist [^{3}H]dihydroalprenolol (DHA) and the inverse agonist ICI-118,551 with the same affinities as the native expressed receptor. However, the affinities of the engineered construct for agonists (isoproterenol, epinephrine, and formoterol) and partial agonist (salbutamol) were 2−3-fold higher than those of the native receptor. A shift in agonist affinity is associated with constitutive receptor activity, and it is possible that the engineered receptor conformation, at least in membranes, is partially active. The engineered receptor fusion could not bind to heterotrimeric G proteins. However, studies of a receptor construct containing a fluorescent probe linked to Cys265(6.27) at the cytoplasmic end of TM helix 6 were consistent with a partial active conformational equilibrium.[4] Finally, when compared with the structure of the β_2-AR determined in complex with the Fab fragment, one prominent difference is the altered packing of Phe264 at the cytoplasmic end of TM helix 6 in the fusion receptor. In the β_2-AR−Fab complex, the interactions between Phe264 and residues in TM helices 5 and 6 and the C2 loop may be important for keeping the β_2-AR in its basal "off" state (*8*).

TOWARD A FUNDAMENTAL UNDERSTANDING OF LIGAND BINDING SPECIFICITY IN GPCRs

How can the β_2-AR crystal structure be used to gain a better understanding of the thermodynamics of ligand−receptor interactions? What experimental data are available? What are the most important missing links?

Both engineered β_2-adrenergic receptors were crystallized in the presence of the inverse agonist (*S*)-(−)-carazolol, which is seen in the active site of both of the structures, although it is well resolved only in the structure of the receptor fused to T4 lysozyme. Interestingly, (*S*)-(−)-carazolol has the same configuration in its ethanolamine moiety as the classical receptor agonist (*R*)-(−)-adrenaline. One obvious question is whether adrenaline itself would be stable in fundamentally the same receptor structural conformation found when it binds carazolol. At a minimum, the receptor must be able to distinguish between two similar bound structures, those of the inverse agonist and the agonist. Two residues in particular, Asn312(7.39) and Asn293(6.55), have been implicated in explaining ligand binding specificity in the β_2-AR. It has been shown that substitutions of Asn312(7.39) modify the ligand binding specificity of the β_2-AR, resulting in a reduction in binding affinity for typical antagonists in the N312T mutant receptor (*28*). The second Asn residue, Asn293(6.55), is involved in stereospecific recognition of agonist and receptor activation, whereas the stereoselectivity for antagonists was not affected by mutations of this residue (*29*). The stereoselective recognition of (*R*)-(+)- and (*S*)-(−)-carazolol on β-AR in frog erythrocyte membranes has been determined in terms of dissociation rate and equilibrium constants (*30*). The stereoisomers of carazolol demonstrate approximately 5-fold tighter binding of (−)-carazolol to the β-AR in frog erythrocytes (*30*) compared with 20-fold tighter binding for metoprolol and 100-fold tighter binding for propranolol isomers in the human β_2-AR (*29*). Unfortunately, the primary structure of the frog

[4] The term "partial activity" can refer to a shift in the equilibrium between two states, one inactive and one active, or a shift in the distribution of conformational substates that contribute to specific measurable biological activities.

erythrocyte β-AR is not available, and it might be useful to clone this receptor to compare these detailed binding studies on a homology model based on the crystal structure.

Alternatively, one could characterize binding of both carazolol isomers on the β_2-AR−T4 lysozyme protein (β_2-AR−T4L). An indication that carazolol binding to the frog receptor might be different from its binding to β_2-AR−T4L is the set of dissociation rates, which are approximately 6 times faster for the frog receptor (*30*) than for β_2-AR−T4L (*8*).

Originally, Asn312(7.39) was thought to form a hydrogen bond with the phenoxy oxygen of common antagonists (propranolol and alprenolol) (*28*). Exchange of the Phe at a homologous position in the α_2-AR(F412N) dramatically enhances the sensitivity toward β-antagonists and reduces the sensitivity toward the α-antagonist yohimbine. The α_2-AR(F412N) mutant shows an unchanged binding affinity of isoproterenol and a reduced affinity of adrenaline (*31*). Interestingly, the homologous substitution in the α_1-AR, F312N, does not improve the poor sensitivity toward β-antagonists (propranolol and alprenolol) (*32*). Analysis of the binding of a series of propranolol analogues to the $5HT_{1D\beta}$ receptor and its T355N(7.39) mutant (T342 in human $5HT_{1D}$) demonstrates the role of this Asn residue in recognizing the β-hydroxyl group of these antagonists (*33*). However, simultaneous binding of this residue to the phenoxy oxygen seems improbable considering the crystal structure. It would be very interesting to obtain dissociation constants for these propranolol analogues on the β_2-AR, or preferentially, β_2-AR−T4L. Such an experiment could form the basis for a detailed molecular dynamics (MD) computer simulation study on the key pharmacophores used as therapeutic β-blockers.

Despite the importance of Asn7.39 in the recognition of the β-hydroxyl group of propranolol-type antagonists, the stereospecific recognition of the β-hydroxyl group of adrenaline analogues has been shown to be due to Asn293(6.55). The β_2-AR(N293L) mutants show indistinguishable stereospecificity for antagonists (propranolol, alprenolol, and metoprolol), but there is an approximately 6-fold reduction in the stereoselectivity toward the agonist isoproterenol (*29*). These mutagenesis results were used to guide earlier receptor computational modeling studies that used rhodopsin as a structural template for the β_2-AR (*34*). A model of *p*-(bromoacetamido)benzyl-1-[^{125}I]iodocarazolol (IpBABC) in the binding pocket of the β_2-AR incorrectly placed the β-hydroxyl group hydrogen near Asn293(6.55) (*34*). The orientation of carazolol in this model is upside down compared with its orientation in the crystal structure presented here, probably a result of an attempt to position the β-hydroxyl group in a position similar to that of epinephrine, also shown in the same paper. Likewise, Rosenbaum et al. tried to model isoproterenol in the binding pocket of the β_2-AR with its β-hydroxyl group hydrogen bound to Asn312(7.39), similar to the binding mode of carazolol in the crystal structure (*8*). In this binding mode, the catechol hydroxyl groups are too far from Ser203(5.42), Ser204(5.43), and Ser207(5.46), which are known to be involved in catechol ring hydroxyl group binding (*35, 36*).

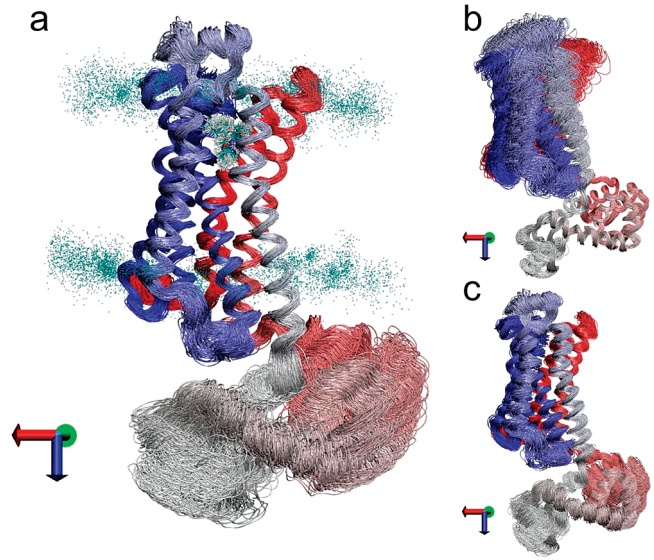

FIGURE 2: MD simulations of a model derived from the crystallographically resolved structure of β_2-AR−T4L. The all-atom model contains the inverse agonist carazolol, a POPC bilayer membrane, and a salt solution with a physiological ionic strength, a total of ~50000 atoms. The DaliLite-generated structural alignment was used to place the β_2-AR at the position of rhodopsin in a previously equilibrated model including a POPC bilayer membrane (*81*). Four independent copies of this system were simulated for 60 ns each using NAMD2.6 (*82*). Shown are structural superpositions of the resulting protein backbone conformations, sampled every nanosecond, using the β_2-AR (a), T4 lysozyme (b), or both domains (c) as reference structures for the rotational fit. This representation indicates that the β_2-AR and T4 lysozyme are rather rigid structures by themselves. Aligning both domains together gives an impression of unrealistically high mobility. In panel a, the cyan spheres show the membrane−water interface by sampling the positions of the glycerol backbone of the surrounding phospholipids. The binding pocket for carazolol (shown as sticks) in the receptor is relatively deep within the bilayer.

MD SIMULATION OF ADRENALINE (EPINEPHRINE) BINDING

To investigate the mode of binding of catecholamines in the context of the new structures, we employed MD simulations. First, we carried out an MD simulation to place the receptor bound to carazolol in a typical bilayer membrane environment (Figure 2). Next, to address the question of whether adrenaline itself would be stable in fundamentally the same receptor structural conformation as when it binds carazolol, we carried out additional simulations (Figure 3 and Figure 11 of the Supporting Information). Our MD model of adrenaline in the binding pocket begins with a conformation restrained to match the carazolol binding mode of the ethanolamine moiety. After a short simulation run to relax any steric overlap, the restraints were removed. To our surprise, our simulation revealed no stable contacts between Asn312(7.39) and the β-hydroxyl and amino groups sufficient to fixate or stabilize the ligand. The hydrogen bond between Asn312 and the β-hydroxyl group breaks almost immediately, and the catechol hydroxyl groups move toward TM helix 5. Many conformations are probed by the ligand, which is rather mobile in the ligand-binding pocket. Ser204(5.43) sometimes binds a water molecule, which allows secondary indirect hydrogen bonds to reach into the binding pocket. This water is further stabilized by Tyr209(5.48). Ser204(5.43) in this way comes close to the *m*-hydroxyl

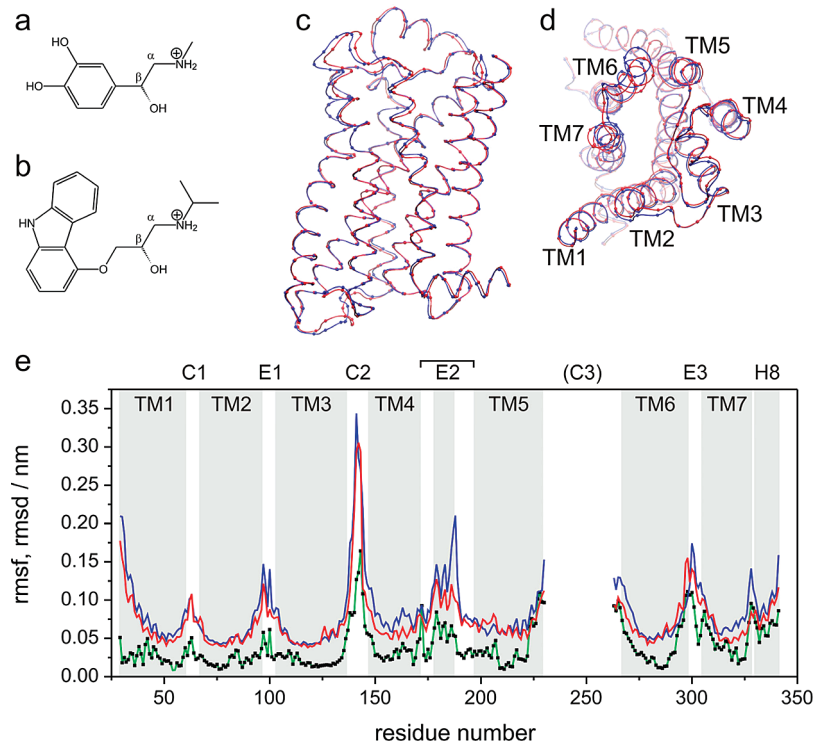

FIGURE 3: MD simulations of agonist- and antagonist-bound receptor models. Chemical structures of (a) the natural agonist (*R*)-(−)-adrenaline (epinephrine) and (b) the antagonist (inverse agonist) (*S*)-(−)-carazolol. The ethanolamine moieties of both compounds have the same stereochemical configuration. The basic β-aryl-ethylamine moiety is common to the endogenous ligands of the monoaminergic GPCRs (including the acetyl group of acetylcholine as a minimal aryl). The topologies including hydrogens for the ligands carazolol and epinephrine were generated using the MOLEFACTURE1.1 module of VMD1.8.6. The force field parameters for the ligands were estimated making standard transferability assumptions (see the Supporting Information). (c and d) Ensemble-averaged receptor backbone conformation of 170 ns simulations of the carazolol form (blue) and 500 ns simulations of the adrenaline form (red). Changing the inverse agonist to the agonist results in outward movement of TM helix 7 and inward movements of TM helices 5 and 6. In addition, the E2 loop, including the short helix, exhibits some movement. (e) Comparison of the root-mean-square fluctuations of the carazolol-bound (blue) and adrenaline-bound (red) receptors which indicates similar mobility of both states. The root-mean-square deviations between the two forms are shown as black squares and a green line net and demonstrate the movement of the backbone induced by the agonist. The movement of TM helices 6 and 7, including the E3 loop, is as large as 0.1 nm. In addition, the Cα atom of Ser207(5.46) in TM helix 5 moves ~0.05 nm toward the catechol hydroxyl groups of adrenaline.

group of the catechol ring of the agonists, which it is supposed to recognize (*36*). The missing α-helical hydrogen bond at Pro211(5.50) appears to generate a water binding site together with the side chain of Glu122(3.41), which was modeled in the protonated form in the MD model. This water-binding site initiates a string of water molecules hydrating Ser207(5.46) and Ser203(5.42) and potentially mediating hydrogen bonds to the *p*-hydroxyl group of the agonists.

DYNAMICS OF RECEPTOR ACTIVATION

The nanosecond time scale of our MD simulations is certainly not sufficient for capturing the slow transition to the active receptor conformation. However, a principal component analysis of the movements of the backbone Cα atoms demonstrates a global change of the receptor structure in its transition from the carazolol-bound to adrenaline-bound form. The change is consistent with the elongated structure of the antagonist pushing the extracellular ends of TM helices 2 and 6 outward into the bilayer and of TM helix 7 toward water. In the fluorescently labeled β_2-AR [tetramethylrhodamine-5-maleimide labeled at Cys265(6.27) or monobromobimane labeled at Cys271(6.33)] in detergent, noradrenaline induces a change in fluorescence with biphasic kinetics with half-life times of 2.8 and 70 s for the fast and slow phases, respectively (*37*−*39*). However, the activation kinetics in cyan fluorescent protein/yellow fluorescent protein (CFP/YFP) fusion constructs of the α_2-AR in vivo with comparable ligand concentrations is significantly faster. Noradrenaline induces a conformational change within ~40 ms (*40*), but the time traces appear to contain a second slow phase on a time scale of seconds. Possible reasons for this difference in activation rates compared to that of the β_2-AR have been discussed, for example, the presence of the cell membrane and G proteins in vivo (*41*). The obvious control experiment, activation of α_{2A}-AR−CFP/YFP fusion constructs in vitro under conditions comparable to those of the β_2-AR experiments, has not been reported in the literature.

The other possible control experiment has been reported. Investigation of the construct consisting of C3 loop-FlAsH/carboxyl-terminal (CT)-CFP-β_2-AR in vivo suffers from a poor signal-to-noise ratio and does not allow detailed kinetic analysis. However, additions of (−)-isoproterenol, a synthetic agonist, and ICI 118,551, a selective antagonist, show a rapid response within seconds (*42*). The insufficient quality of these data appears not to be a problem of the FlAsH/CFP pair, since studies on the adenosine A_{2A} receptor demonstrated 5-fold better performance compared with the CFP/YPF pair (*43*). It is possible that a slow association phase in the α_{2A}-AR was not detected in the CFP/YFP studies, since experi-

mental evidence (*44*) indicates that it occurs on a time scale much longer than 30 s, as studied by fluorescence resonance energy transfer (FRET).

Surface plasmon waveguide resonance studies (PWR) of the β_2-AR incorporated into phospholipid membranes indicate multiphase agonist binding kinetics with time constants ranging from seconds to a few minutes (*45*). The kinetic similarity with the detergent studies suggests that phospholipids do not accelerate the agonist action to the millisecond time scale. Phospholipids added to the β_2-AR in detergent increase its thermal stability (*46*), and the β_2-AR in detergent shows normal G protein coupling, as shown for example by formation of a ternary complex on bead-immobilized agonists (*47*). These experiments indicate that GTPγS activation of the ternary complex proceeds with identical kinetics for full and partial agonists, while the extent of complex formation is lower for partial agonists (*47*), indicating that once formed the ternary complex has a similar structure for agonists and partial agonists.

It is noteworthy that a fluorescent analogue of noradrenaline has been described previously (*48*), but the compound appears to be nonfunctional (*49*). The problem might be that the amino group in this compound is converted to an amide, making the ionic interaction with Asp113(3.32) impossible. On the other hand, β_2-AR-specific fluorescent antagonists that maintain the basic nature of the amino group have been synthesized (*50, 51*).

THERMODYNAMICS OF LIGAND BINDING

A classical study of thermodynamic differences between binding of agonists and antagonists has suggested that agonist binding is enthalpy-driven whereas antagonist binding is entropy-driven (*52*). The primary data set was obtained from binding studies in turkey erythrocyte membranes, where the β-AR was originally thought to lack GTP-sensitive high-affinity binding sites. Fortunately, the primary structure of the turkey β-AR is known (*53*), and GTP sensitivity was later demonstrated (*54*). Moreover, follow-up studies have investigated the thermodynamics of ligand binding in the human β_2-AR, including high- and low-affinity states of the receptor (*55, 56*). The results of these studies suggest that passive hydrophobic interactions characterize antagonist binding. It was noted that pindolol-like antagonists deviate from the purely entropy-driven rule. This led to the proposal that the pindolol-type antagonists, which exhibit unusual intrinsic sympathomimetic activity, might actually be partial agonists, and therefore, the favorable enthalpy component would follow the rule (*56*). An alternative explanation for the more negative enthalpy of these ligands could be that the indole nitrogen forms a hydrogen bond with Ser203(5.42)/Ser204(5.43) in TM helix 5. Support for this explanation is the fact that carazolol, which contains almost completely the structure of pindolol, is hydrogen-bonded to the TM helix 5 serines as observed in the crystal structure. MD simulations could test this hypothesis on the basis of binding free energy differences, including their enthalpy/entropy decomposition, for a homologous series of antagonists. Beyond this, the key question that follows is whether this structure can be used to understand agonist binding to the β_2-AR.

BACK TO BASICS

The basic problem can be formulated as follows. The central dogma in GPCR molecular pharmacology is that receptor activation and desensitization are both mediated via strictly stimulus-dependent interaction of the 7-TM receptor with another protein (heterotrimeric G protein, G protein-coupled receptor kinases, and β-arrestins) (*3*). The ternary complex of agonist, receptor, and G protein is believed to trigger allosterically nucleotide exchange on the G protein. The consequence is that agonist binding to the receptor should exhibit allosteric modulation by the G protein. In the absence of the G protein, the binding affinity for agonists is typically reduced (*57*). The question is whether the agonist−receptor complex can formally switch to the active conformation without the G protein.

In rhodopsin, photon capture isomerizes the inverse agonist form of the covalently bound 11-*cis*-retinyledene ligand to the all-*trans* agonist form. Chromophore isomerization results in large conformational changes as demonstrated by several biophysical methods (*58*). It should be noted that rhodopsin also functions as a receptor for diffusible ligands, such as all-*trans*-retinal (*59*), even though its standard function is mediated by the covalently bound ligand, which allows detection of photons. Moreover, in a K296G mutant receptor, an artificial amine ligand binds and activates as a strictly diffusible ligand (*60*).

Experimental evidence of conformational substates of fluorescein maleimide-labeled β_2-AR (Cys265-FM-β_2-AR) has been obtained from single-molecule photon burst measurements, showing agonist-dependent changes. These experiments further suggest that there are at least two distinct substates of the native receptor (*61*). However, these substates of the native receptor could also be explained by the rotamer populations of the fluorescent label. This alternative interpretation is supported by recent MD simulation models of fluorescent labels on proteins that indicate multiple conformations stable on a nanosecond time scale (*62*). Nevertheless, since activation of the receptor changes the substate distribution, the experiments are useful for probing the functional state of the receptor. Changes in the substate distribution were also inferred from fluorescence lifetime distributions of Cys265-FM-β_2-AR, which are sensitive to the pharmacology of drugs (*63*). The labeling position in Cys265-FM-β_2-AR is the endogenous Cys265, located at the cytoplasmic end of TM helix 6.

Receptor activation is accompanied by movement of the cytoplasmic end of TM helix 6, as demonstrated by restraints imposed by engineered metal ion binding sites (*64*) and disulfide bridges (*65*), and by interaction of spin-labels on double cysteine mutants (*65, 66*). Our MD simulations on the nanosecond time scale show no reorganization of the hydrogen bonding network involving the internal water molecules around Asn51(1.50) and Asp79(2.50) upon ligand exchange from the inverse agonist carazolol to the agonist epinephrine. The positions of these water molecules in the MD model are slightly different from those of the crystallographic water molecules initially used to place the internal waters (Figure 4). (A movie showing the location of bulk and internal water molecules, as well as the lipid bilayer, from the MD simulation of the adrenaline-bound receptor is available as Supporting Information.) One possibility for this

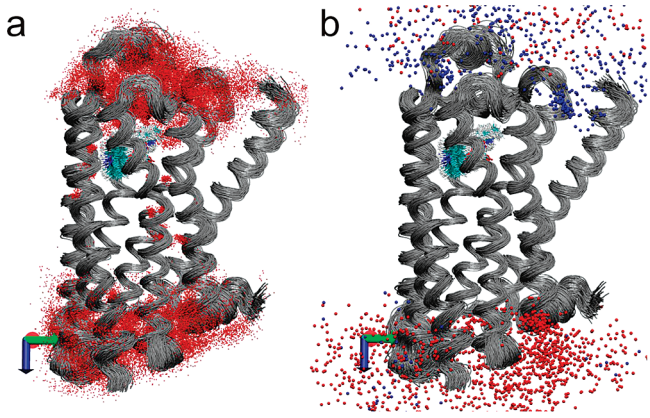

FIGURE 4: Internal water molecules and ionic atmosphere of β_2-AR−T4L from MD simulations. (a) The red spheres represent the position of water oxygens that are separated well from bulk water. The carazolol ligand is show in stick representation and the receptor backbone as a spline through the Cα positions. (b) Sodium (blue) and chloride (red) ions are unevenly distributed, with a preference for sodium for the negatively charged extracellular domain of the receptor. Chloride is enriched around the positively charged cytosolic domain. The structures are sampled in 1 ns intervals of an ensemble with a total length of 170 ns, and the structures are superimposed with the receptor backbone as a reference structure for the rotational fit. The results demonstrate well-localized internal hydration sites and localized sodium density in a putative entrance pathway on the extracellular domain between TM helices 1 and 7. (Stereo presentations are available as Supporting Information. A movie and a set of structural coordinates of the MD simulation of the adrenaline-bound receptor are also available as Supporting Information.)

difference is that Asp79(2.50) was modeled with a protonated side chain. However, this choice appears justified by FTIR experiments, which indicate that the homologous residue in rhodopsin, Asp83(2.50), is protonated in both the inverse agonist and agonist-bound states of rhodopsin and metarhodopsin II, respectively (*67, 68*). The D83N mutation in the β_2-AR has no effect on antagonist binding but reduces agonist affinity by 1−2 orders of magnitude (*69, 70*). Additional MD simulations are certainly necessary for analysis of the effect of the protonation state of Asp79(2.50) on the structure of the internal water molecules.

Asp(2.50) was believed to be part of a putative sodium binding site of the α_2-AR (*71*) and D_2-dopamine receptor (*72*), as mutations abolish regulation of these receptors by sodium. The sodium effect appears to be specific for $G\alpha_i$-coupled receptors (*73*), but as the β_2-AR is known to couple not only to $G\alpha_s$ but also to $G\alpha_i$ (*74*), one might expect to see sodium binding, as well. However, our MD simulations show no evidence of sodium binding to Asp79(2.50), which is not unexpected, since the residue is buried deeply in the TM domain.

Despite little or no change in the cytoplasmic domain, our MD simulations show changes in the extracellular domain. Specifically, TM helix 7 moves outward as Asn312(7.39) abandons its interaction with the amino and β-hydroxyl groups of the ligand. The amino group remains localized at its counterion, Asp113(3.32), but the β-hydroxyl group moves toward TM helix 6. TM helix 6, with Asn293(6.55), moves into the binding pocket, but no long-lived direct hydrogen bonds with the β-hydroxyl group of epinephrine are formed; only water-mediated hydrogen bonds are observed. The catechol hydroxyl groups bind directly or via water coordinated to the serine residues on TM helix 5, which moves slightly inward toward the binding pocket. In summary, these changes revealed in the MD simulation are consistent with mutagenesis data on recognition of a series of homologous agonist structures (*36*).

STRUCTURAL BASIS OF THE ACTIVE STATE

On the basis of the discussion given above, does the crystal structure correspond to that of a constitutively active mutant trapped in the basal state by the inverse agonist carazolol? What are the consequences for ligand binding affinities of computational studies and comparison with literature data? A constitutively active receptor is expected to have higher agonist binding affinities in the absence of G proteins. The β_2-AR−T4L fusion protein, compared with the β_2-AR, exhibits higher affinities for agonists, such as (−)-isoproterenol and (−)-epinephrine (*8*). The experiments were performed in Sf9 membranes in the absence of GTP. In Sf9 membranes, the β_2-AR does not contain sufficient G protein concentrations to exhibit a GTP-sensitive high-affinity receptor form (*57*). The higher agonist affinity is consistent with the constitutive activity of the fusion protein. Moreover, the ligand-dependent changes in fluorescence of bimane-labeled Cys265 indicate an altered basal state (*8*). Interestingly, the crystal structure shows that Glu268(6.30) interacts with T4L instead of Arg131(3.50) in the conserved DRY motif at the cytoplasmic end of TM helix 3 (*8*). Arg131(3.50) interacts with Asp130(3.29) and a sulfate ion, and in the MD simulations, there is evidence of local density of chloride ions. It has been shown that the E268A(6.30) substitution in a β_2-AR−$G\alpha_s$ fusion protein results in higher agonist affinity for the low-affinity state (*75*). On the basis of homology with rhodopsin, the "ionic lock" formed by Asp130(3.49), Arg131(3.50), and Glu268(6.30) is thought to stabilize the inactive receptor conformation. We share the consensus view of Shukla et al. (*76*) and Rosenbaum et al. (*8*) that the crystal structures may in fact represent an "active-like state" or a "partial constitutively active" receptor.

CONCLUDING REMARKS

Opsins, melatonin receptors, and monoaminergic receptors appear to form a local cluster of homologous receptors within rhodopsin-like family A of GPCRs (*16*). Clearly, monoaminergic receptors, which are targets for several major classes of cardiovascular and psychotropic drugs (*77*), will remain a central focus in pharmaceutical research and development (*10, 18*). The challenge now is to develop a systematic approach to rationalizing the specific contributions to the ligand binding free energy of the amino acyl side chains and functional groups of a bound inverse agonist ligand. Medicinal chemistry could contribute substantially with a targeted homologous series of ligand analogues. Molecular biology could contribute with a set of receptor mutants in which the binding site residues are systematically replaced with other amino acids. Classical pharmacology could provide ligand competition data for the various pairs of receptor mutants and ligand analogues. Computational biophysics could simulate the binding energy differences for each substitution and predict potential water binding sites created or removed with each change. The result would be a comprehensive description of the interaction of a prototypi-

cal GPCR with a prototypical aminergic receptor blocker drug. But, of course, none of this is possible without the high-resolution structures now available, and with the hope that additional structures are on the way. Fifty years since Sutherland and Rall, we are finally beginning to understand how it all really works.

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SUPPORTING INFORMATION AVAILABLE

Stereo presentations of the structure figures, a movie, and a set of structural coordinates of the MD simulation of the adrenaline-bound receptor. This material is available free of charge via the Internet at http://pubs.acs.org.

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