Molecular Evolution of Adrenoceptors and Dopamine Receptors: Implications for the Binding of Catecholamines

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We derived homology models for all human catecholamine-binding GPCRs (CABRs; the α-1, α-2, and β-adrenoceptors and the D_1-type and D_2-type dopamine receptor) using the bovine rhodopsin−11-*cis*-retinal X-ray structure. Interactions were predicted from the endogenous ligands norepinephrine or dopamine and from the binding site and were used to optimize receptor−ligand interactions. Similar binding modes in the complexes agree with a large "binding core" conserved across the CABRs, that is, D3.32, V(I)3.33, T3.37, S5.42, S(A/C)5.43, S5.46, F6.51, F6.52, and W6.48. Model structures and docking simulations suggest that extracellular loop 2 could provide a common attachment point for the ligands' β-hydroxyl via a hydrogen bond donated by the main-chain NH group of residue xl2.52. The modeled CABRs and docking modes are in good agreement with published experimental studies. Complementarity between the ligand and the binding site suggests that the bovine rhodopsin structure is a suitable template for modeling agonist-bound CABRs.

Introduction

G-protein coupled receptors (GPCRs)[a] are cell-surface receptors that, upon binding of an extracellular ligand or photoisomerization of bound 11-*cis*-retinal, activate an intracellular heterotrimeric G-protein with concomitant activation of stimulatory or inhibitory signaling pathways as the case may be. The catecholamine-binding GPCRs (CABRs) form a group of rhodopsin-like GPCRs, composed of adrenoceptors (ARs) and dopamine receptors (DRs). The CABRs play key physiological roles in the nervous and cardiovascular systems, and one or more members of the family are often the protein targeted by molecules designed to alleviate health problems in humans.

ARs are endogenously activated by epinephrine (adrenaline) and norepinephrine (noradrenaline) (here, we will refer to norepinephrine and epinephrine, with a methyl group attached to the positively charged amine, collectively as (nor)epinephrine). ARs are divided into three main classes (Figure 1A), and in human they are further divided into three subtypes each: α_1 (α_{1A}, α_{1B}, α_{1D}), α_2 (α_{2A}, α_{2B}, α_{2C}), and β (β_1, β_2, β_3). DRs, activated by dopamine, are divided into two classes in human (Figure 1A): D_1-type receptors, herein collectively referred to as D_1's, composed of D_{1A} and D_{1B} (also named D_1 and D_5), and D_2-type receptors (referred to as D_2's), having three subtypes, D_2, D_3, and D_4 (as yet there is no standard designation for these classes). The total subtypes for some receptor classes differ in nonmammals (e.g., fish), and duplicated CABRs have been found in fish.[1−3] In human, splice variants have been observed for the α_1-ARs, and for D_2 and β_3-AR.[4−6]

In the only three-dimensional (3D) structures of a GPCR solved so far, bovine rhodopsin in the inactive form,[7,8] the ligand 11-*cis*-retinal is located within a pocket surrounded by TM3−TM7 and in direct contact with the second extracellular loop (XL2) that connects TM4 and TM5. XL2 folds as a β-hairpin, covering the extracellular surface of the binding cavity, and is connected to TM3 via a disulfide bridge.[7] Other GPCRs share in their structure a bundle of seven transmembrane α-helices (TM1−TM7). In the CABRs, the binding cavity would be similarly located,[9−11] and the disulfide bridge between XL2 and TM3 would also be present, constraining the position of XL2 on top of the binding cavity.[12,13] It is generally accepted that catecholamines are anchored at the receptor binding site by two polar regions common to all CABRs: (i) the protonated amine of catecholamines forms ionic interactions with the negatively charged aspartate at position 3.32,[84] and (ii) the two catecholic hydroxyls hydrogen bond with serine at positions 5.42 and 5.46 of TM5 and, when present, with serine at 5.43.

The β-hydroxyl group is found only in (nor)epinephrine and is not present in dopamine, and therefore the β-hydroxyl group is expected to form unique interactions with the adrenoceptors. The chirality of the β-hydroxyl is key to selectivity because the *S*-enantiomer of norepinephrine binds to the adrenoceptors with an affinity similar to dopamine in contrast to the tight binding of the *R*-enantiomer (e.g., see ref 14). In the β-ARs, the effects of the mutants N6.55A and N6.55L suggest that the determinants of binding specificity are located nearby.[15,16] No amino acid has, however, been identified so far that would account for specific interactions of the β-hydroxyl of (nor)-epinephrine with the α_1-ARs or α_2-ARs.

Different orientations have been proposed for the catecholic ring for individual receptors (e.g., see refs 17−19), reflecting the information available at the time. The level of similarity in the binding modes of ligands across related CABRs has received far less attention. In the present study, our goals were (i) to make a comparative analysis of the binding cavities of the CABRs; (ii) to compare and contrast likely binding modes across the receptors; and (iii) to propose an origin of the selectivity among the ARs and DRs for binding (nor)epinephrine and dopamine, which differ only by one hydroxyl group. To achieve these goals, we constructed a set of structural models using a single methodology, computed binding modes for catecholamines while using atomic restraints based on experimentally validated interactions, and used maps that predict favorable

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[a] Abbreviations: AR, adrenoceptor; DR, dopamine receptor; CABR, catecholamine binding G-protein coupled receptor; GPCR, G-protein coupled receptor; RMSD, root mean-squared deviation; TM, α-helical transmembrane segment; 3D, three-dimensional

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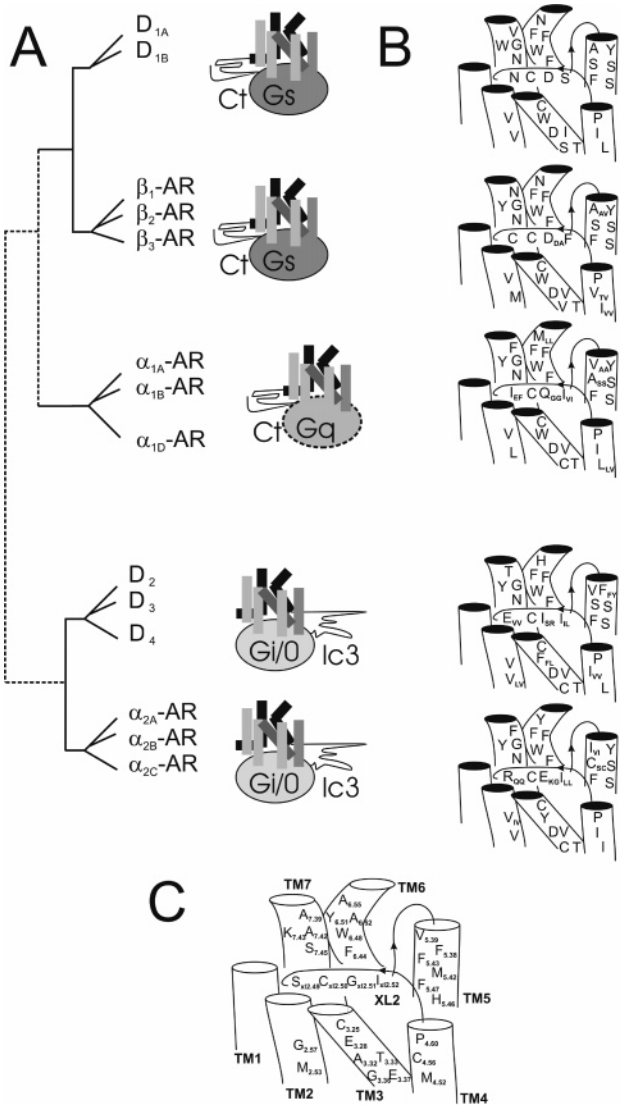

Figure 1. Structural comparisons among human CABRs. (A) Human CABRs are divided into five classes and 14 subtypes. (A, left) Phylogenetic tree computed using the neighbor-joining method and based on amino acid sequences aligned over the TM regions. The α_2-ARs and D_2's cluster together, as do the β-ARs and D_1's (plain branches), while the relative relationship of α_1-ARs with the other clusters ambiguous (dashed branches) possibly because divergence occurred within a relative short evolutionary time scale. This tree is not to scale. (A, right) Features characteristic of each classes are indicated: the coupled G-protein, $G_{s/olf}$, G_q, or $G_{i/0}$, and the presence of an unusually long loop (Ic3, third intracellular loop; Ct, C-terminus). (B) Schematics representing the binding cavities, lined by the seven TMs and covered by XL2, the loop connecting TM4 and TM5, as derived from model structures. Amino acids are indicated by their one letter code and are listed sequentially when the residue varies among the subtypes; for example, for the β-ARs, position 5.39 varies as AAV: A (β_1-AR), A (β_2-AR), and V (β_3-AR). (C) Amino acids at equivalent positions in the rhodopsin X-ray structure, numbered using the Ballesteros and Weinstein convention[80] expanded to XL2.[13]

interactions computed from the ligand and from the binding site to adjust the geometry of the receptor complexes.

Result and Discussion

Sequence Alignment of Bovine Rhodopsin and CABRs Is Unambiguous over the TM Regions. The bovine rhodopsin X-ray structure[8] was used as a structural template to model each of the 14 human subtypes from the five classes of CABRs: the dopamine receptors $D_{1A,1B}$ and $D_{2,3,4}$, and the adrenoceptors $\alpha_{1A,B,D}$, $\alpha_{2A,B,C}$, and $\beta_{1,2,3}$. In the rhodopsin structure, 11-*cis*-retinal acts as an inverse agonist; thus, the bovine rhodopsin structure should represent an inactive "ground" state of the receptor.[7,8] We will discuss below the implications of using this conformational state to model agonist-bound CABRs.

CABRs share within their TM regions 20−26% sequence identity with respect to rhodopsin (Table 1). Despite this modest level of sequence identity, rhodopsin, CABRs, and other rhodopsin-like GPCRs share a pattern of conserved aligned residues in each of their seven transmembrane segments,[20] including 2 or 4 cysteine residues forming disulfide bridges, that provides strong support for the sequence alignment used for modeling these regions and for the assignment of residues lining the putative binding cavities of the CABRs. Other homologous integral membrane proteins share sets of identical and similar residues distributed along the transmembrane sequences that permit alignment of the sequences with high accuracy, matching the alignment of the 3D structures. Furthermore, the level of structural similarity when the sequence identity is low is sufficient to provide reasonable templates for modeling the other homologous members. For example, human aquaporin 1 (PDB code 1H6I) and the bacterial glycerol facilitator (1FX8) share ~30% sequence identity, and de Groot et al.[21] reported that their helical backbone regions can be superimposed with a root mean-squared deviation (RMSD) of 1.4 Å; notably, similar side-chain conformations are also found. Subunits I, II, and III of both ubiquinol oxidase (1FFT) from *E. coli* and of cytochrome *c* oxidase (1QLE) from *Paracoccus denitrificans* can be superimposed with an RMSD of 1.6 Å over 781 C_α-atoms (e.g., for subunit I, the sequence identity is ~32%).[22] In both of these examples, we were able to align the sequences without use of the structures and the alignment matched well the reported structural alignment (not shown). Even though alignment of the sequences of glycerol-3-phosphate transporter (1PW4) and lactose permease (1PV6), both from *E. coli*, was up to 30% wrong, the sequence identity is 21%,[23] superposition of the entire structures (2.46 Å, 206 C_α-atoms) and separately the N-terminal (2.25 Å, 103 C_α-atoms) and C-terminal (2.30 Å, 138 C_α-atoms) domains shows that the domain structures are well-conserved[24] and either structure would be a useful template for modeling the other structure if a valid sequence alignment could be obtained.

The Binding Pocket of CABRs Share Conserved Features with Rhodopsin. In the bovine rhodopsin X-ray structure, the endogenous ligand 11-*cis*-retinal is located in a pocket lined by TM3−TM7, and in direct contact with XL2 that folds as a β-hairpin above the binding cavity.[7,8] The ligand binding pocket of the CABRs (Figure 1) was predicted long ago to correspond to the location where 11-*cis*-retinal is bound in rhodopsin, for example, see refs 9−11, and much supporting evidence has since been gained from biophysical experiments (e.g., refs 12,25) as well as from the effects of mutations (e.g., D_1's, ref 17; D_2's, refs 26−29; α_1-ARs, refs 18,30; α_2-ARs, refs 14,31−33; β-ARs, refs 9,34,35). Moreover, about one-half of the amino acids lining the binding pocket in the rhodopsin structure are identical or vary conservatively in the CABRs (Figure 1) and are mainly located at the intracellular surface of the binding pocket and at the interfaces of TM3, TM4, TM5, and XL2. Conservation of these regions provides support for the proposed location of these residues in the structural models of CABRs as well as placing constraints on the conformations accessible to the side chains.

Of the 30 side chains that are predicted to face the binding cavity, 14 positions are totally conserved among all human CABRs (Figure 1). F5.47, F6.44, and W6.48 (located toward

Table 1. Pairwise Percentage Sequence Identity (Rounded to the Closest Integer) Computed over the Aligned TM Regions from Bovine Rhodopsin (Rhd) and the CABRs[a]

	Rhd	α_{1A}	α_{1B}	α_{1D}	β_1	β_2	β_3	D_{1A}	D_{1B}	α_{2A}	α_{2B}	α_{2C}	D_2	D_3	D_4
Rhd	100	24	24	25	21	21	24	20	21	22	22	21	25	25	26
α_{1A}	24	*100*	*75*	*66*	43	40	41	42	41	43	43	43	42	41	42
α_{1B}	25	*75*	*100*	*73*	44	43	44	42	42	43	46	44	45	43	43
α_{1D}	25	*66*	*73*	*100*	44	43	43	41	42	43	46	42	43	42	45
β_1	21	43	44	44	*100*	*70*	*67*	**47**	**48**	43	40	39	38	37	39
β_2	21	40	43	43	*70*	*100*	*62*	**44**	**44**	37	36	37	38	37	34
β_3	24	41	44	43	*67*	*62*	*100*	**41**	**43**	37	39	38	37	38	40
D_{1A}	20	42	42	41	**47**	**44**	**41**	*100*	*79*	42	38	39	39	38	37
D_{1B}	21	41	42	42	**48**	**44**	**43**	*79*	*100*	42	38	40	41	38	39
α_{2A}	22	43	43	43	43	37	37	42	42	*100*	*77*	*78*	**46**	**42**	**44**
α_{2B}	22	43	46	46	40	36	39	38	38	*77*	*100*	*79*	**44**	**43**	**46**
α_{2C}	22	43	44	42	39	37	38	39	40	*78*	*79*	*100*	**43**	**42**	**46**
D_2	25	42	45	43	38	38	37	39	41	**46**	**44**	**43**	*100*	*73*	*49*
D_3	25	41	43	42	37	37	38	38	38	**42**	**43**	**42**	*73*	*100*	*50*
D_4	26	42	43	45	39	34	40	37	39	**44**	**46**	**46**	*49*	*50*	*100*

[a] β-ARs versus D_1's (bold), as well as α_2-AR versus D_2's (bold), form evolutionary closer clusters (Figure 1A) in contrast to the α_1-ARs that cannot be grouped reliably with any of the other CABR classes. Percent identities among subtypes of each class are in italic.

Table 2. Amino Acid Sequence Differences among the Modeled CABRs Corresponding to 16 of the 30 Residues Lining the Binding Pocket in the Bovine Rhodopsin X-ray Structure[a]

	amino acid and location within the binding cavity															
	2.53	2.57	3.28	3.33	3.36	4.52	4.56	5.38	5.39	5.43	6.55	7.39	7.43	xl2.49	xl2.51	xl2.52
Rhd	M	G	E	T	G	M	C	F	V	F	A	A	K	S	G	I
α_{1A}	L	V	W	V	C	L	I	Y	V	A	M	F	Y	I	Q	I
α_{1B}	L	V	W	V	C	L	I	Y	A	S	L	F	Y	E	G	V
α_{1D}	L	V	W	V	C	V	I	Y	A	S	L	F	Y	F	G	I
β_1	M	V	W	V	V	I	V	Y	A	S	N	N	Y	C[b]	D	F
β_2	M	V	W	V	V	V	T	Y	A	S	N	N	Y	C[b]	D	F
β_3	M	V	W	V	V	V	V	Y	V	S	N	N	Y	C[b]	A	F
D_{1A}	V	V	W	I	S	L	I	Y	A	S	N	V	W	N	D	S
D_{1B}	V	V	W	I	S	L	I	Y	A	S	N	V	W	N	D	S
α_{2A}	V	V	Y	V	C	I	I	Y	I	C	Y	F	Y	R	E	I
α_{2B}	V	V	Y	V	C	I	I	Y	V	S	Y	F	Y	Q	G	L
α_{2C}	V	V	Y	V	C	I	I	Y	I	C	Y	F	Y	Q	Q	L
D_2	V	V	F	V	C	L	I	F	V	S	H	T	Y	E	I	I
D_3	L	I	F	V	C	L	V	F	V	S	H	T	Y	V	S	I
D_4	V	V	L	V	C	L	V	Y	V	S	H	T	Y	V	R	L

[a] Of the remaining 14 residues, six are conserved in bovine rhodopsin and in most of the rhodopsin-like GPCRs, C3.25, P4.60, F5.47, F6.44, W6.48, and Cxl2.50; and eight vary in rhodopsin, F6.51 (tyrosine in rhodopsin), F6.52 (alanine), D3.32 (alanine), T3.37 (glutamate), S5.42 (methionine), S5.46 (histidine), G7.42 (alanine), and N7.45 (serine). [b] Part of a second disulfide bridge present in the β-Ars; see ref 78.

the intracellular surface of the binding cavity) and C3.25, P4.60, and Cxl2.50 (at the interface of XL2 with TM3 and TM4) are conserved in bovine rhodopsin and in most of the rhodopsin-like GPCRs, whereas the CABRs differ from rhodopsin at F6.51 (rhodopsin: tyrosine), F6.52 (alanine), D3.32 (alanine), T3.37 (glutamate), S5.42 (methionine), S5.46 (histidine), G7.42 (alanine), and N7.45 (serine). In most of the modeled structures, the carboxyl group of D3.32 was positioned such that weak hydrogen-bonding interactions could form with the edge of the indole ring of W6.48. Both D3.32 and W6.48 play key roles in the process leading to receptor activation, and, if they do form, weak interactions between them may be important for the stabilization of the ground state of the receptor.

The core of the ligand binding region in the human CABRs is otherwise formed by aromatic (3.28, 5.38, and 7.43) and aliphatic (2.53, 2.57, 3.33, 4.52, 4.56, and 5.29) side chains. The seven most variable positions (Table 2, Figure 1) are mainly (5 of 7) located at the extracellular "top" of the binding pocket. Position 3.36 (varies as alanine, valine, cysteine, serine) faces both the polar binding site and the indole ring of W6.48. Position 5.43 varies as alanine/cysteine/serine, faces TM6 near F6.52, and variation at 5.43 may play a role in subtype specificity for the antagonist yohimbine in the α_2-ARs.[36] In the β-ARs, the main candidate for interaction with the β-hydroxyl group on ligands is asparagine at position 6.55,[15,16] but asparagine is also found at the equivalent position in the D_1-type receptors activated by dopamine lacking the β-hydroxyl group. In other CABRs, tyrosine (α_2-ARs), histidine (D_2-type receptors), or methionine/leucine (α_1-ARs) is found at position 6.55. Position 7.39, asparagine (β-ARs), phenylalanine (α_1-ARs and α_2-ARs), valine (D_1's), or threonine (D_2's), has been implicated in antagonist specificity differences observed for the β-ARs and α_2-ARs.[37] Both 6.55 and 7.39 are located at the TM6−TM7 interface. XL2 is the most variable region in contact with the binding site where, with the exception of Cxl2.50, residues at xl2.49, xl2.51, and xl2.52 vary even among receptor subtypes. Variation in XL2 has been implicated in antagonist binding specificity among subtypes of α_1-AR and α_2-AR.[13,38,39]

In summary, considering their different functions and overall modest level of sequence identity, there is a surprisingly high degree of amino acid conservation between rhodopsin and the CABRs within the ligand binding pocket.

Predicted Ligand Interactions across the CABRs. For each CABR model structure, the relevant endogenous ligand, either dopamine or norepinephrine, was automatically docked (Figure 2) using Gold[40] under the distance restraints described in the Materials and Methods (in our hands, it was not possible to obtain reasonable solutions without applying constraints, which may reflect the uncertainties in the model and/or in the docking and scoring methodologies). It was not possible to simulta-

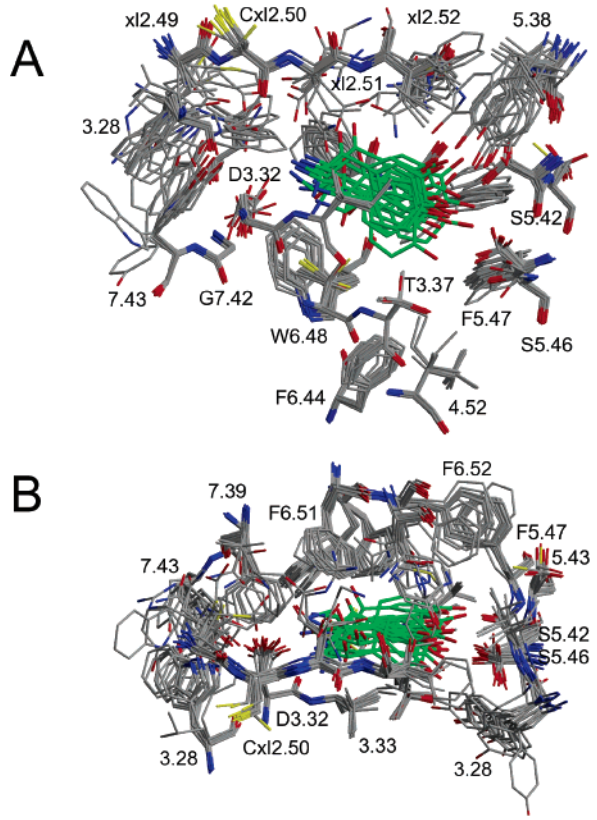

Figure 2. Endogenous catecholamines docked to the 14 model structures of CABRs. All receptor models and their docked endogenous ligand (green), either dopamine or norepinephrine, were superimposed on each other. The view is (A) from TM3 within the plane of the membrane and (B) from above the TM bundle (from the extracellular surface of the binding cavity). For clarity, only the important side chains surrounding the ligands, and not hydrogen atoms, are shown. This figure and Figures 3, 5, and 7 were prepared using Molscript[81] and Raster3D.[82]

neously enforce distance restraints between D3.32 and the protonated amine of the ligand and between the *meta*-hydroxyl and S/C5.43 (in all CABRs but α_{1A}-AR with alanine at 5.43) because position 5.43 is located ~2 Å too far away from D3.32. Minor variations in the orientation and location of docked ligands were found among the CABRs, and these differences will certainly reflect the uncertainties of the models and the procedures that generated them, given that the core region (i.e., D3.32, V(occasionally I)3.33, T3.37, S5.42, S(A/C)5.43, S5.46, F6.51, F6.52, and W6.48) of the catecholamine binding site is almost entirely conserved. In general, the docked ligands are positioned in an extended conformation because D3.32 and polar residues on TM5 (i.e., S5.42 and S5.46) are distant from each other, located at the opposite ends of the binding pocket.

Common to the structure of (nor)epinephrine and dopamine is the aromatic ring, the catecholic hydroxyls on the ring, and, at the opposite end of the molecules, the positively charged amine group. In general, the aromatic catecholic ring is sandwiched between aromatic side chains on TM6 (F6.51 and F6.52) and hydrophobic V/I3.33 on TM3, lies above W6.48, and is located below a β-strand of the β-hairpin of XL2 (as for the rhodopsin−11-*cis*-retinal complex, in the CABRs β-strand 4 of XL2 would be in contact with the ligand). The amine group in all cases is located close to the negatively charged side chain of D3.32. This ion-pair is surrounded by aromatic residues (W6.48, F6.51, W/Y/F3.28, F/W7.36, and F7.39), which by lowering the dielectric constant may reinforce this interaction. The protonated amine of the ligand is suggested by all or some of the docked complexes to form additional stabilizing interactions: with (i) F6.51, through cation-π side-chain interactions; with (ii) the main-chain carbonyl of xl2.50 in some of the modeled complexes; with (iii) the side chain at xl2.49, especially for polar side chains (glutamate, glutamine, aspartate) with a suitable conformation; with (iv) a polar side chain at 7.39 (asparagine, threonine) via a bridging water molecule; and/or with (v) the hydroxyl group of tyrosine at 3.28 or 7.36 when present.

Epinephrine, having a methyl group attached to the positively charged nitrogen of norepinephrine, binds with higher affinity to the adrenergic receptors in comparison to norepinephrine (e.g., Table 3). Based on the binding mode for norepinephrine, the methyl group of epinephrine would form additional interactions with F6.51 as well as with F7.39 in α_1-ARs and α_2-ARs, N7.39 in β-ARs. Two unique features of the β_2-ARs may explain the higher observed selectivity for epinephrine over norepinephrine in comparison to that observed for the α_2-ARs: 22.9-fold (β_2-ARs) versus 3.2−4.5-fold (α_2-ARs) differences. First, the methyl group of epinephrine could form a weak CH···O hydrogen bond with the carbonyl of the amide side chain of N7.39 in the β_2-ARs, because the carbon atom of epinephrine is located adjacent to an electronegative oxygen atom that would increase the acidity of the methyl hydrogen. Second, the β-ARs have a second disulfide bridge located close to the position where the methyl group of epinephrine would be located, which could function to lower the local dielectric and hence increase the strength of interactions, including that of any weak CH···O hydrogen bond. These effects appear to be accentuated when the alkyl group is enlarged; for example, Suryanarayana et al.[37] reported that a swap mutant F7.39N in α_2-AR increased the affinity for the β_2-blocker alprenolol (with a propyl group attached to the charged nitrogen) by 3000-fold and reduced the affinity by 350-fold toward yohimbine.

Interactions with S5.42 and S5.46. The catecholic hydroxyl groups point toward TM5, where S5.42 and S5.46 are conserved across all CABRs. In the modeled CABR structures derived from the bovine rhodopsin structure, positions 5.42, 5.43, and 5.46 of TM5 are exposed to the binding cavity (Figure 2), which agrees with experiments where MTSEA (2-aminoethyl methanethiosulfonate) covalently attaches to cysteines present or sequentially engineered at these positions in α_{2A}-AR, β_2-AR, and D_2.[35,41,42]

S5.42 was often found within hydrogen-bonding distance of both the *meta*- and the *para*-hydroxyl groups because of the staggered organization of the catechol moiety and polar side chains on TM5. Closer to the intracellular surface, S5.46 is positioned such that it would mainly interact with the *para*-hydroxyl group (the main-chain carbonyl of S5.46 could in some docked complexes interact with the *para*-catechol hydroxyl). This mode of binding would allow formation of three hydrogen bonds, reinforced by electronic resonance along the catecholic ring system. Several groups[33,43−45] have reported that S5.42 and S5.46 would respectively hydrogen bond to the catecholic *meta*- and *para*-hydroxyl groups, the presence of these groups having a synergistic effect on receptor activation.

Position 5.43 is variable in sequence and has a less-well-defined role among the CABRs; it appears to be important for binding in α_{2A}-AR,[33] has a role in β_2-AR,[35] whereas only 5.42 and to a lesser extent 5.46 were found important for ligand binding to D_2 and D_3.[18,29] In the docked complexes, there are no clear interactions taking place with 5.43. S5.43 might interact with ligands via a bridging water molecule (as in the X-ray structure of a protein unrelated to GPCRs, anthocyanidin

Table 3. (Nor)epinephrine and Dopamine Binding to α_{2A}-AR and β_2-AR, Taken from the Literature (Inhibition Constants (nM) Are Determined from Competition Binding Assays Using a One-Site Model (K_i); ND, Not Determined)

	ligand binding competition assays			
	α_{2A}-AR			β_2-AR
agonists	[^{3}H]RX821002[a]	[^{3}H]RX821002 ($\sim K_{iL}$)[b]	[^{3}H]UK14,304 ($\sim K_{iH}$)[b]	^{125}I-CYP[c]
R-epinephrine	361 ± 38	3240 ± 390	1.4 ± 0.2	170 ± 10
R-norepinephrine	1650 ± 330	13 900 ± 1080	4.6 ± 1.0	3900 ± 800
S-norepinephrine	7130 ± 79	ND	105 ± 25	174 000 ± 6300
dopamine	4780 ± 722	43 500 ± 2400	34 ± 6	400 000 ± 150 000
agonists	ratio of K_i values			
R-epinephrine:dopamine	13.2	13.4	24.3	2350
R-norepinephrine:dopamine	2.9	3.1	7.4	102.5
S-norepinephrine:dopamine	1.5		3.1	2.3

[a] Reference 14. [b] Reference 33: conditions favorable for the low-affinity ($\sim K_{iL}$) and high-affinity ($\sim K_{iH}$) conformations; apparent K_i's based on a one-site model. [c] Reference 15.

synthase bound to the catechol-containing *trans*-dihydroquercetin; PDB code 1GP5, ref 46) while C5.43 present in α_{2A}-AR and α_{2C}-AR may lower the local dielectric constant, thereby strengthening nearby polar interactions between S5.42 and S5.46 and the catecholic hydroxyls. Alternatively, local conformations of the receptor may be required that are not represented by the bovine rhodopsin X-ray structure and the CABR models based on that structure.

Other conserved polar groups could provide additional interactions with the *meta*-hydroxyl group: the hydroxyl of Y6.55 in the α_2-ARs or of Sxl2.52 in the D_2's. In turn, the *para*-hydroxyl group is located near the side-chain hydroxyl of T3.37 and the main-chain carbonyl of S5.46; the latter group is "free" to accept a hydrogen-bonding partner because the structure of the TM5 helix is interrupted due to the presence of proline at position 5.50. In the rhodopsin structure, the protonated carboxylate of E3.37 forms a hydrogen bond with the main-chain carbonyl of H5.46, and these functional groups are in direct contact with 11-*cis*-retinal, forming a key link between TM3 and TM5.[7] Among the CABRs, P5.50, T3.37, and S5.46 are conserved, and a hydrogen bond corresponding to the link seen in the rhodopsin structure, here between the hydroxyl group of T3.37 and the "free" main-chain carbonyl of S5.46 and/or side chain of S5.46, may be present, too.

The presence across the CABRs of a large conserved "binding core" (i.e., D3.32, V(I)3.33, T3.37, S5.42, S(A/C)5.43, S5.46, F6.51, F6.52, and W6.48 located closest to the docked ligands; see Figure 3F), as well as the results from automated ligand docking, suggests that catecholamines have modes of binding similar to those of the CABRs. This proposal is well supported by the current experimental data,[34,35] but this has not always been the case because the lack of expression/detection of a S5.42 mutant of β_2-ARs[47] had suggested a recognition mechanism different from that operating in α_1-ARs and α_2-ARs where mutants were expressible and tested.[17−19]

The *β*-Hydroxyl Group of (Nor)epinephrine and Position 6.55. Chemically, the difference distinguishing dopamine from norepinephrine is the presence of the β-hydroxyl group in norepinephrine. Of the two enantiomers, *R*-norepinephrine is more selective than *S*-norepinephrine toward the adrenergic receptors, for example, for α_{2A}-AR,[14] binding 4-fold (low affinity site, K_{iL}) and 2000-fold (high affinity site, K_{iH}) better than the *S*-enantiomer. The β-hydroxyl group adopts three preferred staggered conformations in crystal structures of epinephrine (Cambridge Structure Database;[48] access code ADRTAR) and closely related molecules (ISPROT20, JAMMEP, LTXPSE, NADRHC, VAVREP, WELYOB, WELYUH, XPSERC) (data not shown). In (nor)epinephrine, the β-hydroxyl

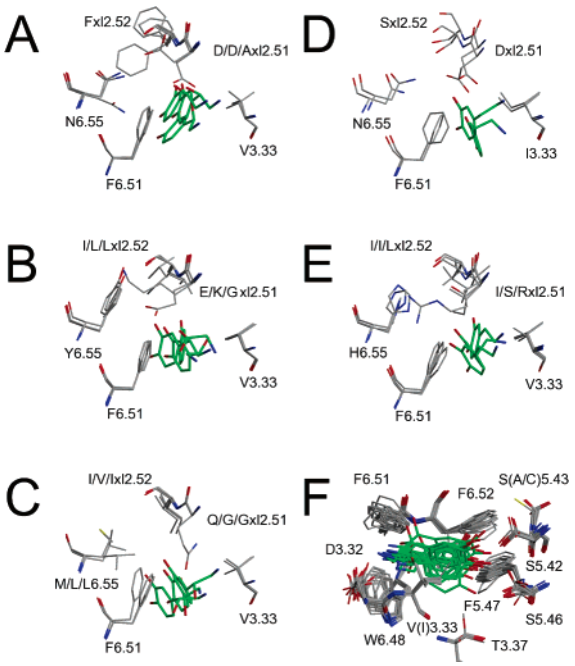

Figure 3. Side-chain differences proposed to be associated with differential specificity for norepinephrine and dopamine. Model structures of (A) β-ARs, (B) α_2-ARs, (C) α_1-ARs, (D) D_1's, and (E) D_2's, showing side chains located near the β-hydroxyl group of docked norepinephrine in the ARs and the equivalent residues in the DRs. Where amino acids differ among subtypes, they are sequentially listed. In (F), conserved side chains among all of the CABR model structures, plus positions 3.36 and 5.43 that are largely conserved, form the core of the catecholamine binding region.

could thus project toward TM6/XL2, toward XL2/TM3, or toward the bottom of the binding site in which the β-hydroxyl is positioned parallel to the catecholic ring.

Norepinephrine bound in the extended conformation seen in the docked complexes positions the β-hydroxyl group close to xl2.51 near 6.55 in TM6 (e.g., Figure 3A) or otherwise near F6.51 (e.g., Figure 3B and C). Asparagine at position 6.55 in human β_2-AR has been proposed to interact with the β-hydroxyl group, but as the mutation N6.55L decreased the binding of both *S*- and *R*-norepinephrine by 10-fold while dopamine binding was unaffected, additional determinants of binding selectivity are suspected.[15,16] In the docked complexes of the β-ARs, the distance between the β-hydroxyl group and the side-chain amide of N6.55 is long (4.5−6 Å), but an interaction could occur if the distance is reduced, that is, either through structural changes or via a water molecule. N6.55 is also found in the dopamine receptor D_1, but it is not conserved in the other ARs:

methionine or leucine present in the α_1-ARs (Figure 3C) cannot form polar side-chain interactions with the β-hydroxyl, and the conserved tyrosine at 6.55 in the α_2-ARs (Figure 3B) may interact with the *meta*-catecholic hydroxyl but would require both a different side-chain conformation and rearrangement of the extracellular top of TM6 to interact with the β-hydroxyl. Moreover, in α_{2A}-AR the triple mutant T6.54A, Y6.55F, T6.56A was not stereospecific for norepinephrine; binding was reduced 10-fold for both *S*- and *R*-norepinephrine and for dopamine, as well.[49] Thus, the residue at 6.55 would not appear to provide interactions with the β-hydroxyl common across all adrenoceptors, again suggesting that additional mechanisms are involved.

Binding of the β-Hydroxyl via the Main-Chain NH Group at xl2.52 of XL2. The main chain of XL2 is positioned in the docked complexes so that it could provide a common interaction with the β-hydroxyl in all adrenergic receptors via a hydrogen bond donated by the main-chain NH group of the residue at xl2.52 (Figures 3 and 4). The β-hydroxyl is positioned in the model structures such that it could also accept weak hydrogen bonds donated by the edge of the aromatic ring of F6.51, a residue present in all CABRs. Some of the docked complexes suggest that additional stabilizing interactions with the β-hydroxyl could take place, for example, with N6.55 in the β-ARs and Dxl2.51 in β_1-AR and β_2-AR (Figure 3A), with Qxl2.51 in α_{1A}-AR (Figure 3C) and Exl2.51 in α_{2A}-AR, and with the conserved Y6.55 in α_2-ARs if some structural rearrangements take place (Figure 3B). Interaction between the XL2 main-chain and the β-hydroxyl would be favored for α_{1B}-AR, α_{1D}-AR, α_{2C}-AR, and β_3-AR by the small side chain (glycine, alanine) present at xl2.51, while larger, often long and flexible, polar side chains in α_{1A}-AR (glutamine), α_{2A}-AR (glutamate), α_{2B}-AR (lysine), and β_1-AR and β_2-AR (aspartate) could point toward TM6 where the side chains would not interfere with binding of the β-OH to the XL2 main chain but would form additional interactions with the receptor.

The β-hydroxyl in α_{2A}-AR has been proposed to bind to S4.53[50] and to S2.61 and S7.44,[49] but these positions are too distant from the bound ligands in the model structures. We were also unable to reproduce the interaction of the β-hydroxyl with D3.32 in α_{2A}-AR proposed by Nyrönen et al.,[14] which used structural models built using the best structural data available at that time, that is, the structural template of Baldwin et al.[51] In comparison with the bovine rhodopsin X-ray structure, that template did not include the XL2 loop and position 3.32 is located more centrally within the binding cavity.

Origin of the Specificity of Binding in Adrenergic versus Dopamine Receptors. Molecular phylogenetic analyses, corroborated by distinct morphological features of the receptors such as an unusually long third intracellular loop (α_2-ARs and D_2's) or a long carboxyl-terminal segment (α_1-ARs, β-ARs, and D_1's), or specificity for coupling certain G-proteins ($G_{i/0}$ for α_2-ARs and D_2's; G_s/G_{olf} for β-ARs and D_1's; G_q for α_1-ARs) show that the ligand-based pharmacological classification does not reflect the evolutionary history of the ARs and DRs (e.g., Figure 1A). The α_2-ARs are evolutionarily closer to the D_2's than to other CABRs, whereas the β-ARs are closer to the D_1's and to the α_1-ARs.[52−54] Thus, over evolutionary time, which saw the emergence of the different CABR classes, the specificity preferences for (nor)epinephrine and dopamine appear to have arisen on several occasions. The highly conserved nature of the binding cavities supports the notion that subtle changes to the active site can shift the ligand affinity preference from dopamine to (nor)epinephrine and vice versa. The evolutionary pathway leading to the present-day receptor classes is not yet well understood, but would have involved switches in specificity, in addition to epinephrine−dopamine, among other biogenic amines, too, for example, octopamine, tyramine, tryptamine, and 5-hydroxytryptamine.[2,53,54] In mammals, synephrine and octopamine, which occur naturally and are co-released with catecholamines, selectively activate α_{2A}-AR.[55] In addition, there is interplay between dopamine and adrenoceptors in vivo: dopamine has been shown to activate adrenoceptors in the preoptic area,[56] and re-uptake of dopamine has been reported at adrenergic neurons.[57]

D_1's are highly similar to the β-ARs and share N6.55, yet seven positions within the binding site differ, three of which are near where the β-hydroxyl could be located (amino acids displayed in Figure 3A and D; see also Figure 4A). In the D_1's, I3.33 (smaller valine in the β-ARs and in all of the other adrenergic and dopamine receptors) would likely have a minor role, increasing the hydrophobic interactions with dopamine and functioning to rigidify the amino-ethyl chain of dopamine. Bulky aromatic Fxl2.52 in the β-ARs is serine in the D_1's, the latter possibly interacting with the side chain at 6.55, as was found for model structures of the D_1's, and/or interacting with the *meta*-hydroxyl of bound dopamine. Position xl2.51 is aspartate in β_1-AR, β_2-AR, and the D_1's, and alanine in β_3-AR; alanine would make the main-chain NH of xl2.52 more accessible for binding to the β-hydroxyl. From the modeled complexes and similarities with the adrenoceptors (Figure 3A and D), it is not surprising that (nor)epinephrine also binds to the D_1's: for example, epinephrine binds to D_{1A} but with about 20-fold less selectivity than dopamine (Table 4).[58] Conversely, dopamine binds to the α_{2A}-AR and β_2-ARs, and for β_2-AR the reported specificity of *R*-norepinephrine over dopamine is about 100-fold (Table 3), while *S*-norepinephrine binds little better, ~2-fold, than dopamine.[15]

The D_2's are most similar to the α_2-ARs. Two residues differ in the vicinity where the β-hydroxyl could bind (Figures 3B,E and 4B): tyrosine at 6.55 in the α_2-ARs is histidine in the D_2's and the residue at xl2.51 varies considerably; large flexible residues such as lysine in α_{2B}-AR and arginine in D_4 could be positioned away from the binding site (as was seen for the model structure of α_{2B}-AR), otherwise they would interfere with ligand binding. In α_{2A}-AR, Exl2.51 could interact with the β-hydroxyl and the positive charge on the ligand, while small residues are found in α_{2C}-AR (glycine) and D_3 (polar serine). In both D_3 and D_4, the binding sites should be able to accommodate (nor)-epinephrine, while the bulky, β-branched hydrophobic side chain of Ixl2.51 in D_2 would effectively block interaction of the β-hydroxyl with the main-chain NH group at xl2.51. Indeed, human D_4 binds epinephrine and norepinephrine with high affinity, activating the receptor, while D_2 does not (Table 4).[59] Except for D_2, no other β-branched amino acids (isoleucine, threonine, or valine) are found at xl2.51 in the CABRs.

In summary, model structures and docking simulations suggest that XL2, positioned "above" the binding cavity, could provide a common attachment point for the β-hydroxyl of (nor)-epinephrine in all adrenoceptors, and that it would also permit (nor)epinephrine binding to dopamine receptors when access to xl2.52 is not blocked; blockage most likely occurs only in D_2. For α_{1B}-AR and the α_{1D}-ARs, there are few other possibilities when it comes to providing a polar group for interaction with the β-hydroxyl. Furthermore, it is striking that for some subtypes of the three classes of ARs, but not for the DRs, a smaller side chain (glycine, alanine) has independently evolved at xl2.51. A small side chain at xl2.51 might allow better access to the main-chain amine of xl2.52; however, for glycine at xl2.51

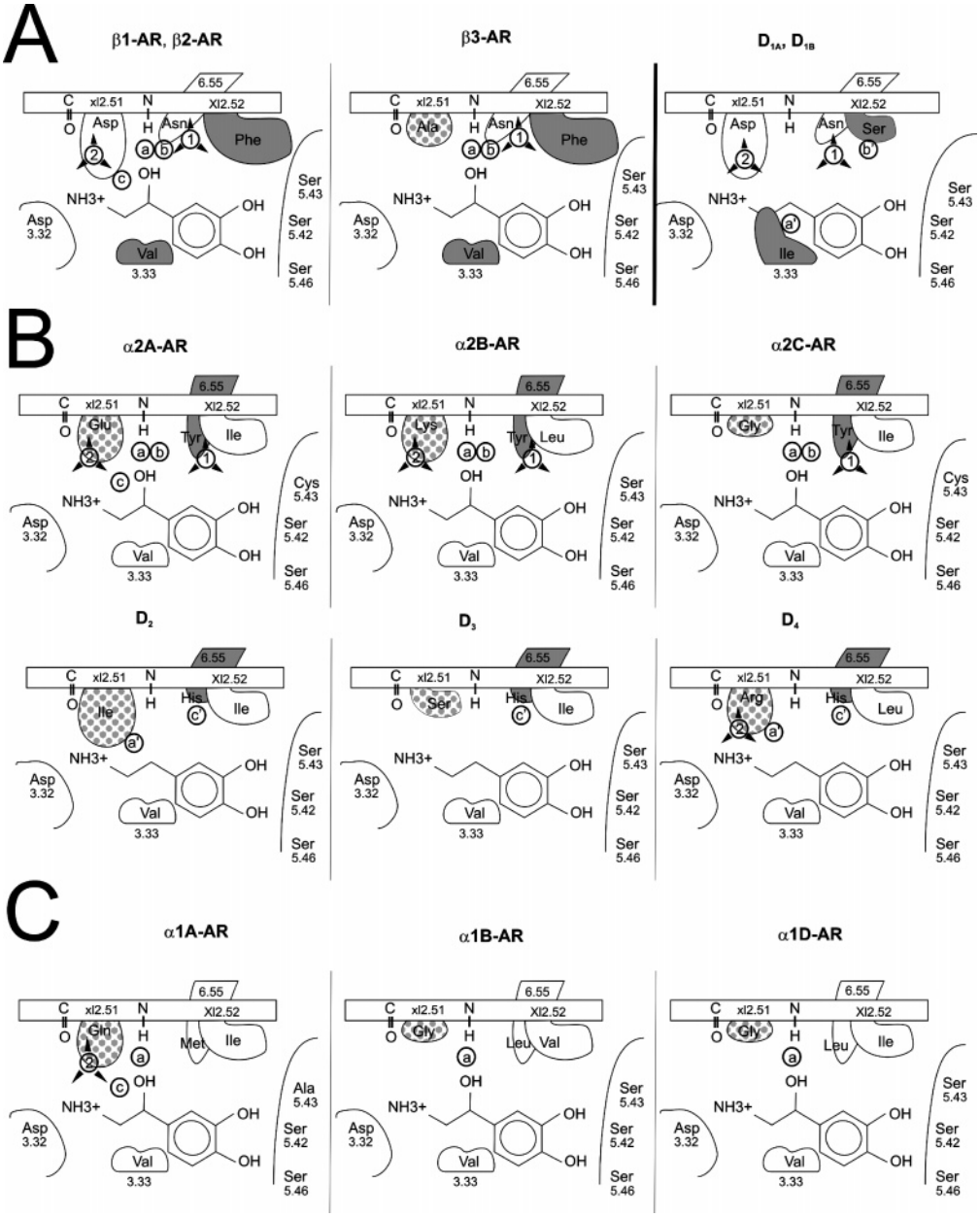

Figure 4. Side-chain differences proposed to be associated with dopamine/(nor)epinephrine specificity. Comparisons are presented for the most closely related classes, (A) β-ARs and D_1's, and (B) α_2-ARs and D_2's, and for (C) α_1-ARs. Gray scale: Residue types identical among subtypes yet differing across receptor classes, gray; residue types that vary across subtypes within a class, spotted gray. For ARs, proposed interactions between the binding site and the β-hydroxyl of norepinephrine are indicated by circled letters: (a), amide backbone; (b) polar side chain at 6.55; (c) polar side-chain group at xl2.51. For DRs, potential specificity determinants are indicated: (a′) increased hydrophobic interactions that might rigidify the amino-ethyl group of dopamine; (b′) serine at 2.52, interaction with N6.55 might prevent hydrogen-bond formation between N6.55 and the β-hydroxyl; alternatively, Sxl2.52 might form an additional stabilizing interaction with the *meta*-hydroxyl; (c′) the side chain of H6.55 in D_2's is shorter than that of Y6.55 in α_2-ARs and cannot reach the β-hydroxyl in the model complexes. Backbone atoms from XL2, the amide of xl2.52, and the carbonyl of xl2.51 are represented. Side chains at 6.55 (circled 1) and at xl2.51 (circled 2) are long and flexible and may adopt one of several conformations.

in α_{2C}-AR, the ratio of the K_i's, dopamine:*R*-norepinephrine, is 5, while for α_{2A}-AR (Exl2.51) and α_{2B}-AR (Kxl2.51), the ratios are respectively 18 and 9.[39] Nevertheless, experimental binding data from the literature show that dopaminergic receptors and adrenoceptors can recognize both dopamine and (nor)epinephrine (Tables 3 and 4). This is not surprising given the close similarity of the D_1's and β-ARs and the D_2's and α_2-ARs.

Interaction Maps Suggest Interactions Common to All CABRs. In Figure 5 is shown a model of β_2-AR with bound norepinephrine, representing the adrenoceptors, and a model of D_2 with bound dopamine as a representative of the dopamine receptors. In these models, the interactions between ligand and receptor have been manually optimized according to an initial set of interaction maps calculated on the basis of the receptors and on the basis of the ligands: small adjustments were made to the side-chain torsion angles of D3.32, V3.33, T3.37, S5.42, S5.43, and S5.46 and to the positions of the ligands. The maps based on the receptor were then recomputed (Figure 6A−D), but this was unnecessary for the ligand-based maps because the ligand conformations were not altered (Figure 6E−H). The interaction maps are predicted on the basis of observed patterns of protein−ligand interactions extracted from X-ray structures in the PDB.[60−62] Distances between key ligand−receptor atom pairs, before and after optimization, are listed in Table 5.

Table 4. Dopamine and (Nor)epinephrine Binding to D_{1A}, D_2, and D_4 Dopamine Receptors, Taken from the Literature (Inhibition Constants (nM) Are Determined from Competition Binding Assays Using Either a One-Site Model (K_i) for D_{1A} and D_2 (with *R*/*S*-Norepinephrine and *R*/*S*-Epinephrine) or a Two-Site Model (K_{iL}, K_{iH}) for D_2 (with Dopamine) and D_4)

	ligand binding competition assays			
	D_{1A}	D_2	D_4	
agonists		[^{3}H]spiperone[b]	[^{3}H]spiperone (K_{iL})[b]	[^{3}H]spiperone (K_{iH})[b]
dopamine	2500	3423 ± 727 (K_{iL})	47.4 ± 9.6	0.9 ± 0.1
R/*S*-epinephrine (racemic mixture)	50 000	1700 (K_i)	240 ± 48	13.8 ± 2.9
R/*S*-norepinephrine (racemic mixture)	55 000	9000 (K_i)	1324 ± 423	33.0 ± 8.0
agonists	ratio of K_i values			
dopamine:*R*/*S*-epinephrine	20	0.5[c]	5.0	15.3
dopamine:*R*/*S*-norepinephrine	22	2.6[c]	27.9	36.7

[a] Reference 58. [b] Reference 59. [c] Represents the ratio of K_i (one-site model) versus K_{iL} (two-site model).

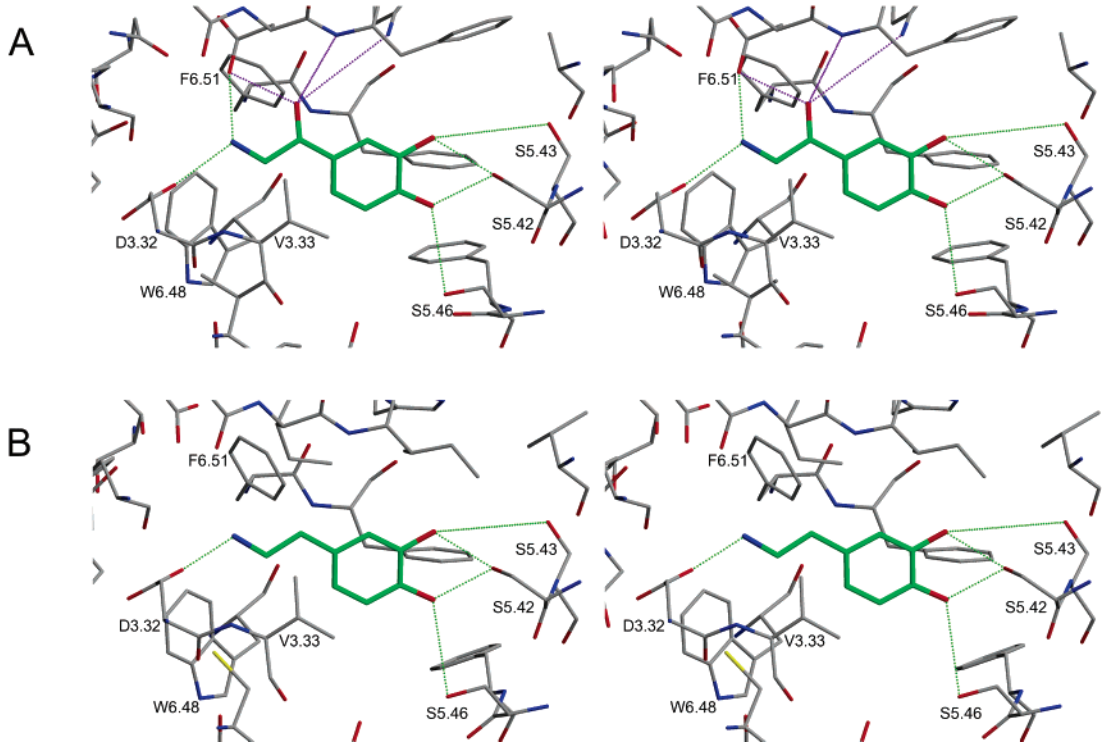

Figure 5. Representative model complexes: β_2-AR with norepinephrine and D_2 dopamine receptor with dopamine. Receptor−ligand interactions were optimized by manual adjustments using the maps predicting favorable interactions (see Figure 6). Interactions were common to both the β_2-AR and the D_2 (green dashed line) and specific to the β-hydroxyl of β_2-AR (purple dashed line). The view is as in Figure 2A. In stereo.

The final interaction maps calculated on the basis of the β_2-AR and D_2 model structures and respectively on the basis of the ligands norepinephrine and dopamine have a high degree of mutual support for the docking of the ligands. With β_2-AR, there are three regions where hydrogen-bond donor/acceptor groups may be placed: near D3.32, parallel to TM5, and near xl2.51 (Figure 6A). In contrast, with D_2, hydrogen-bond donor/acceptor groups are not predicted near xl2.51 (Figure 6C). The hydrophobic interaction maps fill most of the volume of the binding cavities and effectively indicate the space available for ligands, the main difference between the two receptor types being that in β_2-AR there is room for the β-OH group of the ligand (Figure 6B) while in D_2 the presence of Ixl2.51 reduces the size of the cavity in that region (Figure 6D). In both model complexes, the ligand polar and nonpolar groups are docked such that they match well with the predicted interactions based on the binding sites.

Interaction maps computed for norepinephrine (Figure 6E,F) and dopamine (Figure 6G) indicate discrete volumes where hydrogen-bond donors and acceptors groups from the receptors could be placed about the ligands. Three volumes suitable for hydrogen-bond acceptors are located near the docked position of the ligands' protonated amine. A carboxylate oxygen atom of D3.32 is located within one of these volumes in both β_2-AR and D_2. Additionally, in the β_2-AR, a second region is occupied by a carboxylate oxygen atom from Dxl2.51.

From 2.5 to 4.0 Å away from the catechol hydroxyls are located three regions suitable for hydrogen-bond donor and acceptor groups from the receptor. These volumes are positioned within the same plane as the catechol ring. If viewed from the side (extracellular surface above, Figure 6E,G), one predicted interaction volume lies above the *meta*-hydroxyl, one below the *para*-hydroxyl, and one between and to the right of both catechol hydroxyls. These maps correlate well with the observed interaction pattern for catecholic rings found in the X-ray structures of complexes with proteins unrelated to GPCRs (see Supporting Information) and of small molecule structures from the Cambridge Structural Database (data not shown). The hydroxyl group of S5.42 lies within the interaction volume located between the two catecholic hydroxyls. The side-chain hydroxyl and the free carbonyl of S5.46 are close to the interaction predicted below the *para*-hydroxyl. The side chain of xl2.52, serine in the D_1's,

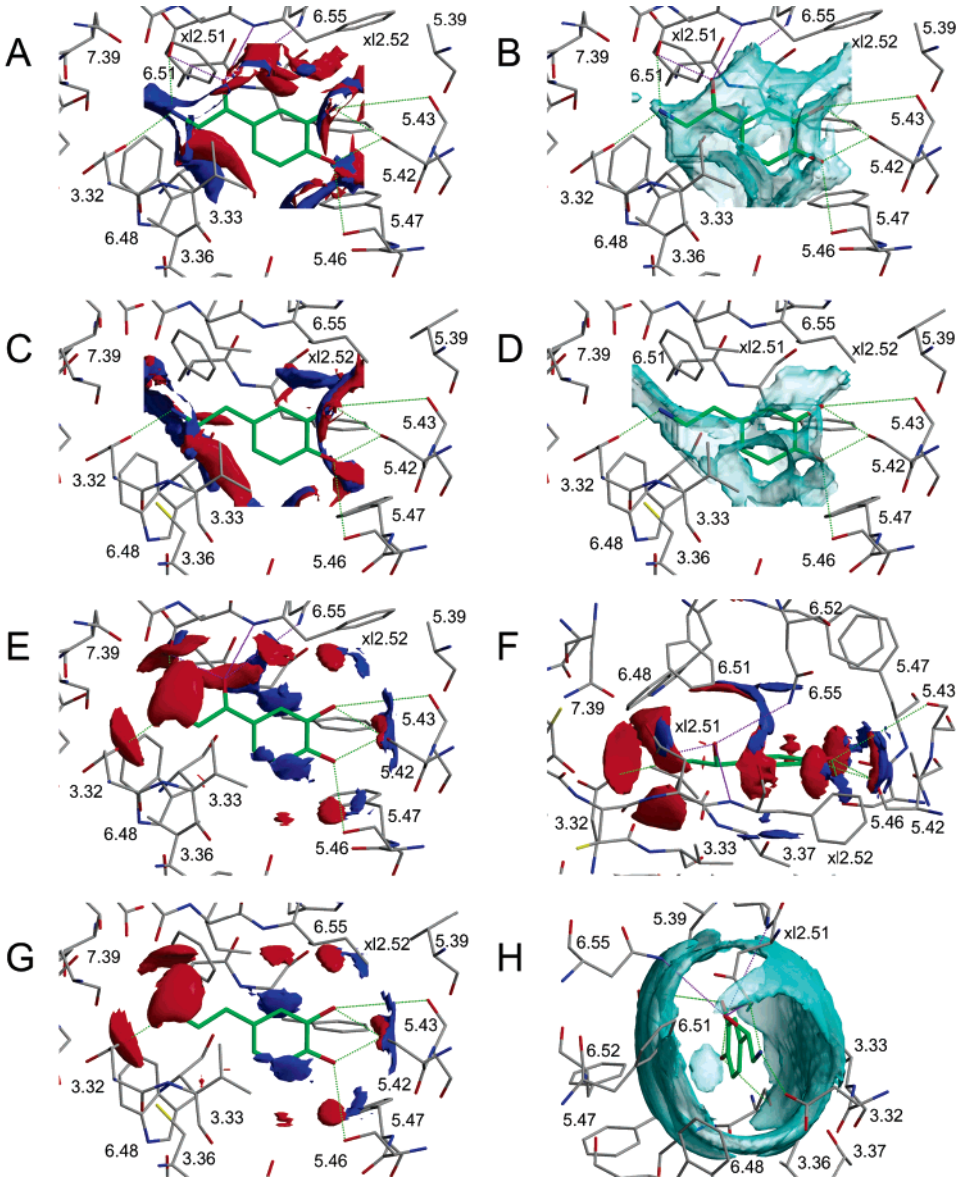

Figure 6. Maps predicting favorable interactions computed for the binding sites of the β_2-AR (A,B) and D_2 dopamine receptors (C,D), and for the ligand structures of norepinephrine (E,F,H) and dopamine (G). Favorable interactions are represented by surfaces: hydrogen-bond donor, blue; hydrogen-bond acceptors, red; and hydrophobic interactions, cyan. The binding modes are the same as in Figure 5. The views displayed in A−E and G are the same as those in Figure 5. For (F), top view, and for (H), view from D3.32. The figure was prepared using Bodil (see ref 83; www.abo.fi/bodil), Molscript,[81] and rendered using Raster3D.[82]

is located near the volume above the *meta*-hydroxyl, as is the side-chain hydroxyl of Y6.55 in the α_2-ARs.

Dopamine and norepinephrine differ only in the β-hydroxyl group present in the latter, and, because the docked conformations are similar, the maps computed on the basis of the ligands only differ about the β-hydroxyl group, too. For norepinephrine, one volume is located every 120° around the β-hydroxyl where hydrogen-bond acceptors can be accommodated, one pointing toward the side-chain amide of N6.55, one toward the aromatic ring of F6.51, and the third pointing toward the main-chain amine of xl2.52 (Figure 6E,F). For hydrogen-bond donors, a semicircle shaped area is predicted near the β-hydroxyl; in the case of β_2-AR, the main-chain amine of xl2.52 is close to this volume (Figure 6E,F). Based on maps computed from the binding site for the β_2-AR with asparate at xl2.51, a polar region favorable for accommodating the β-hydroxyl was predicted, but this is not found in D_2 where the bulky side chain of Ixl2.51 occupies that space (Figure 6B in comparison with Figure 6D).

There is density for possible cation-π interactions located on either side of the catechol ring of norepinephrine (Figure 6E,F) and dopamine (Figure 6G), but no positively charged groups are positioned nearby. Instead, the ligands are surrounded by atoms from hydrophobic and aromatic side chains (e.g., V/I3.33, W6.48, F6.51, F6.52), whose locations match well a hydrophobic spherical shell predicted about the ligands (norepinephrine is shown bound to β_2-AR in Figure 6H).

Overall, the match between maps computed on the basis of the ligand to predict favorable interacting atoms of the binding site, and the reciprocal maps computed from the binding site to predict the ligand, complement each other and support the binding modes where the catecholamines simultaneously interact with D3.32 and S5.42 and S5.46 at the opposite end of the binding cavity. In the norepinephrine−β_2-AR complex, the distances between the β-hydroxyl group and the main-chain amide of xl2.52 and side-chain nitrogen atom of N6.55 are long, ~4.5 Å (Table 5), and minor rearrangements of the structural models may lead to improved interactions. Water molecules,

Table 5. Distances between Ligand and Receptor Atoms in the β_2-AR−Norepinephrine and D_2–Dopamine Complexes after Automated Docking and for Two Successive Rounds of Manual Adjustment[a]

interactions			distances (Å)					
interactions between key atom pairs			β_2-AR			D_2		
ligand	receptor (atom)	type	automated	manual, 1st	manual, 2nd	automated	manual, 1st	manual, 2nd
N^+	D3.32(O^δ)	ionic	2.4	3.2	2.7	2.4	2.8	2.3
meta-OH	S5.42(O^δ)	H-bond	4.2	2.4	2.6	2.5	2.5	2.6
para-OH	S5.42(O^δ)	H-bond	5.8	2.2	2.7	3.7	2.2	2.6
meta-OH	S5.43(O^δ)	H-bond	7.4	5.7	5.4	7.8	6.0	5.6
para-OH	S5.46(O^δ)	H-bond	4.9	3.2	3.4	4.1	3.5	3.7
C[b]	V3.33(C^γ)	hydrophobic	3.7	2.8	3.8	3.4	2.5	3.5
C[b]	F6.51($C^{aromatic}$)	hydrophobic	3.5	3.6	3.7	3.8	4.7	3.7
C[b]	Ixl2.51($C^{\gamma,\delta}$)	hydrophobic	NA	NA	NA	3.3	2.6	2.8
β-OH	Fxl2.52($Nb^{ackbone}$)	H-bond	4.7	3.7	4.4	NA	NA	NA
β-OH	N6.55(N^δ)	H-bond	6.1	4.7	4.5	NA	NA	NA
other interactions referenced in the text			β_2-AR			D_2		
ligand	receptor (atom)	type	automated	manual, 1st	manual, 2nd	automated	manual, 1st	manual, 2nd
N^+	Cxl2.50($O^{backbone}$)	H-bond	5.5	4.1	4.8	4.5	4.0	4.8
N^+	Exl2.51(O^ϵ)	ionic	3.0	6.3	2.6	NA	NA	NA
β-OH	F6.51($C^{aromatic}$)	H-bond (weak)	4.1	3.6	2.8	NA	NA	NA
para-OH	S5.46($O^{backbone}$)	H-bond	3.6	4.6	4.2	4.4	5.1	5.4
para-OH	T3.37(O^δ)	H-bond	7.0	5.6	6.0	5.5	5.7	5.1

[a] The initial complexes were derived from automated docking. Some initial side-chain conformations were far from optimal with respect to accommodating ligand. Two successive rounds of manual adjustment were performed on the modeled complexes, taking advantage of maps predicting favorable interactions. The maps were used to adjust the conformations of the side chains of D3.32, V3.33, T3.37, S5.43, S5.46, and Exl2.51 using the rotamer library of Lovell et al.[79] implemented in Sybyl (Tripos, St. Louis, MO), or of S5.42 by rotating the $C_\alpha-C_\beta$ bond. For each of the binding sites, predictive maps were computed three times in total: after automated docking and after each of the two rounds of manual optimization. The results from the second round of optimization are shown in Figures 5 and 6. Distances are measured between heavy atoms. NA, not applicable. [b] Closest approach between an aliphatic carbon atom from the ligand and the specified carbon atom from the receptor.

which can function as a bridging link between polar groups, have been for the most part ignored in this study, but they certainly will have an important role: in the bovine rhodopsin X-ray structure, water molecules are in contact with 11-*cis*-retinal and form a hydrogen-bonding network involving polar atoms from XL2.

Specificity for the β-Hydroxyl Should Be Best Seen in an Activated State. Selectivity is best seen between (nor)-epinephrine and dopamine, toward adrenergic and dopamine receptors, when the receptors are activated to their high-affinity forms (Tables 3 and 4). Thus, the determinants of β-hydroxyl binding may not be best seen in models based on the ground-state X-ray structure of bovine rhodopsin. Liapakis et al.[45] have shown that the addition of a β-hydroxyl group to any phenethylamine-based ligand having at least two among $N-CH_3$ or catechol−OH groups, substituents known to be important for receptor activation, leads to a 60−120-fold increase in affinity in comparison to the addition of a β-hydroxyl group to phenethylamine without catecholic groups. Furthermore, fluorescence spectroscopy using a reporter molecule attached to C265 of the third intracellular loop near the intracellular end of TM6 of β_2-AR shows that (nor)epinephrine and dopamine induce a "rapid state" (the rate at which the conformational response takes places occurs with a $t_{1/2}$ measured in seconds) capable of activating G_s, but only (nor)epinephrine, not dopamine, can further induce both transition to a "slow state" (with $t_{1/2}$ measured in hundreds of seconds) and agonist-induced receptor internalization.[43] Altogether, these results suggest that additional key interactions are formed during the activation stage and that structural models based on an active structure might provide indications of further interactions with the β-hydroxyl that are common to the adrenoceptors. The difficulty is deciding on what that structure might be in the absence of a representative crystal structure.

Does the Ground-State Structure of Rhodopsin Provide a Suitable Template for Modeling CABR−Agonist Interactions? Before the X-ray structure of bovine rhodopsin was solved, a Cα-atom structural template based on cryo-electron microscopy data for frog rhodopsin and sequence alignments of GPCRs[51] was widely used to construct GPCR model structures. This structural template did not include the "kink" (the face-phase shift) in TM5 due to P5.50. Consequently, in our model structures of α_2-ARs based on the Baldwin template,[14,42,63] position 5.42 is not exposed to the binding cavity in contrast to structures modeled using the bovine rhodopsin X-ray structure (Figure 7). As a consequence, we had postulated that either the model was wrong in that region or TM5 must rotate to expose 5.42 to the binding cavity because a S5.42C mutant was alkylated by sulfhydryl-reactive reagents.[42]

In the field of GPCR modeling, an open question is whether the X-ray structure of bovine rhodopsin in the inactive form (cocrystallized with the inverse agonist 11-*cis*-retinal) would be a suitable template to model agonist-bound GPCRs. Activation of rhodopsin, as is generally accepted for other GPCRs (e.g., for the much studied β_2-AR, see refs 43 and 64), is propagated through a series of conformational intermediates. The first of these intermediates, metarhodopsin I, was recently shown to be similar to the ground state as seen by low-resolution electron microscopy from two-dimensional crystals.[65,66] Thus, even though the agonist all-*trans* retinal is bound to metarhodopsin I, no observable rigid body movement of the helices had taken place.[65,66] For the CABRs, agonists do bind to them under conditions where the receptors are present in the lower affinity inactive conformation.

Model structures based on bovine rhodopsin have usefully explained the binding data for agonists of, for example, α_2-ARs,[33] β_2-AR,[25] and histamine H_1 receptors,[67] while other studies, for example, modeling of the chemokine CCK1,[68] have suggested that the rhodopsin structure is not suitable for modeling of agonist-bound complexes. Bissantz et al.[69] have reported that in using rhodopsin-based models of D_3, β_2-AR, and tachykinin NK receptors, virtual screening failed for small agonists ligands in contrast to relatively good success achieved with antagonists. This apparent unsuitability of rhodopsin-based

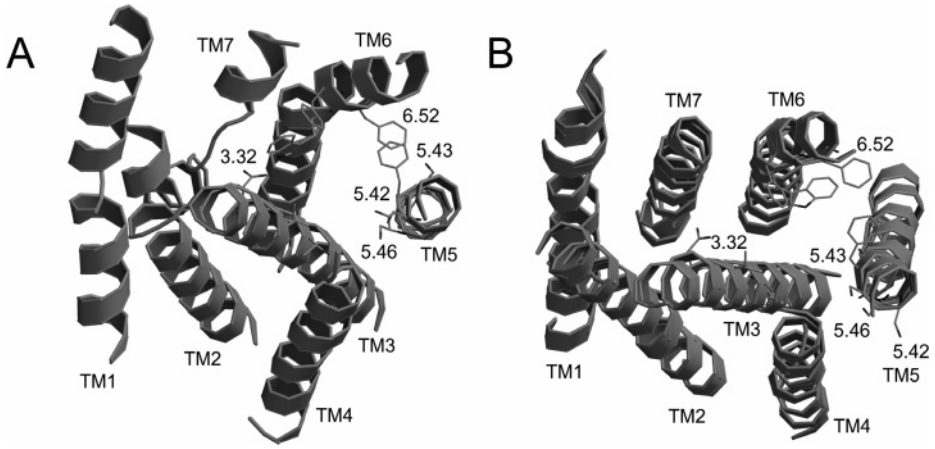

Figure 7. Comparison of the relative orientation of positions 5.42, 5.43, and 5.46 in (A) rhodopsin-based models and (B) a model based on the template of Baldwin (1997). The orientation of TM5 in Baldwin's model differs from that in more recent rhodopsin-based models because kinks were not predicted. Figure shows only the seven TM helices and a few key side chains.

models for agonists has led several groups to construct "activated" forms of the receptor usually by introducing rotation of TM3 and TM6 (e.g., refs 69,70). Furthermore, and because of the low sequence identity shared by the receptor to be modeled with bovine rhodopsin, others have used structural models that significantly deviate from the rhodopsin structure, for example, rhodopsin-based models modified via molecular dynamics simulations[71,72] or constructed ab initio.[73,74]

As always with modeled structures, one needs to be cautious. In the present case, we do not have an X-ray structure for a representative CABR, we do not know to what degree the binding site of the CABRs will differ from bovine rhodopsin, we do not know the locations of water molecules that are likely to have an important structural role within the binding cavity, and we do not know to what degree the binding cavity structure will alter in the process of binding ligand. Nonetheless, using models constructed for the different classes of adrenergic and dopamine receptors directly derived from the bovine rhodopsin structure, it was possible to connect via the ligands the opposite ends of the binding pockets when using distance constraints that are in line with extensive experimental evidence. Moreover, the interaction properties of the ligands in their docked binding modes match well the surrounding residues of the binding site, and, in turn, the interaction properties of the binding site are complementary to the ligands themselves. More generally, the structural models of the CABRs together with the comparison across the diverse classes of CABRs in, for example, Figure 1 and Table 2 can be used to study the specificity of binding for pharmaceutically active ligands. The models should not only explain existing results but also be useful in the design of informative experiments, for example, mutagenesis, and the design of novel specific ligands.

Materials and Methods

Amino Acid Sequence Comparisons. Amino acid sequences of the human adrenoceptors and dopamine receptors, adrenoceptor subtypes $\alpha_{1A,B,D}$, $\alpha_{2A,B,C}$, and $\beta_{1,2,3}$ and dopamine receptor subtypes $D_{1A,1B}$ and $D_{2,3,4}$, were retrieved from the Swiss−Prot database.[75] Together with the amino acid sequence of bovine rhodopsin (extracted from the 2.8 Å resolution X-ray structure (ref 8; PDB entry 1HZX)), a multiple sequence alignment was built using Malign.[76] Regions that correspond to transmembrane segments from the bovine rhodopsin structure are characterized by amino acids highly conserved across most rhodopsin-like GPCRs, and could be aligned without any ambiguities.

Receptor Structure Modeling. Individual structural models of all human CABRs, $\beta_{1,2,3}$, $\alpha_{1A,B,D}$, and $\alpha_{2A,B,C}$ adrenoceptors, and the $D_{1A,B}$ and $D_{2,3,4}$ dopamine receptors were constructed using Modeller v7.1,[77] based on the individual pairwise sequence alignments with the bovine rhodopsin sequence, extracted from the multiple sequence alignment above. XL2 was modeled as a β-hairpin where Cxl2.50 was aligned on its equivalent in rhodopsin, forming the disulfide bridge with C3.25 observed in the rhodopsin structure. Gaps in XL2 from the pairwise sequence alignments were shifted upstream or downstream until a reasonable backbone of the protein main chain was constructed via Modeller. In β_2-AR from hamster, experimental evidence has identified a second disulfide bridge internal to XL2 linking Cxl2.43 and Cxl2.49.[78] Equivalent cysteines are found in all three human β-AR subtypes; this second disulfide bridge was introduced into the β-AR models. Overall, in regions of XL2 away from the disulfide bridge(s), the rhodopsin sequence and the amino acid sequences of the CABRs lack sufficient similarities for the CABRs to be modeled with any degree of confidence, but these regions are located away from the binding cavity and their influence on ligand binding is likely to be minimal.

Ten structural models were built for each receptor, and among those where D3.32 has a side-chain conformation $g+$, $\chi_1 = +60°$, one was chosen for further study. The other alternative conformations for D3.32 are *trans*, $\chi_1 = +180°$, where the carboxylic group of D3.32 points toward TM2 and is not a favorable orientation for ion-pair formation with catecholamines, and $g-$, $\chi_1 = -60°$, which in α-helices is never observed for steric reasons.[13,79]

Automated Docking and Receptor−Ligands Complexes. The relevant endogenous ligand, either dopamine or norepinephrine, was automatically docked to the binding cavities of receptor model structures using Gold v2.2.[40] Gold allows flexibility of the ligand while it keeps the binding site rigid. Standard parameters were used with the exception that during the docking procedure a 3.5 Å distance restraint was used to enforce interactions between (i) an oxygen atom from the carboxylate group of D3.32 and the protonated amine of the catecholamines; and (ii) the catecholic *para* and *meta*-hydroxyls, respectively, with oxygen atoms of S5.46 and S5.42. There is compelling evidence that these interactions do take place (e.g., D_1's, ref 17; D_2's, refs 26−29; α_1-ARs, refs 18,30; α_2-ARs, refs 14,31−33; β-ARs, refs 9,34,35). It was not possible to enforce simultaneously distance restraints between D3.32 and the protonated amine of the ligand and between S/C5.43 (in all CABRs but α_{1A}-AR with alanine at 5.43) and the *meta*-hydroxyl, because position 5.43 is located too far away from 3.32.

Predicting Regions Favorable for Interaction with the Catecholic Ring. Regions favorable for interacting groups (hydrogen-bond donors, hydrogen-bond acceptors, hydrophobic interactions) were computed for norepinephrine and dopamine, and for the binding sites of the β_2-AR and dopamine D_2 receptors. The methodology is described in detail in Rantanen et al.,[60−62] used by Xhaard et al.[13] to map the binding cavity of the α_2-ARs. Briefly, the binding cavity or the ligand structure was divided into molecular

fragments of 3−4 bonded atoms, and surrounding regions included within a 3D grid of points. A library of molecular interactions derived from protein−ligand complexes found in the PDB[60−62] was used to compute a conditional probability density function for molecular fragments that describe the most prevalent interactions observed for each fragment. Multiple contributions from different molecular fragments were pooled using a sum combination rule at each grid point and were used to assign locations suitable for hydrogen-bond donors, hydrogen-bond acceptors, and hydrophobic carbon atoms.

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Supporting Information Available: Interaction patterns for catechol binding to non-GPCRs. This material is available free of charge via the Internet at http://pubs.acs.org. The coordinates of the complete models for all 14 CABRs, the coordinates (limited to residues lining the binding cavity) of the optimized complex of β_2-AR with norepinephrine and the complex of D_2 with dopamine, and the maps predicting favorable interactions computed from both the ligands and the binding sites are available at http://www.abo.fi/fak/mnf/bkf/research/johnson/structures.html.

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(84) In the present study, to ease comparison of positions equivalent across the receptors, we will use the Ballesteros and Weinstein convention.[80] Hence, each position is indexed such that the first number indicates the helix to which the amino acid is located and the second number refers to the position of the amino acid relative to the most conservative position in the helix, which is arbitrarily assigned an index of 50. For example, the conserved aspartate 113 of the human α_{2A}-AR, located on TM3, is referred to as D3.32 (the most conservative position in TM3 among GPCRs is R3.50, part of the "DRY" sequence). In addition, Xhaard et al.[13] have extended the numbering scheme to XL2, referring to the conserved cysteine in this loop as Cxl2.50.

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