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# Ligand Binding, Activation, and Agonist Trafficking

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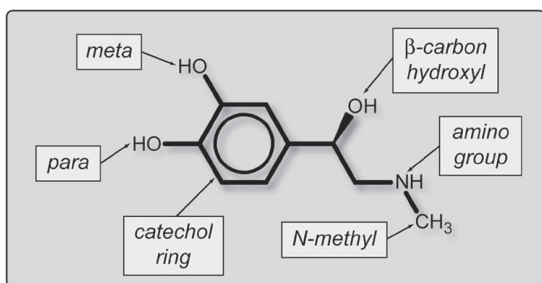
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### Summary

Adrenergic receptors are critical mediators of sympathetic nervous system-regulated physiological responses. Activated by the neurotransmitter and neurohormone, norepinephrine and epinephrine, released from sympathetic nerve endings and the adrenal medulla, respectively, they play a central role in this evolutionarily ancient defense system that regulates many physiological functions, including those involved in circulatory, metabolic, respiratory, and central nervous system homeostasis. In addition, alterations in the regulation and molecular structure of adrenergic receptors have been implicated in a variety of diseases, and drugs targeting these receptors are important and widely used therapeutics. The molecular cloning of the first adrenergic receptor in 1986 revealed structural homology with the functionally related rhodopsin visual transduction system—a finding that led to the realization that these receptors formed a new superfamily of proteins, now known as G protein-coupled receptors. Since that time, a plethora of structure–function studies have provided major insights into the molecular determinants of adrenergic receptor ligand-binding, activation, and signaling, many of which are relevant not only to the adrenergic receptor family but also, more generally, to the broader superfamily of G protein-coupled receptors. These advances in our understanding of adrenergic receptor activation, regulation, and functioning are reviewed in this chapter.

**Key Words:** Activation; adrenergic receptor; agonist trafficking; ligand binding; mechanism; mutagenesis; structure–function.

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**Fig. 1.** Chemical structure of (–)-epinephrine. Individual moieties, including the *meta*- and *para*-hydroxyls, catechol ring, protonated amine, alcoholic chiral  $\beta$ -carbon hydroxyl, and *N*-methyl group are indicated.

## 1. Adrenergic Receptor Ligand-Binding Sites

### 1.1. Binding Contacts of the Endogenous Ligands

In the early 1930s, Easson and Stedman proposed that receptor binding of a compound possessing a chiral center involved interactions between three contact points on the receptor and three moieties of the ligand (*1*). On the basis of experimental data on the activity of the enantiomers of epinephrine, they proposed that epinephrine's triad consisted of the basic group (the amine), the aromatic ring with its hydroxyl groups, and the alcoholic chiral,  $\beta$ -carbon hydroxyl group (*1*) (Fig. 1). The importance of these three chemical groups and their interaction with the adrenergic receptors (ARs) has been borne out by numerous mutagenesis and biochemical studies along with modeling studies performed since the first of the ARs, the mammalian  $\beta_2$ - and turkey  $\beta$ -AR, were cloned in 1986 (*2,3*).

However, as discussed in more detail later in this section, distinct interactions were also defined for both the aromatic ring and its hydroxyls, giving a total of at least four critical receptor/ligand moiety contacts. These studies also defined the binding site for endogenous agonists to be contained within a pocket formed by the clustering of the seven putative transmembrane (TM) helical bundles of the receptor and to be located approx 11 Å below the extracellular surface (*4*). The key interactions (Table 1) are (1) an ionic interaction between the amino group of the catechol with the carboxylate side chain of D3.32<sup>1</sup> of helix 3, (2) hydrogen bonding between the catechol *meta*- and *para*-hydroxyl groups and serine residues in helix 5, (3) an aromatic–aromatic interaction between the phenyl ring of agonists and aromatic residues in helix 6, and (4) hydrogen bonding between the chiral benzylic  $\beta$ -hydroxyl of agonists and a residue in helix 6, which accounts for the stereoselectivity of adrenergic ligands (Fig. 2).

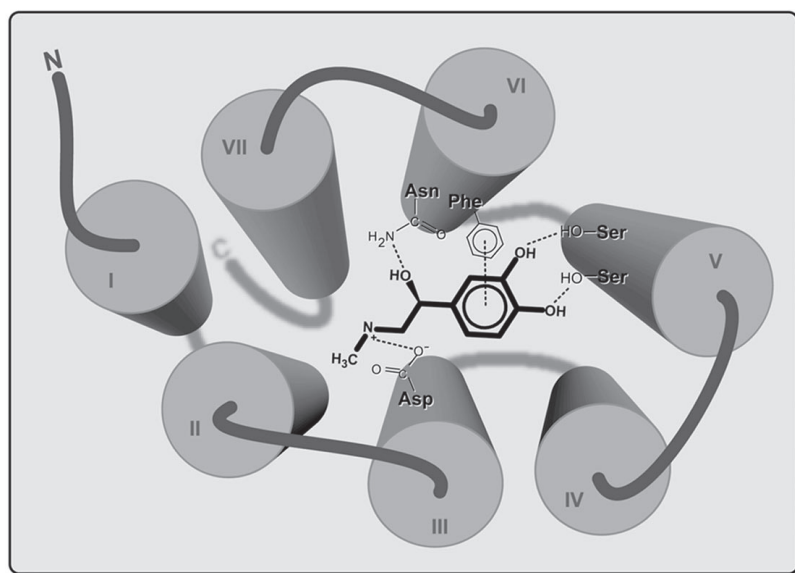
<sup>1</sup>See the Appendix on page 65 for residue numbering.

**Table 1**  
**Binding Contacts of Adrenergic Receptors With Endogenous Ligands**<sup>a,b</sup>

Receptor	Moieties of Endogenous Catecholamine Ligands					
	Amine	<i>para</i> -Hydroxyl	<i>meta</i> -Hydroxyl	Catechol Ring	$\beta$ -Carbon Hydroxyl	<i>N</i> -methyl
$\alpha_{1A}$	D3.32	S5.46	S5.42	F4.62, F5.41		
$\alpha_{1B}$	D3.32	S5.42	S5.42	F6.51		
$\alpha_{2A}$	D3.32	S5.46	—	F6.52, Y6.55	<i>D3.32</i> , <i>S2.61</i> , <i>S7.46</i>	<i>F7.38</i> , <i>F7.39</i>
$\beta_2$	D3.32	S5.46	S5.42, S5.43	F6.51, F6.52	N6.55, <i>D3.32</i> , T4.56	

<sup>a</sup> Interaction demonstrated experimentally.

<sup>b</sup> Residues in italics are those that have been proposed to interact based only on molecular modeling studies.



**Fig. 2.** Major interactions between (–)-epinephrine and its  $\beta$ -adrenergic receptor binding site: (a) ionic interaction between the amino group of epinephrine and the carboxylate side chain of an aspartic acid (Asp) in helix III; (b) hydrogen bonding between the catechol *meta*- and *para*-hydroxyl groups and serine (Ser) residues in helix V; (c) an aromatic–aromatic interaction between the phenyl ring of agonists and an aromatic residue (Phe) in helix VI, and (d) hydrogen bonding interaction between the chiral benzylic  $\beta$ -hydroxyl of epinephrine and a residue (Asn) in helix VI, which accounts for the stereoselectivity of adrenergic ligands. Transmembrane helices are indicated by Roman numerals.

Studies of the structure–activity relationship of adrenergic ligands have established the essential nature of the amine moiety for interaction with ARs (5). It was proposed that a counterion within the receptor was the site of interaction with this moiety. In attempts to identify the residue that provides this counterion, aspartyl and glutamyl residues conserved across all of the receptors that bind cationic amine ligands were identified, including the highly conserved D2.50. However, it was only the loss of D3.32 that dramatically affected the binding of ligands to the  $\beta_2$ -AR (6). D3.32 is conserved across all receptors that bind amine ligands. D3.32N or D3.32G<sup>2</sup> mutants of the  $\beta_2$ -AR had agonist binding below detectable levels; however, agonists were still fully efficacious at these mutated receptors despite their greatly decreased potency (as evidenced by an increase in the half maximal response (EC<sub>50</sub>) by 300- to 40,000-fold) (7). Mutation of D3.32 to Leu in the  $\beta_3$ -AR also resulted in loss of ligand binding (8). When D3.32 was mutated to Ser, the receptor could not be activated by amine-containing ligands. However, compounds in which the amine was replaced by a moiety that can serve as a hydrogen bond acceptor, such as a catechol ester or a ketone, which do not activate the wild-type receptor, activated the D3.32S mutant receptor (9). This finding demonstrated that an interaction between D3.32 and the agonist is required for both optimal binding and receptor activation, but that the nature of this interaction is not critical because it can be either ionic or hydrogen bonding. Subsequently, the salt bridge between the protonated amine of the ligand and the carboxylate side chain of D3.32 was shown to be essential for the binding of the endogenous catechols in all the AR subtypes (6,7,9–11). Of all the interactions involving the receptor and its endogenous ligands, this interaction has been demonstrated as the most important energetically (9).

Seryl residues in TM segment 5 (TM V) are the interaction sites for the hydroxyl groups of the catecholamines, although the exact hydrogen-bonding interactions formed between the agonist hydroxyls and the various TM V seryl residues differ among the various AR subtypes. The  $\alpha_{1A}$ -AR has only two TM V serines (S5.42, S5.46), and both of these form hydrogen bonds with the catechol hydroxyls of endogenous ligands. The contribution of these serines to ligand binding is equal, and each is able to compensate for the loss of the other because mutation of either to alanine does not affect the affinity of epinephrine; however, mutation of both leads to a marked loss of epinephrine affinity (12). S5.42 has been shown to bind the *meta*-hydroxyl of the ligand; the *para*-hydroxyl bonds with S5.46 (13). Although the  $\alpha_{1B}$ -AR has three serines available to interact with the hydroxyl moieties of the catechol ring of the endogenous ligands, only S5.42 has been demonstrated to be involved in ligand binding; in

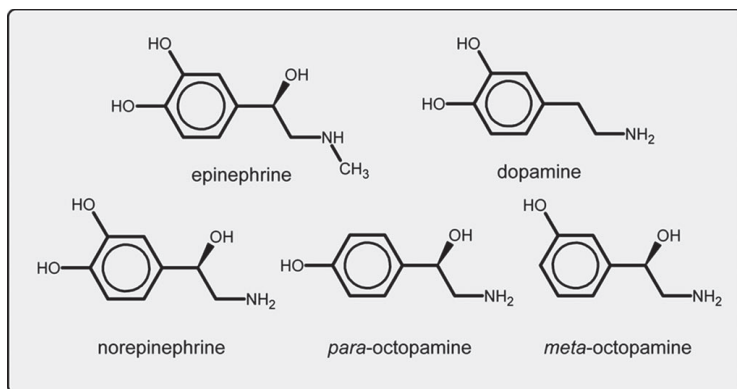
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<sup>2</sup> The nomenclature indicates that the aspartate has been mutated to asparagine or glycine, respectively.

this instance, it forms hydrogen bonds with both the *meta*- and *para*-hydroxyls (10). The  $\alpha_{2A}$ -AR also only utilizes one of the TM V serines (S5.46) to bind the endogenous ligands. Mutation of S5.46 of  $\alpha_{2A}$ -AR suggests a possible role for this serine in hydrogen bond interactions with the *para*-hydroxyl group of the catechol ring (14,15). Mutation of S5.42 of  $\alpha_{2A}$ -AR suggested that this residue does not directly participate in receptor–agonist interactions, in contrast with the corresponding serine residue in the  $\alpha_{1A}$ - and  $\beta_2$ -ARs, which has been postulated to interact with the *meta*-hydroxyl group of catecholamines (15). The interaction of the  $\beta_2$ -AR with the *meta*-hydroxyl of a catecholamine agonist has been postulated to occur via a bifurcated hydrogen bond between the hydroxyl and both S5.42 and S5.43 (16). As in all the other ARs (with the exception of  $\alpha_{1B}$ ), the *para*-hydroxyl of the endogenous ligands binds to S5.46 of the  $\beta_2$ -AR (17). Binding of catechol agonists with the serines of TM V has also been proposed to play an important role in the orientation of the ligand within the binding pocket (11,12,16). Based on the differences in the serines used to interact with the *meta*- and *para*-hydroxyls of the ligand, it has been predicted that the catechol ring lies parallel to the extracellular surface of the  $\alpha$ -ARs; it is rotated 120° from this planar orientation in the  $\beta$ -ARs (12,16).

The third proposed component of the interaction between the endogenous ligands and the ARs is the interaction of the catechol ring with aromatic residues of the receptor. Pharmacophore mapping indicates that the catechol ring is essential for ligand binding (18). Phenylalanines in helices 4, 5, and 6 have been postulated to interact with the catechol ring. In the case of the  $\alpha_{1A}$ -AR, F4.62 and F5.41 have been shown to be involved in independent aromatic interactions with the catechol ring of endogenous ligands (19). Molecular modeling studies of the  $\alpha_{1B}$ -AR, in which ligand has been docked, indicated that the F6.51 side chain is well positioned to interact with the catechol ring (20). There is experimental evidence that the side chain of F6.51 is both solvent accessible and directed into the agonist-binding pocket (21). Direct evidence for the involvement of F6.51 in an aromatic–aromatic interaction with catechol agonists comes from detailed mutagenesis studies coupled with binding and activation studies using a variety of agonists (21). These studies provided compelling evidence that not only is the interaction between F6.51, but not F6.52, and the phenyl ring critical for ligand binding and for positioning the ligand in the correct orientation, but also that F5.61 is a critical switch involved in receptor activation (20). In the  $\alpha_{2A}$ -AR, it has been proposed that the phenyl group of the ligand interacts with Y6.55 and F6.52 in a  $\pi$ – $\pi$  stacking interaction (11). Both F6.51 and F6.52 have been proposed to stabilize the catechol ring of the  $\beta_2$ -AR, along with Y7.53 (21,22), in contrast to the  $\alpha_{1B}$ -AR, in which only F6.51 interacts with the aromatic ring (20).

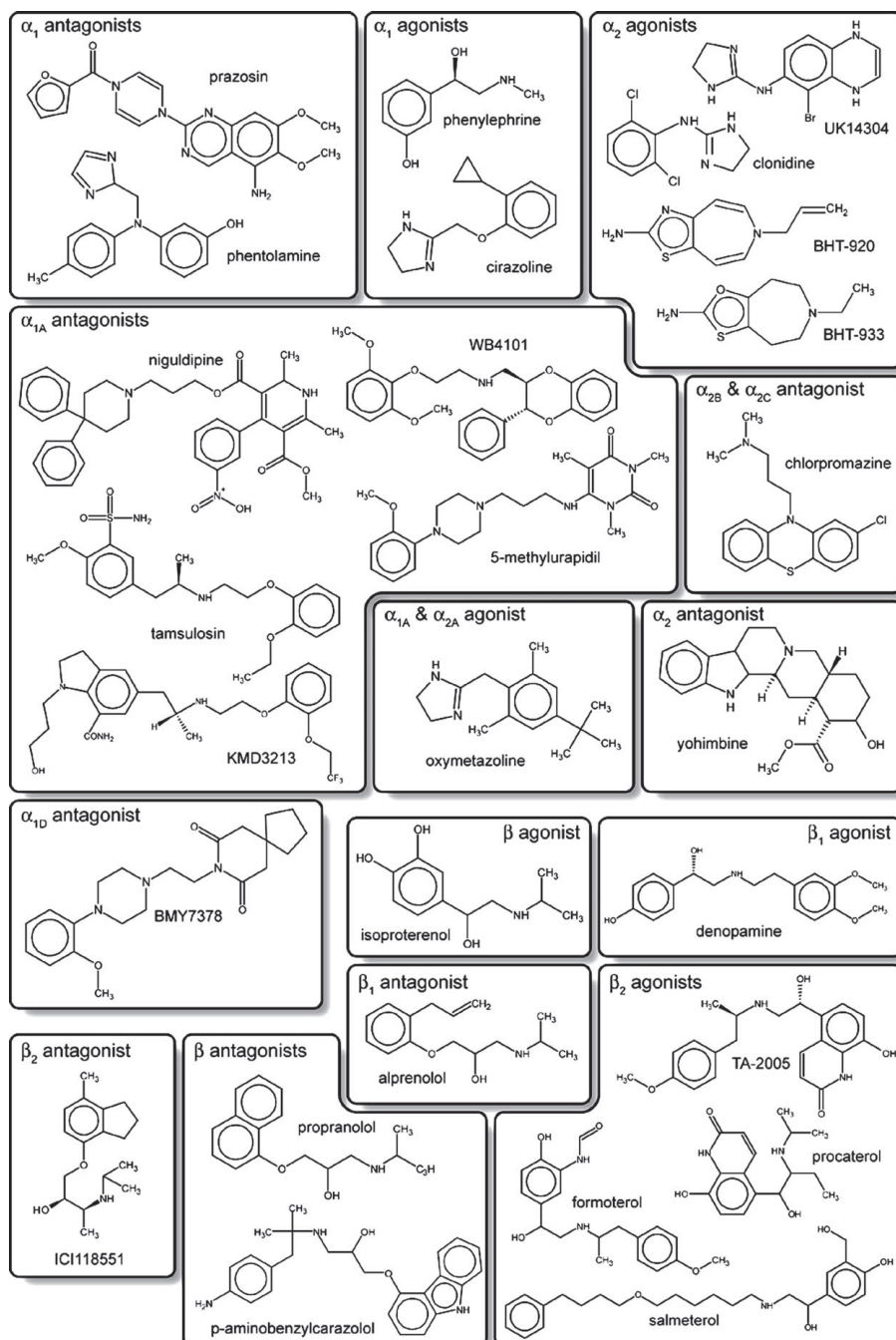
ARs show marked stereoselectivity for the (–)-enantiomers of epinephrine and norepinephrine, in terms of both binding affinity and agonist potency (23,24).



**Fig. 3.** Chemical structure of endogenous catecholamines.

This stereoselectivity is the underlying basis for the Easson and Stedman hypothesis (1). Nevertheless, the receptor contact responsible for this stereoselectivity has not been clearly identified. Early molecular modeling of the  $\beta_2$ -AR proposed S4.57 as the site of interaction between the chiral  $\beta$ -hydroxyl and the receptor (25,26). Mutation of T4.56, the residue directly N-terminal to S4.57, to an isoleucine provided supporting evidence for the involvement of S4.57 in binding the chiral  $\beta$ -hydroxyl (27). This mutant displayed an approximately fourfold reduction in the affinity of epinephrine and norepinephrine for the receptor but had no effect on the affinity of dopamine (which lacks the chiral hydroxyl) (Fig. 3) (27). The authors suggested that this effect is not caused by a loss of a direct interaction between T4.56, but instead that the mutation results in a conformational change that prevents the interaction of the  $\beta$ -hydroxyl with S4.57. In this study, direct evidence for the involvement of S4.57 could not be obtained because its mutation to an alanine resulted in a receptor that failed to be expressed in the plasma membrane, possibly because of global misfolding (17). However, in another study expression of the S4.57 mutant was obtained, and this mutant retained stereoselectivity isoproterenol (Fig. 4) binding (24), hence, eliminating S4.57 as the binding partner for the chiral  $\beta$ -hydroxyl.

Molecular modeling, using membStruk (a computational method to predict atomic level tertiary structure using only primary sequence) and a *de novo* model, has predicted that, in the (–)-enantiomer, the  $\beta$ -hydroxyl interacts with N6.55 (28,29), a prediction that was not confirmed with a homology model based on the crystal structure of rhodopsin (29). Mutation of N6.55 to a leucine resulted in a receptor with a sixfold decrease in its stereoselectivity of the receptor of (–)-isoproterenol vs (+)-isoproterenol (24). However, this loss of binding stereoselectivity was confined to agonists, with the N6.55L mutant showing no change in its stereospecific recognition of neutral antagonists or partial agonists (24). The role of N6.55 has also been examined in the  $\beta_3$ -AR. Mutation of N6.55 to



**Fig. 4.** Chemical structures of synthetic subtype-selective ligands for adrenergic receptors.

alanine did not alter the stereoselectivity of the binding of partial agonists but did result in these compounds displaying reduced efficacy while not altering the efficacy of the full agonists, norepinephrine and isoproterenol (8).

Another molecular model of the  $\beta_2$ -AR also predicts an interaction not only between N6.55 and the  $\beta$ -hydroxyl, but also between the  $\beta$ -hydroxyl and D3.32 (28). This last interaction has also been suggested in a model of norepinephrine binding to the  $\alpha_{2A}$ -AR (30), which proposed that a bidendate hydrogen bond is formed between oxygen atoms of the D3.32 side chain and both the  $\beta$ -hydroxyl and amide groups of the ligand. However, direct evaluation of D3.32 in stereoselectivity of ligand binding has been hindered by the poor expression of an alanine substitution mutant (31,32).

Two seryl residues, S2.61 and S7.46, have also been suggested to play a role in stereoselective ligand recognition by the  $\alpha_{2A}$ -AR because their mutation to alanine resulted in marked reduction in (–)-norepinephrine affinity, with little change in affinity for the (+)-enantiomer or for dopamine (23). However, involvement of S2.61 was not supported by the modeling data of Nyronen et al. (11). In the study of Hieble et al. (23), evidence was obtained to indicate that S4.57, T6.54, Y6.55, and T6.56 are not involved in determining stereoselectivity of agonist binding.

Interactions between ARs and their ligand moieties, distinct from those proposed by Easson and Stedman, have been suggested. For example, molecular modeling of the interaction of the  $\alpha_{2A}$ -AR with epinephrine has indicated that its *N*-methyl group may bond with F7.38 and F7.39 (11). However, experimental support for this interaction from mutagenesis studies is lacking.

## 1.2. Structural Basis of Subtype Selectivity and Antagonist Binding

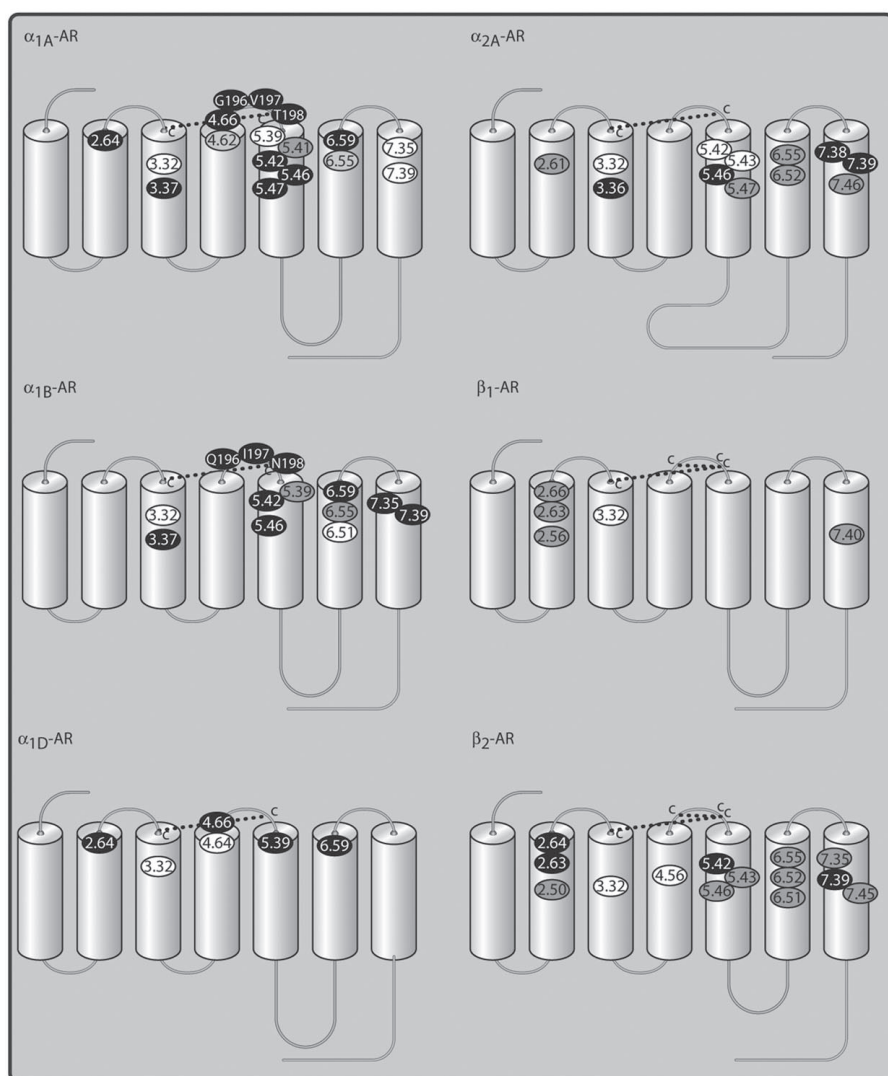
The ARs, particularly those of a particular type (e.g.,  $\alpha_1$ ,  $\alpha_2$ ,  $\beta$ ) share a high degree of amino acid homology, especially within the TM forming the ligand-binding pocket (68–77% identity for  $\alpha_1$ -, 79–82% for  $\alpha_2$ -, and 63–73% for  $\beta$ -ARs). Nevertheless, although all amide-containing ligands bind to D3.32 and many also bind to the serines of TM V, ligand-binding residues unique to each receptor have been identified (Fig. 5) that confer subtype selectivity. In some instances, it has been possible to identify residues responsible for subtype selectivity (Fig.5); in other cases, the lack of ligands that are sufficiently selective for particular subtypes has limited such analyses.

In a detailed study of the residues responsible for subtype selectivity, Hwa et al. (33) demonstrated that just 2 of the approx 172 residues forming the TM segments of the  $\alpha_{1B}$ -AR account for its agonist-binding selectivity as compared to the  $\alpha_{1A}$ -AR. The approach used to identify these two residues, at positions 5.39 ( $\alpha_{1B}$  = A,  $\alpha_{1A}$  = V) and 6.55 ( $\alpha_{1B}$  = L,  $\alpha_{1A}$  = M), is instructive. Based on the reasoning that the residues potentially involved in subtype selectivity should be distinct between the two receptor subtypes, only the 48 nonconserved residues forming the

TM segments were considered. Of these, candidate residues were selected based on the following criteria: (1) location in the ligand-binding extracellular half of the TM segments, and (2) orientation of side chains toward the putative ligand-binding pocket. Residues excluded were those on TM I because this helix was known not to be involved in ligand binding and those for which a lack of conservation was merely caused by interspecies differences given that  $\alpha_1$ -subtype ligand-binding profiles are conserved across species. This allowed the initial set of 48 nonconserved residues to be reduced to only 7 that fulfilled all criteria, which were then subjected to mutational analysis. Mutation of A5.39 in the  $\alpha_{1B}$ -AR to a valine, the residue found at this position in the  $\alpha_{1A}$ -AR, resulted in an 80% conversion of agonist-selective binding from that of the  $\alpha_{1B}$ - to that of the  $\alpha_{1A}$ -subtype; the remaining 20% were accounted for by the additional substitution of L6.55 in the  $\alpha_{1B}$ -AR to a methionine, the residue at this position in the  $\alpha_{1A}$ -AR (33). It was proposed that A5.39V-induced increase in the affinity for  $\alpha_{1A}$ -AR-selective ligands was caused by an increased hydrophobic interaction between valine and the aromatic ring of agonist ligands; the additional 20% increase in affinity provided by L6.55M was caused by increased interactions resulting from the extended chain length of the ortho hydrophobic groups of synthetic ligands (33), a postulate supported by the finding that the complementary substitutions of both V5.39A and M6.55V in the  $\alpha_{1A}$ -AR converted its agonist selectivity entirely to that of the  $\alpha_{1B}$ -AR.

Interestingly, neither the single mutations at the 5.39 or 6.55 positions nor the double mutations at both positions altered antagonist binding, demonstrating the agonist-specific nature of these interactions (33). However, either single mutation (A5.39V or L6.55M in the  $\alpha_{1B}$ -AR or V5.39A or M6.55L in the  $\alpha_{1A}$ -AR) resulted in constitutive activity that could be rescued by additional substitution of the complimentary residue responsible for agonist selectivity (33).

In contrast to the TM V and VI residues involved in subtype-selective agonist binding, residues in extracellular loop 2 ( $e_2$ ) have been found to be responsible for the selective binding of antagonists by the  $\alpha_{1A}$ - and  $\alpha_{1B}$ -ARs. Thus, substitution of Gly<sup>196</sup>, Val<sup>197</sup>, and Thr<sup>198</sup> of the  $\alpha_{1B}$ -AR to the corresponding residues (Gln, Ile, and Asn, respectively) in the  $\alpha_{1A}$ -AR, or vice versa, leads to a change in the antagonist-binding profile to that of the other subtype (34) (Fig. 5). This finding, and those of several other studies, has provided evidence that  $\alpha_1$ -selective antagonists bind at a site closer to the extracellular loops, which is above the plane of the binding site and skewed toward TM VII (35,36). The binding of antagonists to  $e_2$  also raises the question of the spatial arrangement of this loop with respect to the TM domains. In rhodopsin,  $e_2$  covers the binding site like a lid. It is tethered by a disulfide bond (found in almost all G protein-coupled receptors [GPCRs]) connecting it to  $e_1$  at the N-terminal end of TM III and forms multiple interactions with the chromophore ligand, 11-*cis* retinal (37,38). It has been postulated that this structural arrangement traps the chromophore and prevents its extrusion into



**Fig. 5.** Residues involved in determining subtype and ligand selectivity. Residues important for antagonist binding are shown in black, for agonist binding in grey, and for both in white. Residue numbers are indicated by the Ballesteros and Weinstein system (see the Appendix on page 65).

- 2.50 Mutation of D2.50 of the  $\beta_2$ -AR results in decreased affinity for agonists but not antagonists (6)
- 2.56 In the  $\beta_1$ -AR, L2.56 makes a major contribution to binding interactions for those agonists containing a dimethoxyphenyl group (43,44)
- 2.61 S2.61 is involved in the stereoselectivity of  $\alpha_{2A}$ -AR for (–)-enantiomers of catecholamines; It has been proposed that this site represents an important point for attachment of the  $\beta$ -hydroxyl group of catecholamines (287)
- 2.63 In the  $\beta_1$ -AR, T2.63 constitutes a major binding interaction for those agonists containing a dimethoxyphenyl group (43,44); in the  $\beta_2$ -AR, S2.63 interacts with *p*-aminobenzylcarazolol (288)

- 2.64 M2.64 of  $\alpha_{1D}$ -AR imparts its selectivity for niguldipine vs  $\alpha_{1A}$ -AR (F2.64) (34,289,290); H2.64 of  $\beta_2$ -AR interacts with *p*-aminobenzylcarazolol (291)
- 2.66 V2.66 of the  $\beta_1$ -AR contributes to agonist binding (43,44)
- 3.32 D3.32 of the adrenergic receptors is important for binding of the amine moiety of ligands (6,7,291)
- 3.36 C3.36 of the  $\alpha_{2A}$ -AR interacts with antagonist phenoxybenzamine; this position is important for binding of imidazoline derivatives (292)
- 3.37 T3.37 has been predicted to interact with the 2-methoxy group of the quinazoline ring of prazosin in the  $\alpha_{1A}$ - and  $\alpha_{1B}$ -AR (36)
- 4.56 The T4.56I mutation in the  $\beta_2$ -AR results in loss of affinity for agonists with a  $\beta$ -hydroxyl group (27)
- 4.62 F4.62 is involved in agonist binding to the  $\alpha_{1A}$ -AR (19)
- 4.64 W4.64 of the  $\alpha_{1D}$ -AR is involved in agonist and antagonist binding (293)
- 4.66 E4.66 of  $\alpha_{1D}$ -AR imparts selectivity for KMD3213 vs  $\alpha_{1A}$ -AR (Q4.66) (36)
- e<sub>2</sub> The selectivity between  $\alpha_{1A}$ -AR and  $\alpha_{1B}$ -AR for the binding of the antagonists phentolamine and WB4101 is caused by differences in e<sub>2</sub>; Q196 is predicted to hydrogen bond with the imidazoline nitrogen of phentolamine or the dioxan oxygen of WB4101; I197 is proposed to have hydrophobic interactions with the methyl of the phenyl ring or the ring itself of phentolamine and WB4101; N198 is thought to hydrogen bond with the nitrogen of the linker of these antagonists (34)
- 5.39 V5.39 of the human  $\alpha_{1A}$ -AR imparts  $\alpha_{1D}$  (A5.39) selectivity for oxazole antagonists and selectivity vs  $\alpha_{1B}$  (A5.39) for agonists (33,294)
- 5.41 F5.41 is involved in agonist binding to the  $\alpha_{1A}$ -AR (19)
- 5.42 S5.42 of the  $\alpha_1$ -AR has been proposed to interact with the 1-nitrogen of the quinazoline of prazosin (36); it has also been implicated in the binding of the *meta*-OH of phenethylamine ligands in the  $\alpha_{2A}$ -AR (295); removal of the hydroxyl moiety of S5.42 of the  $\beta_2$ -AR leads to loss of affinity of antagonists with nitrogen in their heterocyclic ring and isoproterenol; pindolol's heterocyclic ring has been predicted to hydrogen bond with S4.52, but this position has no effect on the affinity of  $\beta_2$ -AR for propranolol and alprenolol (16)
- 5.43 S5.43 of the  $\alpha_{2A}$ -AR is responsible for the selectivity of UK14304 but not chlorpromazine and interspecies variation in yohimbine affinity (296); in the  $\beta_2$ -AR, S5.43 is involved in isoproterenol binding (297)
- 5.46 S5.46 of the  $\alpha$ -ARs has been predicted to interact with 2-methoxy of the quinazoline ring of prazosin (36); in the  $\beta_2$ -AR S5.46 is involved in isoproterenol binding and binding of  $\beta$ -carbon hydroxyl of native ligands (297)
- 5.47 F5.47 of  $\alpha_{1A}$ -AR has been demonstrated to interact with antagonists 5-methylurapidil, HEAT, and WB4101 but not niguldipine (35)
- 6.51 F6.51 has been shown to be an interaction site between agonists and antagonists and the  $\alpha_{1B}$ -AR and has been suggested to be involved in ligand binding to the  $\beta_2$ -AR (20,22)
- 6.52 F6.52 of the  $\alpha_{2A}$ -AR interacts with the catechol ring of agonists and has been suggested to be involved in ligand binding to the  $\beta_2$ -AR (11,22)
- 6.55 M6.55 of the  $\alpha_{1A}$ -AR imparts selectivity vs  $\alpha_{1B}$ -AR (L6.55) for agonists (33); Y6.55 of the  $\alpha_{2A}$ -AR interacts with catechol ring of agonists (11); in the  $\beta_2$ -AR, N6.55 plays a role in the stereospecificity of agonists (24)
- 6.59 S6.59 of the  $\alpha_1$ -ARs has been predicted to hydrogen bond with the carbonyl group between the piperazine and furan rings of prazosin (36)
- 7.35 F7.35 of  $\alpha_{1A}$ - and  $\alpha_{1B}$ -AR is a major contributor to the affinity of the antagonists prazosin, WB4101, niguldipine, and 5-methylurapidil; however, it is not involved in the binding of phenethylamine agonists (i.e., epinephrine) but is involved in the binding of imidazoline agonists such as oxymetazoline and cirazoline (35); F7.35 is also responsible for high-affinity binding of TA-2005 to the  $\beta_2$ -AR (45) and is predicted to interact with the ether oxygen in side chain of salmeterol, formoterol, and procaterol and is important for  $\beta_2$ -AR selectivity (46)
- 7.38 F7.38 is important in the interaction of phenethylamines with  $\alpha_{2A}$ -AR (11)
- 7.39 F7.39 of the  $\alpha_1$ -ARs contributes to the interaction with antagonists and imidazoline agonists but not phenethylamine agonists (35); F7.39 is also critical for the binding of yohimbine to the  $\alpha_{2A}$ -AR (48); in the  $\beta_2$ -AR, N7.39 is important for the binding of the antagonists propranolol and alprenolol; if this residue is substituted for Gln or Thr, an increase in affinity for the  $\alpha$ -AR ligand yohimbine is seen (47)
- 7.40 W7.40 is proposed to play a role in agonist binding to the  $\beta_1$ -AR (298)
- 7.45 N7.45 of the  $\beta_2$ -AR is involved in agonist affinity (6)
- 7.46 S7.46 is involved in the affinity of the (–)-enantiomers of the catecholamines for the  $\alpha_{2A}$ -AR but plays no role in the affinity of (+)-enantiomers (23)

the extracellular environment as rhodopsin undergoes the conformational rearrangement associated with its activation or may stabilize rhodopsin's active state. It is unlikely, however, that this structural rearrangement is entirely analogous in ARs because this would impede ligand entry from the extracellular milieu into the binding pocket. In the case of rhodopsin, the chromophore enters (and also exits after activation to be reisomerized back from the all-*trans* to the 11-*cis* conformation) from the cytoplasmic side (39).

Other residues identified to be involved in antagonist binding by the  $\alpha_{1A}$ -AR are two aromatics (F7.35 and F7.39) located at the extracellular end of TM VII (35). Mutation of these residues, which are also conserved in other  $\alpha_1$ -subtypes and in all  $\alpha_2$ -ARs but not in  $\beta$ -ARs, markedly reduced the affinity of the receptor for all antagonists with no change in the affinity of phenethylamine-type agonists (35). However, both residues were also shown to be involved in the binding of all imidazoline-type agonists (35)—a finding indicating that this class of agonists binds differently to phenethylamine agonists, as predicted by the Easson-Stedman hypothesis (1). In this regard, imidazoline agonists are more antagonistlike, which may account for many of them being partial agonists (40,41).

Phenylalanines (4.62 and 5.41) in TM IV and V of the  $\alpha_{1A}$ -AR have also been identified as forming potentially novel contacts involved in agonist binding but not receptor activation. Interestingly, whereas substitution of both of these  $\alpha_{1A}$ -residues with the corresponding Gln and Ala of the  $\beta_2$ -AR markedly reduced agonist affinity, the reverse substitutions in the  $\beta_2$ -AR increased agonist affinity (19). Although antagonist binding was unaltered with these substitutions (19), given that 4.62 is not conserved in other  $\alpha_1$ -subtypes, it remains unclear if the affinity changes observed are caused by these residues forming direct ligand contacts or rather are indirect as a result of mutation-induced local conformational changes.

Macromolecular modeling studies have been undertaken to predict which receptor residues form critical interactions with the various moieties of  $\alpha_1$ -antagonists, and contacts have been suggested with residues in TM III, IV, V, VI, and VII (35,36,42). For the non-subtype-selective  $\alpha_1$ -antagonist, prazosin (Fig. 4), for example, the 4-amino group and 1-nitrogen atom of its quinazoline ring have been suggested to interact with the carboxyl group of Asp 3.32 and hydroxyl group of Ser 5.42, respectively. Interaction of the two methoxy groups of its quinazoline ring with the hydroxyl groups of Thr 3.37 and Ser 5.46 and the carbonyl group between the piperazine and furan rings and Ser 6.59 have been identified for all  $\alpha_1$ -AR subtypes (36). For tamulosin, an  $\alpha_{1D}$ -selective compound, more interactions were found for this subtype than for the other two  $\alpha_1$ -subtypes, whereas for KMD-3213 (Fig. 4), an  $\alpha_{1A}$ -selective compound, more interactions were found with the  $\alpha_{1A}$ -AR (36). However, these docking interactions have yet to be confirmed by formal structure–function studies.

Although TM V and VI are important for  $\alpha_1$ -AR ligand discrimination, the selectivity of  $\beta$ -AR agonists is because of interactions with TM II and VII.  $\beta_1$ -AR selective agonists (e.g., denopamine; Fig. 4) interact with L2.56, T2.63, and V2.66, and it is these residues that have been demonstrated to impart selectivity (43,44). Y7.35 at the extracellular end of TM VII has been demonstrated to be critical for the binding of several  $\beta_2$ -AR agonists (43–46). The subtype selectivity of antagonist binding by the  $\beta_2$ -AR is also caused by an interaction with a TM VII residue, N7.39, which has been shown to be important for effective binding of  $\beta_2$ -AR antagonists, such as propranolol (Fig. 4) (47). Interestingly, not only is the asparagine side chain required for the binding of  $\beta_2$ -antagonists, but also its substitution with Gln or Thr increases the affinity of the  $\beta_2$ -AR for the  $\alpha_2$ -AR-selective antagonist yohimbine (Fig. 4) (47) despite the native residue at 7.39 in all  $\alpha$ -AR being a phenylalanine. Conversely, if F7.39 of  $\alpha_2$ -AR is replaced with Asn, the  $\alpha_2$ -AR now displays decreased affinity for yohimbine and increases affinity for the  $\beta$ -antagonist alprenolol (Fig. 4) (48).

## 2. Receptor Activation

### 2.1. Theory and Models

The concept of proteins as drug targets was proposed at the end of the 19th century. Ehrlich and Langley both contributed to the notion that compounds evoked biological activity by binding to cellular constituents (Ehrlich stated “*corpora non agunt, nisi fixata*,” i.e., agents cannot act without binding; [49]) that were soon named *receptors* (Langley’s “receptive substances”; [50]). Analogous to Fisher’s lock-and-key theory of enzyme action (51), these concepts led to a model of a receptor that is a rigid structure switched on by the turning of a key (ligand). A more dynamic picture of receptor activation evolved from about the middle of the twentieth century, with models failing to provide for more than one affinity state of the receptor (e.g., the collision coupling model of Tolkovsky et al. [52] and the random matrix hit model of Bergman et al. [53]) giving way to those that could because the existence of more than one affinity state was clearly evident experimentally. The last included the two noninterconvertible site model (53); the cyclic (allosteric) model, initially proposed by Katz and Theleff (54) in 1957 and then used by Weiland and Taylor (55) to successfully model the binding of agonists and antagonists by cholinergic receptors; the divalent receptor model (56); and eventually the ternary complex model (57). The last was developed to explain the interaction of the agonist-bound receptor with its cognate G protein, with high agonist affinity observed when the receptor was complexed with its G protein, but low affinity when the receptor–G protein interaction was disrupted (58).

This finding of modulation of affinity by a cognate interacting component, the G protein, was a major contravention of the accepted dogma of the time, which posited that receptor activation involved merely a binary interaction with ligand.

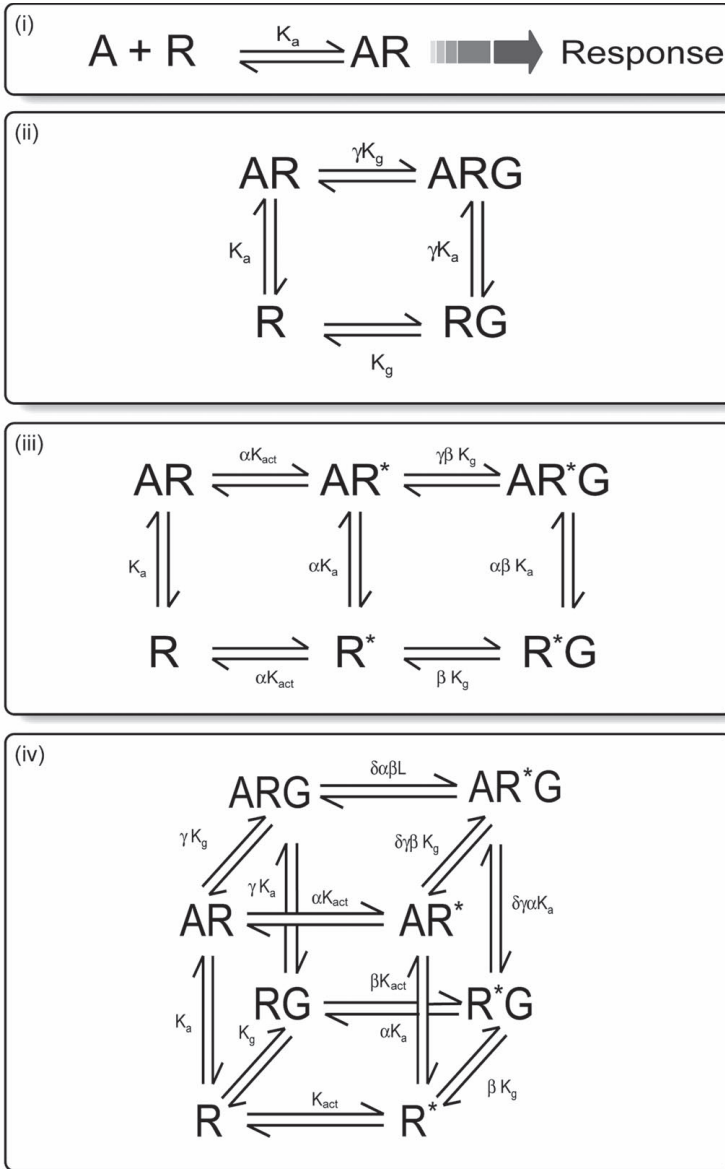
Nevertheless, intrinsic to all of these models, including the ternary complex model (Fig. 6), was the assumption that the binding of agonist is essential for receptor activation and signaling caused by a large energy barrier between the basal state  $R$  and the activated state  $R^*$ . Accordingly, these models invoked an inductive step (ligand induction model) by which the free energy of agonist binding is required to allow the energy barrier between the inactive and active state to be surmounted.

The finding that receptors exhibit constitutive signaling, which is signaling in the absence of agonist (increased basal activity)—a property of GPCRs that was first revealed with the availability of cloned receptors that could be markedly overexpressed (59)—coupled also with the finding that mutations could render receptors constitutively active (60–62) led to proposal of an extended ternary complex (ETC) model (Fig. 6) (63). Akin to the conformational selection model proposed by Koshland and Neet for enzymes (64), central to this model is that receptors can spontaneously isomerize between  $R$  and  $R^*$ , with the binding of agonist merely selecting or stabilizing the active state. In addition to explicitly allowing for spontaneous isomerization between  $R$  and  $R^*$ , the ETC model also accounts for the G protein-independent high agonist (but not antagonist) affinity displayed by constitutively active mutant receptors and for the effects of different classes of drugs (full agonist, partial agonists, inverse agonist, neutral antagonists) (65–67).

Although the ETC model explains most GPCR behavior, a more thermodynamically complete model, the cubic ternary complex (CTC) model (Fig. 6), was subsequently proposed (68–70). This model is merely an extension of the ETC model (that is, the ETC model is one of the subsets making up the CTC model) that allows for the existence of an inactive ternary complex, ARG, although both models similarly predict GPCR behavior. However, at the time of development of the ETC and CTC models, neither specifically accommodated experimental

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**Fig. 6.** (*Opposite page*) Evolution of receptor occupancy models. (i) The first models of receptor occupancy were based on the binding of an agonist  $A$  to the receptor  $R$ , leading to the generation of a response. However, this model could not account for experimental data showing high agonist affinity when the receptor was complexed with its G protein ( $G$ ), but low affinity when the receptor–G protein interaction was disrupted (58). (ii) This led to the development of the ternary complex model. (iii) The finding that receptors can exhibit constitutive signaling resulted in the extended ternary complex (ETC) model, which allows for spontaneous isomerization between inactive ( $R$ ) and active ( $R^*$ ) receptor. However, the ETC assumes that only the active state of the receptor interacts with the G protein, and hence (iv) the cubic ternary complex model builds on the extended ternary complex model with the additional interaction of the inactive receptor with the G protein. (*Figure caption continued on next page.*)



**Fig. 6.** (Figure caption continued from previous page) Modeling parameters:  $K_a$ , equilibrium constant for ligand binding;  $K_g$ , equilibrium constant for binding of G protein to inactive receptor;  $\alpha$ , effect of ligand binding on receptor activation, effect of activation on ligand binding;  $\beta$ , effect of receptor activation on G protein binding, effect of G protein coupling on activation;  $\gamma$ , effect of ligand binding on G protein coupling;  $\delta$ , measure of synergism between any two of receptor activation, G protein coupling, or ligand binding on the level of the third. (Based on a figure from ref. 299.)

evidence predicting the formation of more than one active state (e.g.,  $R_1^*$  and  $R_2^*$ ), each of which can activate a distinct G protein and effector. Both the ETC and CTC models can accommodate agonist-specific receptor active conformations because the thermodynamic constants  $\alpha$  and  $\gamma$  for ETC and  $\alpha$ ,  $\gamma$ , and  $\delta$  for CTC allow for the microaffinity constant of the ligand-bound receptor to be specific to the ligand; that is, the affinity of ligand-bound receptor for G protein can vary (71–73).

Intrinsic to all ternary complex models is the premise that agonists bind with higher affinity to the  $R^*$  state, and this is required to stabilize or select the active conformation. Kobilka and colleagues argued that this feature of the models does not accommodate the fact that agonists bind rapidly to  $R$ , whereas the kinetics of the agonist-induced conformational change in the absence of G protein is slow (74). Moreover, both agonists, which supposedly stabilize the  $R^*$  state, and inverse agonists, which stabilize the basal state  $R$ , protect the receptor from thermal denaturation and from proteolytic degradation (75). This implies that the conformations stabilized by both agonists and inverse agonists are distinct from that of the unliganded receptor. Accordingly, Gether and Kobilka (76) suggested that the unliganded receptor  $R$  represents a unique conformation that can undergo transition to either of at least two other states,  $R^\circ$  and  $R^*$ , stabilized by inverse agonists and agonist, respectively. This model is consistent with findings from studies of various GPCR mutants, which can best be explained only by invoking the existence of conformations that are intermediate between  $R'$  and  $R^*$  (20). Further, these studies suggest that whereas conformational selection can be invoked to account for a degree of basal activity—consistent with spontaneous isomerization from  $R$  to some intermediate  $R'$ —to get to the fully active  $R^*$  state requires an inductive step.

Of interest in this regard is a comparison of the transducin (the G protein coupled to rhodopsin) activation response resulting from photoactivation of rhodopsin vs that resulting from treatment of the protein opsin with the agonist all-*trans* retinal (77). With the former, the chromophore 11-*cis* retinal is bound to opsin and functions as an inverse agonist that stabilizes the inactive state, even in the presence of mutations that would otherwise lead to constitutive activity (78). Thus, photoactivation results in the complete transition from the inactive state ( $R^\circ$  in the dark rhodopsin shows no appreciable binding to transducin) to the fully activated state ( $R^*$ ). By contrast, all-*trans* retinal presumably only induces or stabilizes the transition of opsin from an intermediate state  $R$  (the unliganded state) to  $R^*$ . It is surprising, nonetheless, that the transducin response with all-*trans* retinal is a mere 14% of that observed with light activation (77). Further, this extraordinarily low degree of activation by all-*trans* retinal occurs despite the fact that it binds much more rapidly to opsin than does 11-*cis* retinal (77). Although rhodopsin is distinct from other GPCRs in having its basal state stabi-

lized by a covalently bound inverse agonist, its activation mechanisms and the conformational changes that occur with activation are nonetheless very similar to those observed with other GPCRs, especially ARs. Thus, by analogy, activation of ARs by agonists, in which isomerization may be restricted only to a transition from  $R$  to  $R^*$ , may only be realizing a fraction of the activation potential that might be possible with transition from  $R^\circ$  to  $R^*$ . These considerations also reinforce the notion that multiple conformations,  $R'$ ,  $R''$ , and so on, probably exist between  $R^\circ$  and  $R^*$ . Such conformational intermediates are likely revealed by various mutations, which presumably lower the energy barrier required for the spontaneous adoption of a partially activated conformation (cf. refs. 20 and 79).

It is evident that each successive model of receptor activation has been formulated to account parsimoniously for new experimental findings as they have been advanced. All, however, are based on two concepts. First, receptor activation involves a conformational change in the receptor protein that is imparted to its coupled intracellular machinery rather than some other mode of activation, such as relay of redox changes, as is operative, for example, with *Rhodospseudomonas viridis*, the first membrane protein receptor system yielding to high-resolution X-ray crystallographic structural analysis (80). Evidence that activation of GPCRs indeed involves a conformational change in the receptor protein is now overwhelming, as exemplified, for example, by the spectroscopic identification of conformational intermediates with activation of rhodopsin (81,82) and, more recently, with activation of the  $\beta$ -AR (83). In addition, direct evidence for helical movements with activation, initially obtained from studies of photocycle intermediates of the archeobacterial proton pump bacteriorhodopsin (84) and from site-directed spin-labeling studies of both bacteriorhodopsin and rhodopsin, have given rise to the helix movement model of GPCR activation (85–87).

The second concept underlying receptor activation theory is the notion that it involves a single quiescent species that is activated by agonist; that is, there exist but two receptor states,  $R$  and  $R^*$ . As indicated above, this notion has been challenged by evidence that receptors can isomerize to more than one active state (e.g.,  $R^{*1}$ ,  $R^{*2}$ , etc.), and that the output of receptor activation is not only limited to G protein and effector activation, but also involves a spectrum of effects: internalization, phosphorylation, and interaction with other membrane and cytosolic components, such as receptor activity-modifying proteins, arrestins, and the like, that can alter ligand-binding characteristics, relative potency, and thus the final receptor-mediated response. In other words, as indicated by Kenakin (88), receptors can no longer be regarded as simple on/off switches, but must be considered more three-dimensionally as bipolar (or better still multipolar) recognition (or transduction) units. Accordingly, the binding of ligand at one recognition site governs its interaction with a variety of cognate “effectors” (used here broadly to cover not only the enzymes or channels activated by their cognate

receptors, but also GPCR kinases (GRKs), arrestins, receptor activity-modifying proteins, etc.) at other recognition sites, and thus that ligand efficacy not only is a quantitative parameter, but also has a qualitative dimension.

This concept of a bipolar recognition unit can perhaps be best understood by consideration of Sakmar's elegant exposé of rhodopsin activation at the molecular level (89). He proposed that activation involves conformational changes at a variety of distinct topological receptor locations, such that those, for example, involved in forming the ligand-binding pocket may be in the "on" state (in other GPCRs, this would be evidenced by high-affinity agonist binding) without necessarily locking the G protein-activating domain into its active conformation and vice versa. Thus, he argued, a concerted transition of individual amino acid subsets may generate the overall active conformation but not all transitions may actually be essential for activation of the downstream effector (here used in the restrictive sense to designate the cognate enzyme or channel activated by a receptor). For example, a minimal subset of amino acids (in Kenakin's parlance, this would constitute a "recognition unit") may allow either high-affinity agonist binding, interaction with arrestins, receptor phosphorylation, and so on irrespective of the binary states (on/off) of all possible subsets constituting the active conformation. Further, the functional hierarchy of individual group transitions can be revealed by mutations in which the side chain of an individual amino acid within a particular recognition unit is influenced or locked into the on or off state. This simple binary model of group transitions allows one to reconcile the fact that mutations can result in constitutive activity without causing an increase in agonist affinity (90–92) or vice versa; can result in the binding but not the activation of the cognate G protein (93); can result in constitutive activation for one but not another receptor-coupled effector pathway (94); or can result in the dissociation of internalization, desensitization, phosphorylation, or dimerization from activation of the G protein-coupled response (72,95). Similarly, it provides an understanding of the identification of ligands that are seemingly antagonists (do not cause activation), and yet promote internalization (96,97), for ligands that result in dissociation of agonist efficacy vs phosphorylation (98) or even for series of ligands able to activate a single receptor but with different kinetics (99).

## 2.2. Agonist Trafficking

As indicated in Section 2.1, a growing body of experimental data supports the notion of more than two distinct receptor conformations. Further, it has been suggested that agonists can stabilize different receptor active states, which then selectively activate specific G protein signaling pathways. This phenomenon of promiscuous G protein coupling by distinct agonist-specific active states has been termed *agonist trafficking* (100); a process that extends beyond G protein activation and includes functional outcomes such as receptor phosphorylation

and internalization. For the  $\beta_2$ -AR, it has been demonstrated not only that different ligands induce different conformations (74,101,102), but also that on agonist binding the receptor undergoes a temporal change in conformation (83). Also, consistent with the notion of agonist trafficking is the finding of differences in ligand potency/efficacy-order for the activation of different signaling pathways by a single receptor (71).

### 2.2.1. Multiple Activation States

In detailed studies of detergent-solubilized, purified  $\beta_2$ -AR, Kobilka and colleagues used several spectroscopic techniques to demonstrate not only that receptors exist in multiple conformations, but also that ligands induce conformational changes that vary depending on the ligand type, that is, agonists, partial agonists, or antagonists (83,102–105). By derivitization of cysteine residues with *N,N'*-dimethyl-*N*-(iodoacetyl)-*N'*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine, a fluorescent probe sensitive to its solvent environment, they demonstrated that agonists and partial agonists caused decreases in fluorescence that were proportional in magnitude to ligand efficacy, whereas antagonists increased fluorescence (102). Using the technique of single-molecule spectroscopy, they further showed that, in the absence of ligand, the  $\beta_2$ -AR exists in multiple substates, suggesting that the receptor is spontaneously oscillating (or “breathing”) between different conformations, and that on application of the agonist isoproterenol, a different subset of conformations was apparent (105).

Using another environmentally sensitive probe (i.e., fluoresceine maleimide) in lifetime spectroscopy studies, discrete conformational states were evident within a population of receptors (104). Moreover, whereas the unliganded receptor existed in a single flexible state and neutral antagonist, alprenolol, reduced conformational flexibility; agonists and partial agonists promoted the formation of two separate species with different fluorescence lifetimes (104). The last two species are indicative of at least two distinct conformations, with the agonist-bound one representing the active conformation. Because the fluorescence lifetime (and thus the microenvironment around the fluorophore) differed with each of the agonists, it is likely that these species represent distinct agonist-specific active states (104).

In additional studies, it was demonstrated that norepinephrine can induce at least two conformational states in the  $\beta_2$ -AR: one capable of activating  $G_s$  and another required for interaction of the receptor with G protein-receptor kinase (GRK) or arrestin and hence agonist-induced internalization (83). In contrast, dopamine, which can stimulate  $G_s$  activation of the  $\beta_2$ -AR but not internalization, induced only one of the conformational states observed with norepinephrine (83).

Further evidence of conformational variance in the ligand-bound  $\beta_2$ -AR has been provided by plasmon-waveguide resonance studies. These studies evaluated changes in mass density and mass distribution of receptors incorporated into a preformed (artificial) lipid bilayer, with changes in mass distribution caused by changes in structural anisotropy. Again, evidence for agonist-specific conformational states was apparent with the plasmon-waveguide resonance studies, and changes in resonance differed for agonists compared to antagonists (103). However, shifts in resonance were detected for all ligand classes, with those for agonists and partial agonists multiphasic and those for antagonists monophasic (103). This finding is consistent with receptor activation occurring through discrete conformational intermediates.

### 2.2.2. Pathway-Selective Mutants

Mutation of C3.35 in the  $\alpha_{1B}$ -AR to phenylalanine results in a receptor that is constitutively active for the  $G_q$ /inositol-1,4,5-triphosphate ( $IP_3$ ) pathway, but not for the  $G_i$ /arachidonic acid-coupled pathway (94). Given that C3.35 is directed toward TM II and is immediately below D3.32, which is critically involved in stabilizing the inactive state via an interaction with K7.36 (cf., Sections 1.1. and 2.3.2), pathway-specific activation observed with the C3.35F mutation is likely because of the bulky phenylalanine side chain sterically altering the correct juxtapositioning of TM III with TM II; a conformational perturbation that presumably resembles the activated state for  $G_q$ - but not for  $G_i$ -signaling. Interestingly, this mutation also leads to increased affinity of the receptor for the endogenous catecholamines and other phenethylamines, but not for imidazoline agonists or for antagonists (94).

Mutation of N6.55 of  $\beta_2$ -AR, a residue located at the extracellular end of TM VI that has been implicated in determining stereoselectivity of catechol agonist binding to an aspartate, results in a receptor that cannot respond to agonist activation of  $G_s$ /adenyl cyclase or to agonist stimulation of receptor phosphorylation, but nonetheless it continues to display wild-type-like basal activation (106). Again, in keeping with distinct activated intermediates between R and R\*, this suggests that the active-state conformation generated by spontaneous isomerization from the basal state is distinct from that achieved with agonist stimulation of the receptor (106).

Like the C3.35F  $\alpha_{1B}$ -AR mutant, an  $\alpha_2$ -AR D2.50N mutant shows pathway-specific signaling. Thus, this mutant displays loss of  $G_q$  and  $G_s$  coupling but can still couple to  $G_i$ , albeit with reduced potency and despite retaining the same affinity for the agonist UK14304 (Fig. 4) as wild type  $\alpha_2$ -AR (107). This mutant is unable to activate  $K^+$  channels but shows unimpaired inhibition of cyclic adenosine 5'-monophosphate (cAMP) production or voltage-sensitive  $Ca^{2+}$  currents (108,109).

### 2.2.3. Ligand-Specific Signaling

For both  $\alpha_{1A}$ - and  $\alpha_{1B}$ -ARs, *meta*- and *para*-octopamine have been shown to maximally stimulate one G protein-coupled pathway (pertussis toxin-sensitive  $PLA_2$  activation) but are only partial agonists for another ( $G_q$ -mediated  $IP_3$  production) (110). In terms of  $PLA_2$  activation, *para*-octopamine is a full agonist at the  $\alpha_{1A}$ -AR but a partial agonist for the  $\alpha_{1B}$ -AR, whereas *meta*-octopamine is a partial agonist at  $\alpha_{1A}$ - and a full agonist at the  $\alpha_{1B}$ -AR. However, norepinephrine is a full agonist for both pathways with both receptor subtypes (110). Stimulation of the  $\alpha_{1A}$ -AR by epinephrine also leads to activation of the  $IP_3$  signaling pathway but, in contrast to  $\alpha_{1B}$ -AR, does not cause receptor phosphorylation or internalization. However, the  $\alpha_{1A}$ -AR is internalized by the imidazoline agonist oxymetazoline (Fig. 4). This suggests that a distinct conformation is required for receptor internalization, and that the generation of this conformation is ligand specific (111).

Many examples of ligand-specific signaling have been documented for  $\alpha_2$ -ARs. Thus, although  $\alpha_{2B}$ -AR couples to both  $G_s$  and  $G_i$ , some agonists (e.g., UK14,304) show preference for coupling to  $G_i$  over  $G_s$  (112–115). Also, despite a dissociation constant ( $K_d$ ) of 670 nM for UK14,304 binding at the  $\alpha_{2A}$ -AR, its  $EC_{50}$  varies widely: 0.09 nM for  $G_i$  signaling, 50 nM for  $G_q$  signaling, and 70 nM for  $G_s$  signaling (107). *meta*-Octopamine selectively activates  $\alpha_{2A}$ -AR coupling to  $G_i$  but promotes coupling of  $\alpha_{2B}$ -AR and  $\alpha_{2C}$ -AR to both  $G_i$  and  $G_s$  (115). Further, at the  $\alpha_{2C}$ -AR, *meta*-octopamine is one order of magnitude less potent than norepinephrine with respect to  $G_s$  coupling but is equipotent to norepinephrine for coupling to  $G_i$  and displays increased efficacy at stimulating this G protein when compared to norepinephrine (115).

For all three  $\alpha_2$ -ARs, the efficacy of  $G_s$  coupling is dependent on the structure of the agonists. Thus, compounds that act as full agonists with respect to  $G_i$  coupling do not necessarily display full efficacy for  $G_s$  coupling (114). This is especially the case for the  $\alpha_{2C}$ -AR, for which it has been suggested that oxymetazoline, BHT-920, and BHT-933 (Fig. 4) activate  $G_i$  but not  $G_s$  signaling (114). However, this differential signaling activity of agonists acting on the  $\alpha_{2C}$ -AR was not observed by others and has been attributed to the presence of spare receptors (116).

It has also been shown that some ligands function as antagonists with respect to one receptor-coupled pathway but as partial agonists at the same receptor for another signaling pathway (117). For example, for the  $\alpha_{2A}$ -AR the imidazoline derivative dexefaroxan is a neutral antagonist for  $Ca^{2+}$  signaling via  $G_{\alpha_{15}}$ , an inverse agonist for  $G_{\alpha_o}$  signaling by the mutant Cys<sup>351</sup>Ile, and a partial agonist for signaling by a constitutively active mutant  $\alpha_{2A}$ -AR that couples via  $G_{\alpha_{15}}$  (117).

Ligand-specific signaling is also evident with  $\beta$ -ARs. For example, at the  $\beta_2$ -AR, ICI118551 and propranolol (Fig. 4) have been found to act as inverse ago-

nists for  $G_s$ -stimulated adenylyl cyclase but as partial agonists for  $G_{s/I}$ -independent extracellularly responsive kinase 1/2 (ERK1/2) activation. Because these two ligands promote  $\beta$ -arrestin recruitment to the  $\beta_2$ -AR, this receptor-coupled response is not an exclusive property of agonists, and ligands normally classified as inverse agonists have been shown to require  $\beta$ -arrestin for their signaling activity (118).

### 2.3. Molecular Determinants of Activation

#### 2.3.1. Helical Movements and Disruption of Helical Interactions

Experimental data and consideration of the crystal structure of rhodopsin suggest that intramolecular interactions stabilize the inactive conformation of GPCRs. Removal or rearrangement of these constraining interactions results in receptor activation as a result of movements of the TM helices, which are then relayed to the G protein-interacting intracellular loops. In the activated conformation, receptors display structural instability and enhanced conformational flexibility, as evidenced by the thermolability of constitutively active mutants (75).

Of the GPCRs, the activation mechanism of rhodopsin has been most extensively studied using a variety of biophysical approaches, including tryptophan ultraviolet absorbance spectroscopy, Fourier transform infrared resonance spectroscopy, and site-directed spin-labeling studies. With the last, electron paramagnetic spin of pairs of cysteine-substituted residues labeled with sulfhydryl spin probes is monitored (119–121). These studies have provided evidence that activation involves a small movement of TM III coupled with significant rigid body movement and counterclockwise (when viewed from the extracellular surface of the receptor) rotation of TM VI, leading to movement of the cytoplasmic end of TM VI away from TM III (86). This finding was also supported by mutagenesis studies in which either an engineered disulfide bond (122) or the binding of zinc to a site engineered between two histidine-substituted residues (87), one in TM III and one in TM VI, has been used to lock rhodopsin into the inactive state.

Studies of TM residue accessibility to water-soluble sulfhydryl-reactive compounds has allowed Javitch and coworkers to gain evidence that  $\beta_2$ -AR activation also involves a conformational rearrangement of TM VI (123), whereas detailed fluorescence spectroscopy studies by Kobilka and coworkers indicated that the helical movements occurring with  $\beta_2$ -AR activation are almost identical to those for rhodopsin, that is, a counterclockwise rotation (when viewed from the extracellular surface of the receptor) of both TM III and TM VI, with a tilting of the cytoplasmic end of the latter toward TM V (74,124). The importance of the orientation of TM VI, which is stabilized by interhelical interactions with TM V, comes from studies of the  $\alpha_1$ -ARs, which showed that mutation of either the

residue at 5.39 in TM V or 6.55 in TM VI of the  $\alpha_{1B}$ -AR to that of the  $\alpha_{1A}$ -AR or vice versa results in constitution activation, and further that the basal state could be restored by mutation of both residues in one subtype to those of the other subtype (double reciprocal mutation) (125). Evidence for involvement of TM VI in  $\alpha_{1B}$ -AR activation also comes from the delineation of F6.51 as a key switch residue involved in both agonist binding and receptor activation by this receptor, albeit that this residue appears only to be involved in the isomerization from R' to R\* and not from the basal state to R' (20). In other GPCRs, such as the NK-1 substance P receptor, interhelical stabilization of TM VI by TM V has been demonstrated from studies showing that an engineered zinc-binding site linking the extracellular ends of these two helices prevents activation (126). Activation may also involve movement of other helices, such as TM VII, as demonstrated in studies of rhodopsin (127) and other GPCRs, such as the thyroid-stimulating hormone receptor (128).

### 2.3.2. Molecular Basis for Helical Movements and G Protein Activation

In the case of rhodopsin, the ground state is stabilized by a salt bridge linking E3.28 in TM III with the protonated Schiff base formed by the covalent binding of the chromophore 11-*cis* retinal with K7.43 in TM VII (129–131). Photoisomerization of the chromophore to the all-*trans* conformation disrupts the salt bridge as a result of deprotonation of the Schiff base (132). Thus, movement of TM III is likely the first step in the rhodopsin activation process. Similarly, studies by Perez and coworkers (5,133,134) have provided evidence that a salt bridge between D3.32 and K7.36 stabilizes the ground state of the  $\alpha_{1B}$ -AR, and that its disruption also is essential and probably the proximate step in activation. Thus, mutagenesis of either D3.32 or K7.36 to an alanine results in constitutive activation that can be rescued by reciprocal mutation of these residues, and activation can be induced even with triethylamine, a compound that mimics the protonated amine moiety of catecholamines (5,133,134). However, other ARs, including the  $\alpha_{2A}$ -,  $\alpha_{2B}$ -, and all  $\beta$ -AR subtypes, lack a lysine at the 7.36 position and are thus unable to form a salt bridge with D3.32. Indeed, lack of such a strong bonding interaction to stabilize the basal state may explain why some receptors, such as the  $\beta_2$ -AR, are more likely to demonstrate constitutive activity because the energy barrier for spontaneous isomerization from R to R\* would be lower.

Based on detailed fluorescence spectroscopy studies of the  $\beta_2$ -AR, it has been suggested that binding of the various moieties of catecholamine agonists is sequential (83), and although interactions between the receptor and the catechol ring and amine group are rapid, whereas that with the chiral hydroxyl is slow, formation of the catechol ring interactions with TM VI and TM V precedes that between the amine and D3.32 in TM III, with the latter required only to stabilize the interactions with the catechol ring (83). These findings could be interpreted

to imply that, for the  $\beta_2$ -AR, movement of TM III may occur after that of TM VI, and thus that movement of TM VI, rather than TM III, is the initial step in receptor activation.

Another interhelical interaction that is disrupted, and likely plays a role in the activation process, is that between a glutamic acid at the cytoplasmic end of TM VI and residues of the (D/E)RY motif in TM III. This interaction is discussed in Section 2.3.3.

Movement of TM III and TM VI, which are contiguous with the G protein-interacting second and third intracellular loops ( $i_2$  and  $i_3$  loops), is consistent with their central involvement in the activation process. However, it remains unclear exactly how the binding of agonist to a site in the outer third of the TM domain is transmitted to the G protein-interacting loops on the cytoplasmic surface of the receptor—a distance of some 30–40 Å (135). The crystal structure of rhodopsin revealed that TM VI is kinked because of the presence of a proline (6.50) located approximately at the junction of the outer and middle thirds of the helix (135). This proline is highly conserved in GPCRs, including all ARs. In addition, the six residues N-terminal to 6.50 are highly homologous between ARs and rhodopsin. Given that the residues N-terminal of proline have been shown to be the determinants of proline-induced kinks, as well as other nonhelical elements in TM proteins (136), it is likely that a kink in TM VI is a conserved architectural feature of most GPCRs. The putative Pro-kink in ARs is surrounded by a cluster of aromatic residues (F6.44, W6.48, F6.51, F6.52). Because computational simulation studies indicated that Pro-kinks form flexible molecular hinges that can act as conformational switches in TM  $\alpha$ -helices (137), it has been suggested that the aromatic residues of the  $\beta_2$ -AR that are clustered about 6.50 act as a toggle switch that modulates the TM VI kink (123). Specifically, based on both mutagenesis data and computer simulations of activation-induced structural changes in TM VI, Shi et al. (123) proposed that activation results in a switch in the rotamer conformations of C6.47, W6.48, and to a lesser degree F6.52, which result in a sweeping movement of the cytoplasmic end of TM VI away from TM III.

Although of interest, further experimental studies will be required to validate or refute this rotameric switch model, which runs contrary to data indicating that activation of rhodopsin involves rigid body movement of TM VI (86). In addition, in the study of Shi et al. (123), the molecular simulations of TM VI were performed with the helix isolated from the likely constraining influences of other TM helices; the rotamers assigned to the TM VI residue were those determined from studies of  $\alpha$ -helices in soluble proteins, which may not pertain for residues in membrane-embedded helices. In agreement with the rhodopsin studies, site-directed fluorescence-labeling studies of the  $\beta_2$ -AR are entirely consistent with TM VI moving as a rigid body (124).

Based on engineered cysteine sensor studies of the  $\beta_2$ -AR, which provided evidence for movement not only of the cytoplasmic end of TM VI below the Pro-kink, but also of the extracellular end of the helix above P6.50, Chen et al. (20) suggested that rather than adding flexibility to TM VI, P6.50 rigidifies the helix into a stiff, kinked helical rod, a proposal supported by studies of other proteins showing increased thermostability with the substitution of an alanine by a kink-inducing proline (138). As a result, the Pro-kink acts as a fulcrum to allow TM VI to pivot and thus amplify the conformational change associated with agonist binding. This results in productive propagation of the agonist signal from the agonist binding site to the G protein-binding site on the cytoplasmic face of the receptor.

Coupled with the findings of modeling studies of the  $\beta_2$ -AR, Chen et al. (20) further proposed mechanical momentum transfer as a plausible mechanism for the propagation of activation-induced movements over a distance of 30–40 Å, a mechanism suggested not only by the presence of a cluster of aromatic residues, but also by their interaction in a typical herringbone arrangement interspersed by strongly dipolar side chains (Asn 6.55, 7.39, 7.44, 7.49). Because buried amide side chains, or even charged ones, form thermodynamically stable dipolar/aromatic clusters as a result of  $\pi$ -cation interactions with aromatic Trp, Phe, and Tyr residues (139), any local changes to such interactions resulting from ligand-induced residue rearrangements are likely to affect the stability of the whole cluster. This would explain how large-scale movements of side-chain clusters might be propagated along the length of the TM helices.

### 2.3.3. Extracellular Disulfide Bond Disruption

Even before the structure of any GPCR was known from molecular cloning, it was demonstrated that the  $\beta$ -AR was stabilized by a disulfide bond(s) (140). Indeed, based on an analysis of  $\beta$ -AR disulfide bonding, Pedersen and Ross (141) suggested that its structure would be more akin to that of rhodopsin than to that of other types of TM receptors—an astonishingly prescient prediction. In that study, it was shown that treatment of the  $\beta$ -AR with thiol-reducing agents to disrupt disulfide linkages resulted in its activation (141,142). Subsequently, with the cloning of the  $\beta_2$ -AR, the presence of a disulfide bond connecting extracellular loops 1 and 2 ( $e_1$  and  $e_2$ , respectively) was suggested by the presence of cysteine residues in  $e_1$  and  $e_2$ , which are also conserved in all ARs and most other GPCRs, and by structure–function studies (143,144). However, it was not until some years later that the extracellular loop disulfide bond connectivity of the  $\beta_2$ -AR was confirmed and shown to be atypical in that it involved not one bond, as in other ARs, but two disulfide bonds connecting Cys<sup>106</sup> in  $e_1$  and Cys<sup>184</sup> in  $e_2$  with vicinal cysteines (Cys<sup>191</sup> and Cys<sup>190</sup>, respectively) in  $e_2$  (145), both of which stabilize receptor structure, whereas only the Cys<sup>106</sup>–Cys<sup>191</sup> linkage is involved

in activation (145). Further, it was shown that formation of these disulfide bonds likely involves disulfide exchange during biogenesis of the nascent receptor, with initial bonding between Cys<sup>106</sup> and Cys<sup>184</sup>, the cysteine pair conserved in most other GPCRs (145).

In contrast to the solvent accessibility of the  $\beta_2$ -AR disulfide bonds, that of the  $\alpha_{1B}$ -AR (146), like rhodopsin (147), is solvent inaccessible, a finding consistent with their extracellular loop structures being different from that of the  $\beta_2$ -AR. Because the disulfide bond is masked in  $\alpha_1$ -ARs, one cannot test if its reduction by thiols would also lead to activation. Nevertheless, because catecholamine agonists are reducing agents (148) and are inactive in their oxidized form (149), whereas antagonists are redox inactive (148), it has been suggested that receptor activation involves an essential reductive step, a postulate initially made for the  $\alpha_2$ -AR (150) and later extended to  $\beta$ -AR (148,151) and dopamine receptors (152). Further, it has been suggested that, because G proteins and adenylyl cyclase contain active sulfhydryl groups (153), their activation by  $\beta_2$ -ARs may involve TM redox chemistry. Direct evidence, however, is lacking, and given the reducing environment of the cytoplasm (154), one has to postulate that critical sulfhydryls of intracellular receptor link proteins must be masked in their inactive conformation if they are to react on receptor activation because otherwise they would already be reduced.

#### 2.3.4. Involvement of the E/DRY Motif

It has long been known that photoactivation of rhodopsin is not only associated with deprotonation of the Schiff base linking the retinal chromophore to Lys<sup>296</sup>, but also with proton uptake from the aqueous milieu (155). Careful biophysical studies of wild-type rhodopsin and mutants indicated that it is E3.49 of the E/DRY motif at the cytoplasmic end of TM III that becomes protonated with photoactivation, that protonation occurs with significantly slower kinetics than retinal isomerization, and that two spectroscopically indistinguishable activated forms of rhodopsin (*isochromic* species known as metarhodopsin IIa and IIb) are generated by photoactivation. The first (MIIa), which is generated rapidly, is unable to activate transducin but results in E3.49 becoming available for protonation, and the second (MIIb), generated by proton uptake by E3.49, is able to activate signaling. In addition, mutation of E3.49 to an uncharged glutamine results in constitutively active opsin. Thus, E3.49 is involved in stabilizing the inactive state of rhodopsin.

As in rhodopsin, the residue at position 3.49 in adrenergic and other class A GPCRs, an aspartate, has been proposed as an important modulator of the transition from the inactive (R) to the active (R\*) state, and not surprisingly, charge-neutralizing mutations of D3.49 in several such receptors have been shown to result in constitutive activity (66,156–159). With the  $\alpha_{1B}$ -AR, mutagenesis stud-

ies have suggested that, as with rhodopsin, activation involves protonation of D3.49. Further, based on computer simulations, Scheer et al. (159) proposed that, in the inactive state, R3.50 is constrained in a “polar pocket” formed by residues in TM I, II, and VII, with the counterion for R3.50 being D2.50 in TM II. However, for the  $\beta_2$ -AR, Ballesteros et al. (160) predicted the counterion to be the adjacent D3.49 of the DRY motif, with D3.49 protonated in the active state and R3.50 interacting with D2.50. In other studies of the  $\beta_2$ -AR, Ghanouni et al. (161) provided evidence that protonation increased basal activity by destabilizing the inactive state of the receptor.

Based on additional mutagenesis studies, cysteine accessibility data, and computer simulations, Ballesteros et al. (160) indicated that R3.50 forms ionic interactions with both the adjacent D3.49 and E6.30 in TM VI. Further, they suggested that disruption of this “ionic” lock might constitute a common switch governing the activation of many class A GPCRs. Although an attractive hypothesis, given the rhodopsin data showing that isomerization to MIIa precedes protonation and the generation of MIIb, it is unlikely that disruption of the putative D3.49/R3.50/E6.30 ionic lock is a primary step in receptor activation. Moreover, direct evidence for proton uptake from the aqueous milieu by receptors other than rhodopsin has yet to be provided. It is also of interest that the increased basal activity of the  $\beta_2$ -AR observed with reductions in pH could not be abrogated with alanine substitutions of D3.49 or E6.30. This suggests that the residue(s) mediating protonation-induced activation has yet to be identified, albeit that it would be interesting to test if the activating effect of pH reduction could be prevented with charge-neutralizing mutations of more than one residue forming the putative D3.49/R3.50/E6.30 interaction.

It has been suggested that, unlike rhodopsin or the  $\alpha_{1B}$ - and  $\beta_2$ -ARs, the  $\alpha_{2A}$ -AR does not follow the conventional GPCR mechanistic paradigm with respect to the function of the DRY motif; that is, D3.49 is involved in receptor activation, and R3.50 is involved in activation of the cognate G protein. Regarding the lack of involvement of D3.49 in receptor activation, this conclusion is based on the finding that D3.49I and D3.49N mutant  $\alpha_{2A}$ -ARs did not display constitutive activity (15,162). Although the same substitutions of D3.49 in the  $\alpha_{1B}$ -AR do result in constitutive activity (163), this conclusion should be interpreted with caution because the D3.49N mutation of the  $\alpha_{1B}$ -AR induces the weakest constitutive activity of all 19 substitutions at this residue (163). In addition, although the isoleucine substitution of D3.49 resulted in robust constitutive activation of the  $\alpha_{1B}$ -AR, it is possible that its long hydrophobic side chain may interact with residues in other regions of the  $\alpha_{2A}$ -AR receptor that are not conserved in the  $\alpha_{1B}$ -AR and by so doing may prevent expression of an activated phenotype. Thus, additional data are required before it can be confidently concluded that the role of the DRY motif in the  $\alpha_{2A}$ -AR is distinct from that in other class A GPCRs,

albeit that such a proposal has also been made for the  $M_1$  and  $M_5$  mACh receptors (164,165). With both of these muscarinic receptors, however, involvement of D3.49 in receptor folding and expression may have limited the analysis of its contribution to signaling.

### 2.3.5. Role of TM V Serines

Based on studies of the  $\alpha_{1A}$ -AR, it has been demonstrated that the *meta*-hydroxyl of the endogenous agonists preferentially binds to S5.42, and it is this hydrogen bond interaction, and not that between the *para*-hydroxyl and S5.46, that allows receptor activation (12).

In early studies of the  $\beta_2$ -AR, a critical interaction was demonstrated between S5.42 and S5.46 and the *meta*- and *para*-hydroxyls of catecholamine agonists (17). Using thermodynamic analyses of double mutant cycles in which wild-type and mutant receptors with alanine substitutions of S5.42 and S5.46 were evaluated for their ability to bind agonists with differing hydroxyl moieties on the 3,4 positions of the catechol ring, Ambrosia et al. (166) provided evidence that S5.42 and S5.46 not only provide agonist docking interactions, but also control the equilibrium between the inactive (R) and active (R\*) states of the receptor. Thus, alanine substitution of both S5.42 and S5.46 in the wild-type  $\beta_2$ -AR and a constitutively active mutant inhibited basal signaling. In a similar analysis, Liapakis et al. (16) also showed that the catechol *meta*-hydroxyl interacts not only with S5.42, but also perhaps (through a bifurcated H-bond) with S5.43, and that the interaction with the latter may play a role in partial agonism. Given that S5.43 is not conserved in other ARs such as  $\alpha_1$ - and  $\alpha_2$ -ARs and in some cases is replaced by an alanine that lacks H-bonding potential, catechol binding and the orientation of the catechol ring clearly differ significantly between ARs.

## 3. Interaction of Adrenergic Receptors With Signaling Proteins

### 3.1. G Protein and Receptor Coupling

Like other GPCRs, ARs interact with heterotrimeric guanine nucleotide binding regulatory proteins or G proteins. The heterotrimeric nature of these proteins is evident from their  $\alpha$ -,  $\beta$ -, and  $\gamma$ -subunit composition, each subunit is encoded by a distinct gene. The nucleotide-binding signature  $G_\alpha$  subunit is structurally related to small molecular weight G proteins and, like the latter, possesses intrinsic, but catalytically inefficient, GTPase activity (167). The  $G_\beta$ - and  $G_\gamma$ -subunits form a tightly interacting dimer that is bound to the plasma membrane via an isoprenyl moiety covalently attached to the C-terminus of  $G_\gamma$ .

In the guanosine 5'-diphosphate (GDP)-bound state,  $G_\alpha$  associates with  $G_{\beta\gamma}$ . On activation by the cognate GPCR, GDP dissociation (the rate-limiting step) is facilitated and allows G protein activation as a result of guanosine 5'-triphosphate (GTP) binding. The mechanisms by which receptors bind their cognate G

protein and catalyze GDP/GTP exchange are not well understood but involve interaction with the  $i_2$  and  $i_3$  receptor regions (168). One possibility could be that GTP binding by  $G_\alpha$  instigates a conformational change that may lower its affinity for  $G_{\beta\gamma}$  and thereby leads to dissociation of  $G_\alpha$ -GTP from  $G_{\beta\gamma}$  (although some have suggested that, rather than dissociation, the trimeric complex merely undergoes a conformational rearrangement) (169). Both  $G_\alpha$ -GTP and  $G_{\beta\gamma}$  can activate downstream effectors (170). In addition to effector activation,  $G_\alpha$  complexes have numerous signaling functions, including a role in membrane localization and activation of certain GRKs.

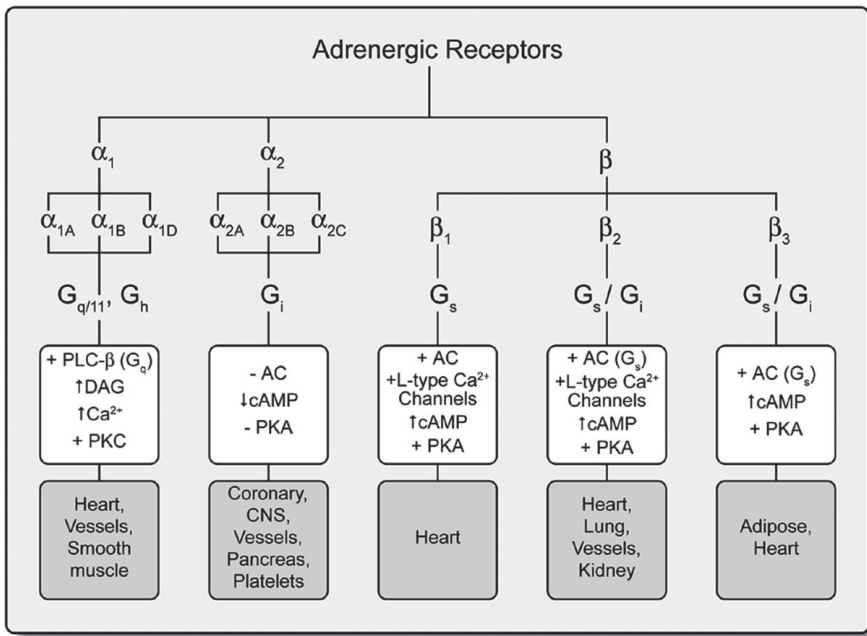
In mammals, there are at least 27  $G_\alpha$ -, 5  $G_{\beta^-}$ , and 13  $G_\gamma$ -subtypes (171); hence, the intracellular propagation of GPCR signaling is orchestrated by myriad  $G_{\alpha\beta\gamma}$  combinations. On the basis of the primary sequence of the  $G_\alpha$ -subunits, G proteins can be divided into different major families, including  $G_s$ ,  $G_i$ ,  $G_q$ , and  $G_{12}$  (172). Each  $G_\alpha$  protein subtype couples to specific effectors. Originally, both the specificity and the selectivity of GPCR signaling were thought to be achieved by the coupling of a given receptor with a single class of G proteins. However, this paradigm was abandoned because several GPCRs, including the  $\beta_2$ -AR, have been shown to be capable of coupling to several different  $G_\alpha$ -subunits, a phenomenon dependent on the specific agonist employed or its concentration, which is referred to, as indicated in Section 2.2, as *agonist trafficking* (100).

As detailed in Sections 3.2–3.4, the G proteins involved in AR signaling (Fig. 7) include  $G_s$ , which couples  $\beta$ -ARs to adenylyl cyclase stimulation, and  $G_i$ , which mainly couples  $\alpha_2$ -ARs to AC inhibition.  $\alpha_2$ -ARs activation can also lead to  $Ca^{2+}$  channel activation via  $G_o$  coupling. For  $\alpha_1$ -ARs, the  $G_{q/11}$  family can mediate receptor coupling to phospholipase C $\beta$  (PLC- $\beta$ ) activation. In addition, the atypical G protein  $G_{12}$ /TGase 2 can mediate PLC- $\gamma$ 1 activation by the  $\alpha_{1B^-}$  and  $\alpha_{1D^-}$ , but not  $\alpha_{1A^-}$ , AR.

### 3.2. $\alpha_1$ -AR G Protein and Effector Activation

Stimulation of  $\alpha_1$ -ARs results in the activation of various effectors, including PLC, phospholipase D (PLD), and phospholipase A<sub>2</sub> (PLA<sub>2</sub>), as well as activation of  $Ca^{2+}$  channels and the  $Na^+/H^+$  exchangers, modulation of  $K^+$  channels (173), and activation of other signaling pathways, such as that involving activation of mitogen-activated protein kinases, or leading to transcriptional activation of early and late response genes.

The main signaling pathways activated by  $\alpha_1$ -ARs are depicted in Fig. 8. All three  $\alpha_1$ -AR subtypes can activate phosphoinositide turnover and calcium signaling. Indeed, although the  $Ca^{2+}$  influx (174,175) response was initially thought to be specific for the  $\alpha_{1A}$ -AR, it was later found to be mediated also by the other subtypes. Both voltage-dependent and -independent  $Ca^{2+}$  channels have been implicated in these responses. Using antisense technology, Marcerez-Lepetre and

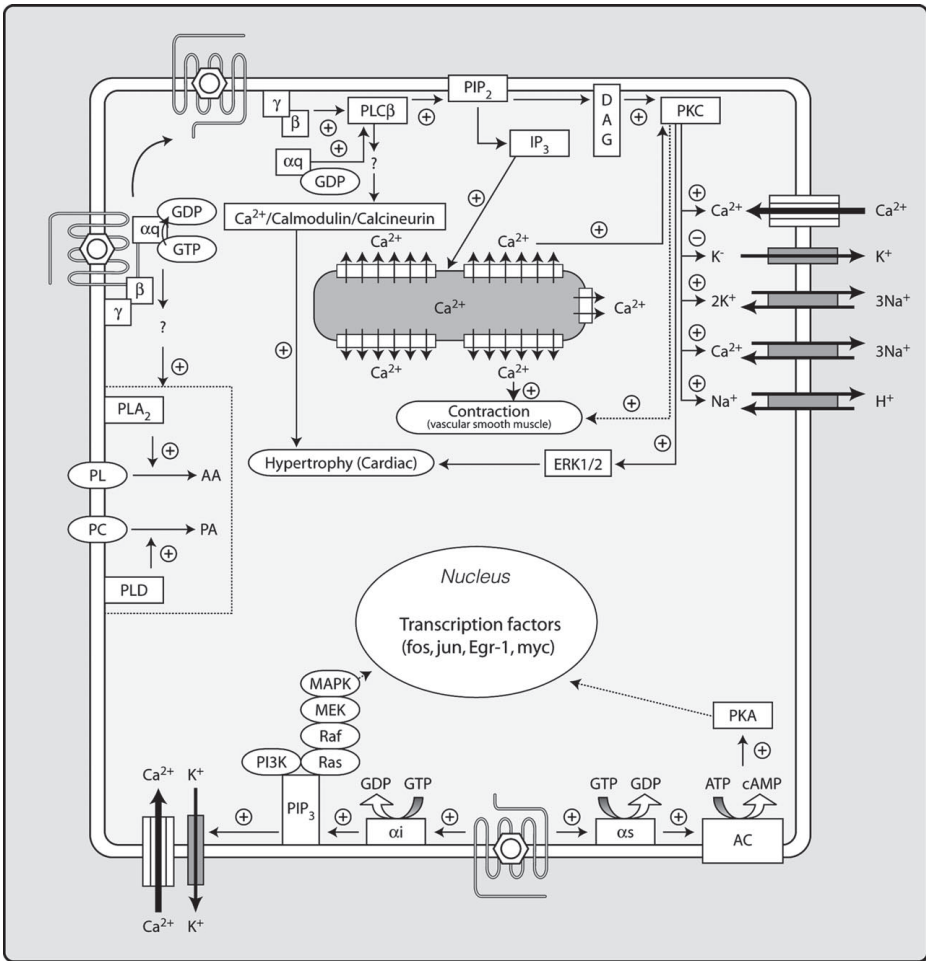


**Fig. 7.** Adrenergic receptor subtypes and their coupled G proteins and effectors. AC, adenylyl cyclase; c-AMP, cyclic adenosine-3',5'-monophosphate; DAG, diacylglycerol; PKA, c-AMP-dependent protein kinase A; PKC, protein kinase C; PLC- $\beta$ , phospholipase C $\beta$ ; +, activation; -, inhibition;  $\uparrow$ , increase;  $\downarrow$ , decrease.

coworkers have provided evidence that  $G_{\alpha_q}$  participates in  $\alpha_1$ -AR-mediated phosphoinositide turnover and intracellular calcium mobilization, whereas  $G_{\alpha_{11}}$  is involved in  $\alpha_1$ -AR-mediated calcium influx (176).

Like many other  $Ca^{2+}$ -mobilizing receptors,  $\alpha_1$ -ARs mainly couple to the  $G_{q/11}$  family of G proteins to increase intracellular free  $Ca^{2+}$  concentration. The  $G_{q/11}$  family includes five  $\alpha$ -subunits:  $\alpha_q$ ,  $\alpha_{11}$ ,  $\alpha_{14}$ ,  $\alpha_{15}$ , and  $\alpha_{16}$  (177). Transient overexpression of both receptor and G protein  $\alpha$ -subunit in COS-7 cells has shown that  $\alpha_q$  and  $\alpha_{11}$ , which are expressed in most cells, can mediate PLC activation by all  $\alpha_1$ -AR subtypes (178). This results predominantly from  $G_{q/11}$ -mediated activation of PLC- $\beta$  (173). In contrast,  $\alpha_{14}$  and  $\alpha_{16}$ , which are expressed in a limited subset of tissues but can also mediate PLC- $\beta$  activation, couple differentially to  $\alpha_1$ -AR subtypes. Thus, whereas the  $\alpha_{1B}$ -AR signals efficiently via coupling to  $G_{\alpha_{14}}$  or  $G_{\alpha_{16}}$ , the  $\alpha_{1A}$ -AR signals via  $G_{\alpha_{14}}$  but not  $G_{\alpha_{16}}$ , and the  $\alpha_{1D}$ -AR poorly interacts with either (178).

The activation of PLC- $\beta$  by  $\alpha_1$ -ARs results in the hydrolysis of a specific membrane lipid, phosphatidylinositol-4,5-bisphosphate, to release the diffusible



**Fig. 8.** Signal transduction pathways activated by  $\alpha_1$ -ARs. The upper portion of the figure shows the classical receptor-linked phosphoinositide pathway and secondary effector activation. The lower portion shows additional  $\alpha_1$ -AR-linked pathways, which include modulation of ion channels and activation of MAPK pathways via  $G_i$ -mediated PI3K stimulation.  $\alpha_1$ -AR-mediated activation of adenylyl cyclase via  $G_{os}$  has also been reported. AA, arachidonic acid; AC, adenylyl cyclase; ATP, adenosine 5-triphosphate; cAMP, cyclic adenosine 3',5'-monophosphate; DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; GDP, guanosine diphosphate; GTP, guanosine triphosphate; IP<sub>3</sub>, inositol-1,4,5-triphosphate; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; PA, phosphatidic acid; PC, phosphatidylcholine; PI3K, phosphatidylinositol-3 kinase; PIP<sub>2</sub>, phosphatidyl inositol-4,5-bisphosphate; PIP<sub>3</sub>, phosphatidylinositol 3,4,5-triphosphate; PKA, protein kinase A; PKC, protein kinase C; PL, phospholipids; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; PLC- $\beta$ , phospholipase C $\beta$ ; PLD, phospholipase D.

second messengers  $\text{IP}_3$  and diacylglycerol (DAG) (173).  $\text{IP}_3$  then activates the release of  $\text{Ca}^{2+}$  from intracellular stores, such as the endoplasmic reticulum, resulting in a rapid increase in cytoplasmic  $\text{Ca}^{2+}$  concentration. The increased free  $\text{Ca}^{2+}$  binds to calcium-dependent regulatory proteins such as calmodulin. The complex formed with calmodulin regulates the activities of a variety of enzymes and other cellular proteins, which leads to a variety of physiological responses in different tissues. DAG, on the other hand, is an allosteric activator of PKC, which in turn phosphorylates seryl and threonyl residues on a variety of cellular protein substrates, including various ion channels ( $\text{Ca}^{2+}$  and  $\text{K}^+$  channels),  $\text{Na}^+$ - $\text{H}^+$  exchanger, and  $\text{Na}^+$ - $\text{Ca}^{2+}$  pump (173). Regulation of these proteins by PKC results in a variety of cellular responses, including altered cardiac function.

There are at least 10 mammalian PLC isozymes, which can be divided into three types:  $\beta 1$ -4,  $\gamma 1$ -4,  $\delta 1$ -4 (179). PLC- $\beta$  can be activated by the  $\alpha$ -subunits of all four members of the  $\text{G}_{q/11}$  family. PLC- $\gamma$  is mainly activated by receptor tyrosine kinases; the regulators of PLC- $\delta$  isoforms have not yet been clearly defined (179). Feng et al. (180) have provided evidence that the  $\alpha_{1B}$ -AR may couple to  $\text{G}_h$  to regulate PLC- $\delta$  activity, and that a  $\text{G}_h$ /PLC- $\delta 1$  complex is formed on  $\alpha_{1B}$ -AR activation. Also, PLC- $\delta 1$  can be stimulated by activated  $\text{G}_h$  following reconstitution, suggesting that PLC- $\delta 1$  is a downstream effector of  $\text{G}_h$  (181). However, Murthy et al. (182) suggested that  $\text{G}_h$  may negatively regulate PLC- $\delta 1$  activity. They showed that the activity of PLC- $\delta 1$  is inhibited when it forms a complex with  $\text{G}_h$  in the empty or GDP-bound state, whereas dissociation of  $\text{G}_h$  from PLC- $\delta 1$  as a result of receptor-stimulated GTP/GDP exchange activates PLC- $\delta 1$ . Both the  $\alpha_{1B}$ - and the  $\alpha_{1D}$ -ARs can utilize the atypical G protein  $\text{G}_h$ /TGase 2 to activate PLC- $\delta 1$  or maxi- $\text{K}^+$  channels (180,183,184).

Stimulation of  $\alpha_1$ -ARs has also been shown to induce arachidonic acid (AA) release in a variety of cells, including FRTL5 cells (185), spinal cord neurons (186), MDCK cells (187), vascular smooth muscle cells (186,188), striatal astrocytes (189), and transfected COS-1 and CHO cells (190). In most of these studies, evidence has been provided that activation of  $\text{PLA}_2$ , either directly or indirectly, was involved in AA release.  $\text{PLA}_2$ s are a family of enzymes that cleave the ester linkage in membrane glycerophospholipids at the *sn*-2 position of the glycerol moiety, producing a free fatty acid and a lysophospholipid (191-193). There are three major types of  $\text{PLA}_2$ s: a 14-kDa secretory form, an 85-kDa cytosolic  $\text{Ca}^{2+}$ -dependent (c $\text{PLA}_2$ ) form, and an intracellular  $\text{Ca}^{2+}$ -independent  $\text{PLA}_2$ s (i $\text{PLA}_2$ ) form. Although all three  $\text{PLA}_2$ s mediate AA release, c $\text{PLA}_2$  possesses characteristics suggesting that it is the enzyme mainly involved in receptor-activated signaling (194). The enzyme does not need  $\text{Ca}^{2+}$  for activation but is able to translocate to membranes in response to increases in intracellular  $\text{Ca}^{2+}$  to micromolar levels. It possesses a preference for AA-containing phospholipids as its sub-

strate, and its activity is regulated by mitogen-activated protein kinase (MAPK)-mediated phosphorylation.  $\alpha_1$ -ARs activate AA release through cPLA<sub>2</sub> (195).

However, it is still unclear how the activity of PLA<sub>2</sub> is regulated. Studies of various cloned  $\alpha_1$ -AR subtypes expressed in different eukaryotic expression systems suggested that their regulation of cPLA<sub>2</sub> activity is complicated and is probably cell type specific (175). Some studies have shown that  $\alpha_1$ -AR-mediated AA release as a result of PLA<sub>2</sub> activation secondary to stimulation of other signaling pathways, such as Ca<sup>2+</sup> influx or PKC and MAPK activation. But, there is also evidence that  $\alpha_1$ -ARs couple directly to PLA<sub>2</sub> activation. For example, Perez and coworkers (190) showed that stimulation of AA by either the  $\alpha_{1B}$ - or  $\alpha_{1D}$ -AR expressed in COS-1 cells is dependent on Ca<sup>2+</sup> influx via dihydropyridine-sensitive L-type calcium channels, whereas in CHO cells, which lack a voltage-dependent calcium channel,  $\alpha_1$ -AR-mediated AA release does not involve stimulation of PLC, Ca<sup>2+</sup> influx, PKC, or DAG lipase or increase of intracellular Ca<sup>2+</sup> but rather involves direct activation of PLA<sub>2</sub>.

In addition to PLA<sub>2</sub>-mediated mechanisms, it is worth noting that AA can also be generated from the activation of other pathways, including PLC, which forms DAG that could be cleaved to generate AA via the action of mono- or diglycerol lipase (194). PLD-mediated cleavage of phosphatidylcholine may also generate phosphatidic acid (PA), which can be further metabolized by phosphatidic acid phosphohydrolase to DAG, and activation of AA release by  $\alpha_1$ -ARs via the PLD pathway has been reported (196).

As with other receptors,  $\alpha_1$ -ARs may regulate PLA<sub>2</sub> activity via G proteins (197,198). Thus, PLA<sub>2</sub> activity can be regulated by guanine nucleotides and in most cases is inhibited by pertussis toxin treatment—thus suggesting involvement of a G<sub>i</sub>- or G<sub>o</sub>-like G protein (197,199,200). However, it is still unclear how activation of G proteins is linked to PLA<sub>2</sub> stimulation, although there is evidence that it may be by via a direct G $\beta\gamma$ -mediated effect (201).

The role of the released AA in cell functions is not completely understood, and AA itself can act as a second messenger to activate PKC (202,203) and PLC $\delta$  (204) and to influence membrane ion channel activity (205,206). AA can also inhibit smooth muscle myosin light chain phosphatase activity (207) or can be converted through the lipoxygenase or cyclooxygenase pathways to a number of bioactive eicosanoids, including prostaglandins, thromboxanes, leukotrienes, epoxides, and hydroxyeicosatetraenoic acids, which are involved in inflammation and cell proliferation (194,208). Moreover, lysophospholipid, which is concomitantly released from phospholipid as a result of PLA<sub>2</sub> activation, can influence cell functions (e.g., proliferation) by acting on its receptor on the cell surface.

PLD mainly catalyzes the hydrolysis of phosphatidylcholine to PA and choline (209). PA may act directly as a signaling molecule or can be converted by

phosphohydrolase to the PKC activator DAG. Activation of PLD via  $\alpha_1$ -ARs has been demonstrated in a variety of tissues and cell lines, including cerebral cortex (210), artery (211,212), parotid (213), ventricular myocytes (214), and MDCK cells (215). Indeed, each of the three  $\alpha_1$ -ARs subtypes, when expressed in rat-1 fibroblast cells, can activate PLD with the following order of efficiency:  $\alpha_{1A} > \alpha_{1B} > \alpha_{1D}$ -AR (196). However, the mechanisms involved in this activation are poorly understood. The activity of PLD can be regulated by multiple pathways, including by PKC or tyrosine kinases, as well as by small G proteins of the ADP-ribosylating factor and Rho families (209). In a few studies that have examined the regulatory mechanisms, it was shown that extracellular  $\text{Ca}^{2+}$  but not PKC activation is required for stimulation of PLD via  $\alpha_1$ -ARs (215). In addition, there is evidence that  $\alpha_1$ -AR-mediated PLD activation can be regulated by the cAMP-PKA (protein kinase A) signaling pathway (196).

$\alpha_1$ -ARs can also signal via interaction with pertussis-sensitive G proteins. Thus,  $G_{\alpha o}$  has been suggested to interact with  $\alpha_1$ -ARs in mediating contraction and inositol phosphate generation in rat aorta (216), a response that may involve effector activation by either the  $\alpha$ - or  $\beta\gamma$ -complexes. Other  $\alpha_1$ -AR-mediated actions, such as  $\text{PLA}_2$  activation (94,197) and modulation of calcium influx (217), are also mediated by pertussis toxin G proteins. Also, in rat aortic smooth muscle (216) or with overexpression of the  $\alpha_{1B}$ -AR in oocytes (218), PLC coupling involves interaction with  $G_o$ . Direct coupling of the  $\alpha_{1B}$ -AR to  $G_s$  to stimulate adenylyl cyclase activity has also been reported in transfected CHO cells (219). In addition to modulation of these classical effector pathways,  $\alpha_1$ -ARs regulate growth responses via activation of the MAPK family, including ERK1/2, c-Jun N-terminal kinases (JNKs), and the p38 kinases. Thus,  $\alpha_1$ -ARs can also activate the MAPK pathway (175). MAPKs are a large family of widely expressed serine/threonine kinases (220), involving three major subfamilies: the ERKs, the JNKs, and the p38 MAPKs. All three MAPKs can be activated  $\alpha_1$ -ARs, albeit to variable extents (175). In particular, their activation plays a critical role in the hypertrophic response of cardiac myocytes to  $\alpha_1$ -AR stimulation (221). The cellular pathways involved have not yet been completely elucidated, but activation of Ras, Rho, and their downstream kinases has been implicated (175). The upstream signals probably involve various receptor-coupled second messengers, such as  $\text{Ca}^{2+}$  and PKC, as well as G proteins (both  $\alpha$ - and  $\beta\gamma$ -subunits) (220). In addition, they may involve tyrosine kinases (Pyk2, Src), adaptor proteins (Shc), and phosphoinositide-3 kinase (PI3K) (175).

As with other GPCRs, the domains of the  $\alpha_1$ -ARs involved in G protein coupling are the intracellular loops, particularly  $i_3$  (222). For example, substitution of a portion of  $i_3$  of the  $\alpha_{1B}$ -AR into the corresponding region of the  $\beta_2$ -AR results in a chimera that can now activate PI hydrolysis but not cAMP generation (223). Moreover, overexpression of the  $i_3$  segment of the  $\alpha_{1B}$ -AR using a minigene

construct inhibits coupling of this receptor to inositol phosphate turnover (224). Finally, selective deletion of a portion of  $i_3$  of the  $\alpha_{1B}$ -AR impairs its ability to couple to  $G_{q/11}$ ,  $G_{14}$ , and  $G_{16}$  (225). The  $i_3$  sequences involved in the activation of G proteins by the  $\alpha_{1B}$ -AR subtype involve residues extending from Lys<sup>240</sup>-His<sup>252</sup> for activation via  $G_{\alpha q}$  and  $G_{\alpha 11}$  but not for activation via  $G_{\alpha 14}$  or  $G_{\alpha 16}$  (225). Two segments in  $i_3$ —one at the amino terminus and another at the carboxy terminus—are required for  $G_{\alpha i}$  activation and to some extent for coupling to  $G_{\alpha 16}$  (225). However, no consensus sequences have been defined that predict selective interaction of the various  $\alpha_1$ -AR subtypes with specific G proteins (222). This is not altogether surprising given that even when receptors belonging to the same family couple to similar G proteins, they share little or no amino acid identity within their G protein-interacting intracellular loops. As a corollary, in addition to primary amino acid sequence, the secondary and tertiary structures of the intracellular loops are likely critical for the specificity of receptor/G protein coupling.

### 3.3. $\alpha_2$ -ARs G Protein and Effector Activation

Signaling by  $\alpha_2$ -ARs mainly involves coupling via  $G_i$  and hence leads to the inhibition of adenylyl cyclase (107,226). Also, Eason and colleagues have demonstrated pertussis toxin-insensitive activation of cAMP in CHO cells expressing high levels of  $\alpha_2$ -AR (226). Moreover, they have also demonstrated a direct agonist-dependent physical coupling of the  $\alpha_{2A}$ -AR to  $G_s$ .  $G_q$  coupling has been demonstrated in HEK293 cells transiently transfected with the porcine  $\alpha_{2A}$ -AR and either murine  $G_{\alpha q}$  or rat  $G_{\alpha s}$  (107). However, coupling of the  $\alpha_{2A}$ -AR to endogenous  $G_i$  was approx 1000 times greater than that to  $G_s$  or  $G_q$ . Hence,  $\alpha_2$ -ARs preferentially couple to the  $G_{i/o}$  families of G proteins (227), and different amino acids are responsible for the activation of  $G_i$  and  $G_s$  (228,229).

Coupling of  $\alpha_2$ -ARs to  $G_i$  leads to inhibition of adenylyl cyclase, which results in decreased cAMP generation. Coupling to several other signaling pathways has also been reported for the  $\alpha_2$ -ARs, including activation of  $K^+$  channels (230); inhibition of calcium channels; activation of the  $Na^+/H^+$  antiporter (231); and mobilization of intracellular  $Ca^{2+}$  (232).

The  $\alpha_{2A}$ - and  $\alpha_{2D}$ -ARs can couple to at least  $G_{i2}$ ,  $G_{i3}$ , and  $G_{o1}$  (233–237), whereas the  $\alpha_{2C}$ -AR couples to  $G_{i1}$ ,  $G_{o1}$ , or  $G_{o2}$  (238,239). After reconstitution in phospholipid vesicles, both the  $\alpha_{2A}$ - and  $\alpha_{2C}$ -ARs have been shown to couple to members of the  $G_{i/o}$  subfamily with the following potency:  $G_{i3} > G_{i1} = G_{i2} > G_{o1}$  (240).

In addition to inhibition of AC, stimulation of both the  $\alpha_{2A}$ - and  $\alpha_{2C}$ -ARs results in direct activation of PLC via a PTX-sensitive G protein (241). Hence, as for many other receptor subtypes,  $\alpha_2$ -ARs can couple to multiple effector systems, indicating heterogeneity of receptor–effector interactions. In addition, the effector pathway utilized is dependent on the specific ligand employed and its concentration.

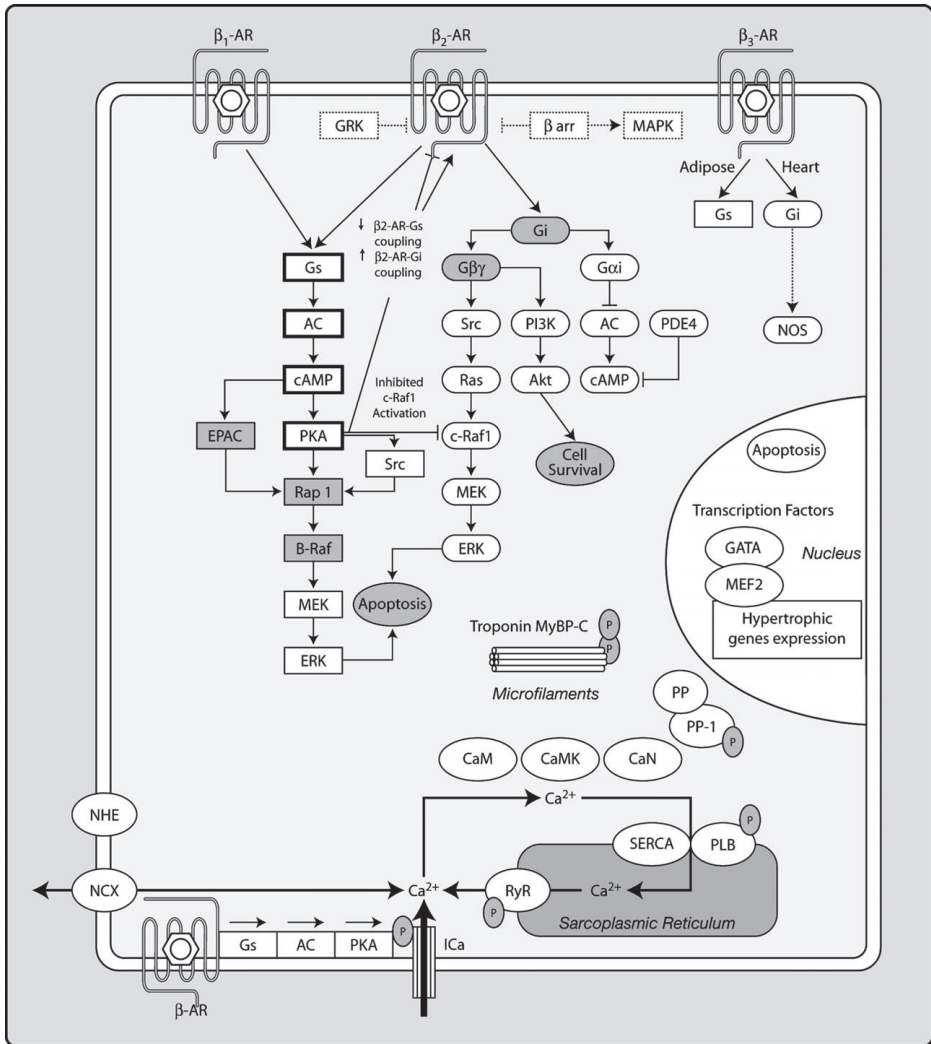
### 3.4. $\beta$ -AR G Protein and Effector Activation

All three  $\beta$ -AR subtypes activate effectors via coupling to  $G_s$ . In cardiomyocytes, stimulation of  $\beta$ -AR by nonselective agonists results in  $G_s$ -mediated adenylyl cyclase activation and enhanced c-AMP generation, which in turn causes activation of PKA. In some cells, enhanced cAMP generation also results in activation of cAMP-gated ion channels. As depicted in Fig. 9, PKA phosphorylates several proteins that are involved in cardiac function, which results in activation of L-type calcium channels (242,243); disinhibition of the sarcoendoplasmic reticular  $Ca^{2+}$ -ATPase as a result of phospholamban (PLB) phosphorylation (244); and activation of troponin I (245), ryanodine receptors (246), and myosin-binding proteins (247). In addition, PKA also phosphorylates and activates protein phosphatase inhibitor-1 (248), which inhibits protein phosphatase-1 and thus prevents dephosphorylation of PLB and other substrates. These  $\beta$ -AR-mediated effects enhance contractility by increasing  $Ca^{2+}$  influx (activation of L-type channels), increasing  $Ca^{2+}$  reuptake into the sarcoplasmic reticulum (PLB/SERCA), and modulating myofilament  $Ca^{2+}$  sensitivity (troponin I, myosin binding protein C). Activation of  $\beta$ -ARs also results in enhanced cAMP-dependent gene transcription (Fig. 9, lower panel).

Although the cAMP pathway is activated by both  $\beta_1$ - and  $\beta_2$ -ARs in both cardiomyocytes (249,250) and transfected cells (251,252),  $\beta_2$ -AR-mediated adenylyl cyclase activation is greater than that by  $\beta_1$ -ARs. These two  $\beta$ -AR subtypes also have opposing actions in regulating cardiomyocyte apoptosis: stimulation of the  $\beta_1$ -AR increases apoptosis, whereas stimulation of the  $\beta_2$ -AR inhibits it (253). Such differences may be because, whereas the  $\beta_1$ -AR couples exclusively to  $G_{\alpha_s}$ , the  $\beta_2$ -AR can also activate effectors via coupling to  $G_i$  (254–256). For example, Daaka and colleagues (254) reported that, in HEK293 cells,

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**Fig. 9.** (*Opposite page*) Classical and cardiomyocyte  $\beta$ -AR signaling pathways. Upper portion: Indicated in white rectangular boxes are the classical signaling pathways mediated by the  $\beta$ -ARs, which mainly involve  $G_s$ -mediated PKA activation. The bold-lined boxes indicate the signaling molecules common to both  $\beta_1$ - and  $\beta_2$ -ARs signaling via  $G_s$ , whereas the thin-lined boxes indicate those activated solely by the  $\beta_2$ -AR. Indicated in oval boxes are the nonclassical signaling pathways. Of particular importance is the PKA-mediated switch in  $\beta_2$ -AR signaling from  $G_s$  to  $G_i$  coupling. Hence, both  $G_s$ - and  $G_i$ -mediated pathways lead to ERK activation by the  $\beta_2$ -AR. Signaling intermediates in gray are unique to either the  $G_s$  or the  $G_i$  pathway, whereas those without color are common to both pathways. Indicated in the dotted boxes are the two major mediators of  $\beta_2$ -AR desensitization: GPCR kinase (GRK) and  $\beta$ -arrestin ( $\beta$ -arr). Interaction of  $\beta$ -arrestin with the  $\beta_2$ -AR leads to its internalization and the activation of new signaling pathways, which result in ERK activation. Lower portion: G protein and effector pathways involved in  $\beta_2$ -AR-mediated enhancement of cardiac inotropy via release of  $Ca^{2+}$  from the sarcoplasmic reticulum. (*Continued on next page*)



**Fig. 9.** (Continued from opposite page) AC, adenylyl cyclase; c-AMP, cyclic adenosine monophosphate; β-arr, β-arrestin; CaM, calmodulin; CaMK, calmodulin-dependent kinase; CaN, calcineurin; EPAC, exchange protein directly activated by cAMP; ERK, extracellular signal-regulated kinase; GRK, G protein coupled receptor kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MEF2, myocyte enhancing factor 2; NCX, sodium-calcium exchanger; NHE, sodium-hydrogen antiporter; NOS, nitric oxide synthase; P, a phosphate group that has modified the target protein as a result of phosphorylation; PDE, phosphodiesterase; PI3K, phosphatidylinositol-3 kinase; PKA, c-AMP-dependent protein kinase A; PLB, phospholamban; PP, protein phosphatase; RyR, ryanodine receptor; SERCA, sarcoendoplasmic reticular calcium ATPase. (This figure has been adapted from refs. 284 and 300.)

activation of the  $\beta_2$ -AR results in initial PKA-mediated receptor phosphorylation. The resulting phosphorylated receptor is no longer able to activate adenylyl cyclase, but switches to activation of MAPK, a response mediated by a pathway involving stimulation of c-Src and Ras by the  $G_{\beta\gamma}$ -subunits of pertussis toxin-sensitive  $G_i$ .

This switch in the coupling of the  $\beta_2$ -AR from  $G_s$  to  $G_i$  signaling leads to the activation of not only ERKs (254), but also Akt/protein kinase B (257), phosphoinositide-3-kinase (257), certain receptor tyrosine kinases (258) and inhibition of adenylyl cyclase (259). In cardiomyocytes,  $G_i$ -mediated stimulation of the PI3K/Akt-dependent cell survival signaling pathway is thought to prevent cardiomyocytes from undergoing  $G_s$ -mediated apoptosis. This  $\beta_2$ -AR-coupled,  $G_i$ -mediated pathway may also result in the transactivation of the epidermal growth factor receptor (254,257–262) and has been demonstrated in a variety of different cell lines, including cultured rat cardiomyocytes (261), HEK293 cells (254), CHO cells (262), and COS-7 cells (258). Both the classical  $\beta_2$ -AR  $G_s$ -mediated ERK activation pathway and the  $G_s/G_i$ -mediated ERK activation pathway are depicted in Fig. 9.

In addition to PKA, the  $\beta_2$ -AR can be phosphorylated by PKC and GRKs as part of the desensitization response. GRKs phosphorylate the ligand-activated  $\beta_2$ -AR and thereby stimulate recruitment of arrestin, which targets the receptor for internalization. In addition, the receptor/ $\beta$ -arrestin complex recruits several proteins that initiate nonclassical signaling pathways. Because this GRK/ $\beta$ -arrestin targeting mechanism is more prominent with the  $\beta_2$ - than the  $\beta_1$ -AR, this could explain why the activation of these nonclassical signaling pathways is more evident with the former subtype. The differing behavior of the  $\beta$ -AR subtypes, in terms of  $\beta$ -arrestin binding and internalization, might also regulate some of their differential signaling properties. It has also been suggested that the receptors may initially be embedded into large signalosomes, which differ for the two subtypes. The hypothesis underlying this proposal is that spatial segregation of receptors allows their association with other sequestered proteins to form specific signaling complexes that mediate subtype-specific responses. In this context, it has been shown that, in neurons, a  $\beta_2$ -AR signalosome containing an entire signaling chain could be isolated (263,264).

Although subtype-specific differences in receptor compartmentalization have been suggested, the mechanisms underlying such differential receptor segregation are not well understood. One possibility is that of differential plasma membrane localization of the two different receptors. Indeed, it has been shown that the  $\beta_2$ -AR could be copurified with caveolae from adult cardiomyocytes, whereas the  $\beta_1$ -AR was much more evenly distributed in these cells (265). However, in rat neonatal cardiomyocytes, the  $\beta_1$ -AR is associated with caveolae (250). cAMP signals might also be spatially regulated via their site of generation (receptor

localization) and destruction (phosphodiesterases). In particular, activation of the  $\beta_2$ -AR leads to  $\beta$ -arrestin-mediated phosphodiesterase (PDE4) recruitment to the plasma membrane, which may regulate the switching from  $G_s$ - to  $G_i$ -mediated signaling (266). Hence, it seems that although signals are usually measured as global changes in second messenger concentrations—which are therefore assumed to change in a uniform manner throughout the cell—compartmentalization of intracellular signaling might be essential, and both spatial and temporal regulation of receptor-induced signaling seems to be operative with both  $\beta_1$ - and  $\beta_2$ -ARs.

Finally, selective coupling of the  $\beta_1$ -AR only to  $G_s$  may not hold in all cell types because ERK activation via  $\beta_1$ -AR and  $G_i$  has been demonstrated in COS-7 cells, a response regulated by the PDZ domain-binding protein: GAIP-interacting protein, carboxy (C) terminus (267). Indeed, both the  $\beta_1$ - and the  $\beta_2$ -AR contain carboxy-terminal PDZ-binding domains that can bind to PDZ domain-containing proteins (268). In this context, the  $\beta_2$ -AR has been shown to interact with  $\text{Na}^+/\text{H}^+$  exchanger regulatory factors in an agonist-dependent manner via its PDZ-binding domain (268), and the  $\beta_1$ -AR interacts with MAGI-2 (269) and PSD-95 in a GRK5-dependent manner (270). Expression of the  $\beta_3$ -AR subtype is mainly limited to the adipose tissue (271), but several groups have reported  $\beta_3$ -AR effects and messenger ribonucleic acid (mRNA) in human, guinea pig, and canine heart and cardiomyocytes (272–274). However, although it couples to  $G_s$  in fat cells, these reports suggest coupling to a nonclassical  $G_i$ /nitric oxide pathway that produces enhanced inotropic effects in human heart (275, 276).  $\beta_1/\beta_2$ -Knockout mice have little or only very slight  $\beta_3$ -AR effects (277–279), whereas with cardiac-specific overexpression of this receptor, enhanced cardiac contractility has been observed (280). Thus, the physiological role of this subtype outside adipose tissue remains to be defined. A fourth receptor subtype, the  $\beta_4$ -AR, has also been postulated to mediate the cardiac effects of the agonist CGP12177, but studies with the  $\beta_1$ -AR and  $\beta_2$ -AR knockout mice revealed that these effects were actually mediated through the  $\beta_1$ -AR (281).

### 3.5. Regulation of G Protein and Effector Coupling

To allow precise homeostatic regulation of cellular functions, signaling pathways that are turned on also need to be turned off, and to this end, agonist occupation of receptors has been shown to initiate a series of molecular processes that control temporal and spatial receptor input. Three such regulatory processes are desensitization, internalization, and downregulation. Desensitization—a phenomenon commonly described as the waning of the receptor response on continuous agonist exposure—is characterized by receptor/G protein uncoupling and occurs rapidly (within seconds to minutes). Two forms, homologous and heterologous, have been identified. The former refers to uncoupling that

occurs with exposure of a receptor to its own agonist, the latter to uncoupling of a receptor as a result of continuous activation of a different receptor.

Although not fully understood, desensitization involves several mechanisms, including receptor phosphorylation and interactions with intracellular protein partners, especially arrestins, which by binding to the G protein recognition site, block the G protein/receptor interaction. Phosphorylation of GPCRs can be mediated by second messenger-dependent protein kinases (PKA or PKC), which phosphorylate receptors whether occupied by an agonist or not. Phosphorylation by these kinases thus is involved in heterologous desensitization. Receptor phosphorylation can also be mediated by GRKs, which selectively phosphorylate agonist-bound receptors and hence participate in homologous desensitization.

Downregulation, by contrast, occurs more slowly (over hours), and is caused by a decrease in the total number of receptor molecules in the cell, resulting from either a decrease in receptor synthesis, destabilization of receptor mRNA, or an increase in receptor degradation (282,283). Recovery from downregulation therefore requires *de novo* protein synthesis. However, as downregulation is not necessarily coupled to receptor internalization, signals different from those involved in endocytosis are likely required.

Internalization or sequestration of receptors away from the cell surface also occurs more slowly (minutes to hours) than desensitization. Although it might be facilitated by receptor phosphorylation, it can also occur in the absence of phosphorylation. In many instances, internalization is dependent on an interaction with arrestin molecules, which then target receptors for endocytosis via clathrin-coated pits. Once internalized, receptors are either recycled to the cell surface (resensitization) or are degraded in lysosomes.

In addition to their roles in the desensitization and internalization of receptors, arrestins also initiate signals from receptors and have been shown to interact with different kinases and other regulatory proteins, such as Src-family tyrosine kinases, and act as receptor-regulated scaffolds for several ERKs, including JNKs and p38 MAPKs. ERKs are activated by a variety of diverse GPCRs (for review, *see ref. 260*) via  $G_s$ ,  $G_i$ ,  $G_q$ , and  $G_o$ -mediated pathways. Thus, it is now well established that internalization, or at least the sequestration of GPCRs, plays a role in their signaling, and that interaction with arrestins is essential for activation of new signaling pathways. These new or nonclassical signaling pathways and paradigms are considered in the chapter "New Signal Transduction Paradigms" by KP Minneman.

Finally, there is increasing evidence for the specific intracellular localization of receptors with distinct signaling pathways, a process that allows spatial segregation of receptors and thus their association/interaction with other proteins to form signalosomes that may be responsible for subtype-specific responses (284).

## Appendix

### Residue Identification

To allow residues to be readily compared between adrenergic receptor subtypes, the standardized numbering system of Ballesteros and Weinstein (285) is used throughout this chapter to identify residues in the TM helices. Each residue is designated by a three-digit number: The first digit (1 through 7) corresponds to the helix in which it is located; the second and third digits identify its position relative to the most-conserved residue in that helix, which is designated as “.50” (Table 2). Residues C-terminal to the conserved residue are designated by successively increasing numbers, whereas those N-terminal are identified by successively decreasing numbers. For example, residues of the “DRY” motif are designated as D3.49, R3.50, and Y3.51.

**Table 2**  
**Residue Designation<sup>a</sup>**

Receptor	1.50	2.50	3.50	4.50	5.50	6.50	7.50
$\alpha_{1A}$	N44	D72	R124	W151	P196	P287	P323
$\alpha_{1B}$	N63	D91	R143	W170	P215	P309	P345
$\alpha_{1D}$	N114	D142	R194	W221	P266	P363	P399
$\alpha_{2A}$	N51	D79	R131	W158	P208	P351	P303
$\alpha_{2B}$	N30	D58	R110	W137	P184	P386	P423
$\alpha_{2C}$	N69	D97	R149	W176	P222	P396	P433
$\beta_1$	N76	D104	R156	W183	P236	P339	P374
$\beta_2$	N51	D79	R131	W158	P211	P288	P323
$\beta_3$	N55	D83	R135	W162	P216	P307	P343

<sup>a</sup>The numbers following the amino acids (identified using the single letter code) indicated below each Ballesteros and Weinstein position are those of the human adrenergic receptor sequences.

## Glossary

The following definitions are based on those recommended by the International Union of Pharmacology, Committee on Receptor Nomenclature and Drug Classification (286).

### Affinity

The equilibrium constant of the reversible reaction of a drug with a receptor to form a drug-receptor complex, dependent on the chemical natures of both the drug and the receptor.

### Agonist

A ligand that binds to a receptor and alters the receptor-state resulting in a biological response. Conventional agonists increase receptor activity, whereas *inverse agonists* reduce it.

**Antagonist**

A ligand that inhibits receptor activation by another ligand, generally an agonist.

$e_1, e_2, e_3$

Extracellular loop 1, extracellular loop 2, extracellular loop 3.

$EC_{50}$

The molar concentration of an agonist that produces 50% of the maximal possible effect of that agonist.

**Efficacy**

The degree to which different agonists produce a response, given the same proportion of occupied receptors. For example a *partial agonist* has a reduced efficacy compared to a *full agonist* for the same receptor

**Full agonist**

An agonist which has the ability to produce the maximal response for a given system. The designation of full vs partial agonist is system-dependent.

$i_1, i_2, i_3$

Intracellular loop 1, intracellular loop 2, intracellular loop 3.

**Inverse agonist:**

A ligand that by binding to receptors reduces the fraction of receptors in the active conformation.

$K_d$

The equilibrium dissociation constant of a ligand determined directly in a binding assay using a labeled form of the ligand.

**Ligand**

A molecule that binds to a receptor.

**Partial agonist**

An agonist that cannot elicit a maximal response (even when applied at high concentrations to ensure full receptor occupancy). The designation of full vs partial agonist is system-dependent.

**Potency**

The activity of a drug, in terms of the concentration or amount needed to produce a defined effect.

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