

The Angiotensin II AT₁ Receptor Structure-Activity Correlations in the Light of Rhodopsin Structure

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Oliveira L, Costa-Neto CM, Nakaie CR, Schreier S, Shimuta SI, Paiva ACM. The Angiotensin II AT₁ Receptor Structure-Activity Correlations in the Light of Rhodopsin Structure. *Physiol Rev* 87: 565–592, 2007; 10.1152/physrev.00040.2005.—The most prevalent physiological effects of ANG II, the main product of the renin-angiotensin system, are mediated by the AT₁ receptor, a rhodopsin-like AGPCR. Numerous studies of the cardiovascular effects of synthetic peptide analogs allowed a detailed mapping of ANG II's structural requirements for receptor binding and activation, which were complemented by site-directed mutagenesis studies on the AT₁ receptor to investigate the role of its structure in ligand binding, signal transduction, phosphorylation, binding to arrestins, internalization, desensitization, tachyphylaxis, and other properties. The knowledge of the high-resolution structure of rhodopsin allowed homology modeling of the AT₁ receptor. The models thus built and mutagenesis data indicate that physiological (agonist binding) or constitutive (mutated receptor) activation may involve different degrees of expansion of the receptor's central cavity. Residues in ANG II structure seem to control these conformational changes and to dictate the type of cytosolic event elicited during the activation. 1) Agonist aromatic residues (Phe⁸ and Tyr⁴) favor the coupling to G protein, and 2) absence of these residues can favor a mechanism leading directly to receptor internalization via phosphorylation by specific kinases of the receptor's COOH-terminal Ser and Thr residues, arrestin binding, and clathrin-dependent coated-pit vesicles. On the other hand, the NH₂-terminal residues of the agonists ANG II and [Sar¹]-ANG II were found to bind by two distinct modes to the AT₁ receptor extracellular site flanked by the COOH-terminal segments of the EC-3 loop and the NH₂-terminal domain. Since the [Sar¹]-ligand is the most potent molecule to trigger tachyphylaxis in AT₁ receptors, it was suggested that its corresponding binding mode might be associated with this special condition of receptors.

I. INTRODUCTION

A. The Renin-Angiotensin System

The renin-angiotensin system (Fig. 1) regulates blood pressure and fluid homeostasis (227) by renin-mediated hydrolysis of angiotensinogen to release ANG I. This inactive decapeptide is further hydrolyzed by the angiotensin converting enzyme (ACE) yielding the vasoconstrictor octapeptide ANG II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), the classical effector of the renin-angiotensin cascade. ANG I may also be hydrolyzed by prolyl-endopeptidase and carboxypeptidases to yield the vasodilator heptapeptide ANG-(1-7). Alternatively, ACE-2, a carboxypeptidase homolog of ACE (46, 285), cleaves a single residue from ANG I to generate ANG-(1-9), which can be hydrolyzed by ACE to yield ANG-(1-7) (Fig. 1). Degradation products of ANG II lacking the NH₂-terminal Asp (ANG III) and the Asp-Arg (ANG IV) residues are particularly involved in brain and pituitary physiology (10, 300).

In addition to the regulation of blood pressure, several other functions have been described for ANG II, leading to the discovery of different receptors in different tissues (43), most of them belonging to the superfamily of G protein-coupled receptors (GPCRs). ANG III shares the same receptors and exerts similar effects as ANG II in the brain, where both peptides have equivalent biological potencies to increase blood pressure; however, because ANG II is converted *in vivo* to ANG III, the identity of the true effector is unknown. ANG IV enhances learning and memory in rodents and was shown to bind to a specific receptor, known as the AT₄ receptor, identified as the transmembrane insulin-regulated membrane aminopeptidase (IRAP), which is not a GPCR (5). However, although

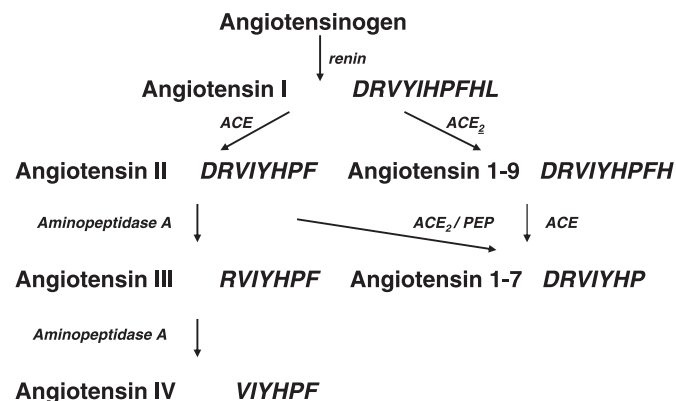


FIG. 1. Renin-angiotensin cascade showing various hydrolytic steps from angiotensinogen to angiotensin-like peptides. ACE, angiotensin converting enzyme; ACE2, ACE-like carboxypeptidase; PEP, prolyl-endopeptidase. Peptide amino acid residues are represented by the one-letter code.

it is inactive on the basal state of the ANG II AT₁ receptor, ANG IV is a full agonist of constitutively activated receptor mutants (145).

Two ANG II receptor types (AT₁ and AT₂) were cloned and sequenced (125, 186, 187, 251), both belonging to the rhodopsin-like family (family A) of GPCRs (AGPCR). ANG-(1-7) was reported to interact with a different GPCR, the mas protooncogene product (250), exerting vasodilator and antiproliferative effects (58, 59).

AT₁ and AT₂ receptor types can be distinguished by their binding profiles to different ligands. Thus, whereas the AT₁ receptor has a high affinity for the nonpeptide antagonist losartan (284), AT₂ binds preferentially to the peptide antagonist CGP42112 (283). The two receptor types trigger distinct (sometimes opposing) signal transduction pathways (113) such as effects leading to growth and antigrowth responses (91, 92). The idea that the AT₂ receptor is an AT₁ receptor antagonist is also suggested by a finding not yet confirmed by others, that the AT₁/AT₂ receptor heterodimer inhibits AT₁ signals (2).

AT₁ and AT₂ receptors also differ in that the former undergoes rapid internalization and desensitization upon agonist stimulation (97, 281), whereas the AT₂ receptor does not internalize and is not desensitized upon interaction with the agonist (87, 108, 186, 288). However, contrasting with these findings, AT₂ receptor was shown to be internalized in response to ANG II and driven to play a novel signaling pathway. This consists of a receptor-mediated translocation of a cytosolic transcription factor, the promyelocytic zinc finger protein (PLZF), to the nucleus and activation of p85 α PI3K and p70^{S6} kinase leading to an increase of protein synthesis (256).

AT₂ receptors have also been shown to play a role in a number of biological processes, such as vascular relaxation, antiproliferation, cellular differentiation, and apoptosis (32, 68, 98, 271), but it is the AT₁ receptor that plays a critical role in signaling pathways that mediate most of the classical physiological and pathological effects of ANG II on target cells, such as vascular smooth muscle cell contraction, aldosterone release, stimulation of sympathetic transmission and cellular growth, salt appetite, thirst, sympathetic outflow, and inhibition of renin biosynthesis and secretion (9, 43, 63, 267, 282). Interestingly, each of these ANG II-induced effects is triggered at different time intervals: seconds in cell signaling, activation of phospholipase C, generation of inositol phosphate (IP) and Ca²⁺ release; minutes in mitogen-activated protein kinase activation; hours in activation of Janus kinase (JNK), signal transducers, and activators of transcription pathway (79).

The AT₁ receptor acts by stimulating protein G_q/G₁₁ and subsequently phospholipase C and IP production or by regulating the level of cAMP by activation of protein G_i (12, 173, 260). In addition to vasoconstriction, regulation

of renal tubular electrolyte handling, aldosterone release, and facilitation of adrenergic release, the AT₁ receptor is one of the most potent stimulators of hypertrophic remodeling of the vascular walls, through ANG II-mediated growth-promoting signals. It has been suggested that the intracellular signaling mechanisms by which the AT₁ receptor exerts hypertrophic and/or hyperplastic effects on targets such as vascular smooth muscle cells are closely associated with receptor and nonreceptor tyrosine kinases (49). Furthermore, it has been demonstrated that G₁₂/G₁₃ can induce vascular smooth muscle cell contraction through a Rho/Rho-kinase (ROCK)-mediated pathway (71). These authors provided evidence that receptor AT₁ couples with G_q and G₁₂ chains to efficiently induce cell contraction via dual regulation of the myosin light chain (MLC) phosphorylation: the Ca²⁺-dependent stimulation of MLC kinase and the Ca²⁺-independent Rho/ROCK-mediated inhibition of myosin phosphatase.

ANG II-stimulated activation of some mitogen-activated protein kinases (MAPKs) has been reported (74, 76), with calcium channels being essential in this mechanism. In fact, activation of the extracellular signal-regulated kinase (ERK₁/ERK₂), tyrosine kinase 2, and Janus-activated kinase 2 (Jak₂), as well as phosphorylation of signal transducer and activator of transcription (STAT₁ and STAT₃), were shown to be inhibited by the calcium channel blocker azelnidipine (152).

Superoxide generation has been found to be mediated by ANG II and to play an important role in vascular smooth muscle cell growth, contraction/relaxation, and inflammation. It was shown that ANG II-induced oxidant activity is increased and generation of reactive oxygen species is enhanced in conditions associated with vascular damage such as in hypertension, ischemia-reperfusion injury, atherosclerosis, and diabetes (30, 49).

AT₁ receptors desensitize following agonist stimulation due to sequestration and endocytosis (281). Differently from this desensitization, acute loss of response upon repeated treatments with ANG II is also observed in smooth muscle cells, characterizing the event of tachyphylaxis (130, 216, 270).

Homodimerization has been described for AT₁ receptors linked to the action of factor XIIIa transglutaminase (3) or as a natural phenomenon occurring in wild-type receptors (233). However, experiments demonstrating oligomerization (or at least cross-talking) in AT₁ receptors consisted of coexpression of a wild-type and a nonfunctional mutant (85, 183). Heterodimerization of AT₁ receptor with AT₂ (2) and bradykinin B₂ (1) receptors, and other AGPCRs such as α_2 -adrenergic receptors (19), has been claimed to occur. Interestingly, it appears that the dimerization of AT₂ receptors might be regulated by disulfide bond exchange (179).

B. Scope of This Review

The ANG II AT₁ receptor is responsible for virtually all of the known peripheral actions of ANG II. It has been most extensively studied by site-directed mutagenesis aiming at the elucidation of ligand binding, signal transduction, phosphorylation, binding to arrestins, internalization, desensitization, tachyphylaxis, and other properties. The main scope of this review is to correlate the AT₁ receptor basic functions (ANG II binding, receptor activation, signal transduction across the transmembrane structure, and some events at the cytosolic ends of the receptor) with the data available about AGPCR sequences, rhodopsin structure, and mechanism of receptor action. Presentation and discussion of related events such as G protein coupling, arrestin binding, desensitization, and tachyphylaxis shall not be fully comprehensive but limited to features considered necessary to enrich the main subject. Other events such as oligo(di)merization of AGPCRs, for which no relationship with receptor activation has been completely confirmed, shall not be discussed.

II. ANGIOTENSIN II STRUCTURE-ACTIVITY RELATIONSHIPS

A. Receptor Binding and Activation

Long before cloning of the receptors was achieved, numerous studies of the cardiovascular effects of synthetic peptide analogs allowed a detailed mapping of ANG II's structural requirements for receptor binding and activation (130, 131, 212, 213, 215). These structural requirements can be assumed to apply to the AT₁ receptor, since the biological responses that were analyzed in those early reports were later found to be mediated by that receptor type.

Analyses of the effects of different ANG II analogs bearing single or multiple modifications indicated that an electrostatic interaction between the ANG II COOH-terminal carboxylate group and the receptor is an important factor for high-affinity binding (100) and that the peptide's Arg², Tyr⁴, and His⁶ side chains are also important for high-affinity binding (31, 178, 239, 240). On the other hand, replacements of Tyr⁴ and mainly Phe⁸ by aliphatic residues were shown to generate competitive antagonists, thus attesting to the importance of these aromatic residues for receptor activation (70, 247). The Val³, Ile⁵, and Pro⁷ residues were found to be neither crucial for binding nor for activation. Asp¹ was not found to be relevant, since analogs with replacements in this position, as well as des-Asp¹-ANG II (ANG III), still retain significant activity. On the other hand, the introduction of a sarcosine

(Sar) residue in position 1 increased the potencies of agonist and antagonist analogs (26, 81, 211, 238, 239). Pharmacological (158) and mutagenesis (249) studies on the AT₁ receptor indicated that [Sar¹]-ANG II has a binding mode different from that of the natural agonist, as discussed in section III, *B4* and *D1*. Also, the presence of Sar in position 1 seems to induce a reaccommodation of other residues involved in binding, such as Arg² (249).

ANG II was shown to be in a random conformation in aqueous solution, various conformers coexisting in equilibrium, which would be displaced towards the "active one" upon binding to the receptor (217). One of these conformers could be that of a horseshoe-shaped structure of ANG II described in a complex of the peptide with antibody (67). Other studies were published in which different conformations were proposed for ANG II or synthetic peptide analogs in aqueous or other media (57, 163, 194, 236), but no rational information was obtained about the "active conformation" that binds to the receptor. Other approaches were based on constraints introduced in the ANG II structure so as to freeze certain conformations of the peptide, thus allowing their biological activities to be checked (122, 192, 306). However, despite the great deal of effort, no conclusive results leading to knowledge about the active conformation of ANG II could be obtained using these approaches.

B. Antagonism by ANG II Analogs

As expected from the structural determinants for signaling described above, one of the first reported antagonists for ANG II was [Phe⁴,Tyr⁸]-ANG II (164), and in the following years, other antagonists were reported, mostly bearing modifications in the Phe⁸ position and usually also with sarcosine in place of Asp¹. A milestone in such studies was the discovery of the analog saralasin ([Sar¹,Ala⁸]-ANG II) as the first high-affinity antagonist (222).

During the first years of investigations on this subject, development of antagonists with modifications at the Tyr⁴ position was less explored, possibly due to the lower potency observed in the first analogs with modifications in this position (246). Later on, sarmesin, a potent antagonist with hydroxymethylated Tyr⁴ ([Sar¹,Tyr(Me)⁴]-ANG II), was described (252). Combined modifications on both Tyr⁴ and Phe⁸ were shown to be nonadditive, generating antagonists of lower potencies (70, 247).

C. Desensitization and Tachyphylaxis

It has been demonstrated in whole animals, isolated tissues, and cultured cells that the AT₁ receptor may be inactivated when left in prolonged contact with the agonist (96, 130, 184, 216, 218, 226, 270, 281). This loss of

response has been linked to two mechanisms, tachyphylaxis and desensitization, which were first recognized by the experimental protocols used to elicit them. Tachyphylaxis is triggered by cycles of stimulation (agonist addition and washing) that have to be repeated twice, thrice, and more times to abolish the response (216, 270). Desensitization is promoted by prolonged contact of receptor with a same dose of agonist.

Differently from desensitization, tachyphylaxis seems to act at the level of agonist-receptor interaction and is dependent on the NH₂-terminal ammonium group and the Arg² guanidinium group of ANG II. [Succinyl¹]-ANG II does not induce and [Sar¹]-ANG II induces an enhanced tachyphylactic phenomenon (211), with this effect being explained by the increased protonation of the sarcosine secondary amino group at physiological pH (214, 216). The importance of the Arg² guanidinium group for tachyphylaxis triggering has also been supported by the fact that [Lys²]-ANG II and even [Sar¹,Lys²]-ANG II cannot induce tachyphylaxis (169, 211). Also differing from desensitization, tachyphylaxis induction depends on the aromatic ring at position 4 of ANG II, but some apparently conflicting aspects need to be analyzed. The phenolic hydroxyl of Tyr⁴ is vital to elicit in vitro tachyphylaxis, a property that is lost with [Phe⁴]-ANG II (185, 262). However, paradoxically, this ANG II analog is able to trigger tachyphylaxis in vivo (286), a condition that was not described for wild-type ANG II.

Thus tachyphylaxis is possibly due to conformational changes on the ANG II-AT₁ receptor complex (126, 216, 263, 264, 270), a supposition compatible with findings showing that this phenomenon can be prevented or reversed by the AT₁ receptor antagonist losartan (241).

Desensitization has been shown to be correlated with phosphorylation of Ser and Thr residues in the cytosolic domains of AT₁ receptor (104, 281). Therefore, rapid desensitization involves primarily phosphorylation (27) of the receptor through two distinct types of Ser/Thr protein kinases: the second messenger-activated kinases, protein kinase A or C (75, 127, 245, 275), and the second messenger-independent G protein-coupled receptor kinases (110).

The installation of both tachyphylaxis and desensitization in AT₁ receptors leads to decays of IP formation and calcium influx (4, 126, 264), but the most flagrant difference between these two states is that only tachyphylaxis is accompanied by reduction of sodium influx (4, 264).

AT₁ receptor sequestration and internalization have been involved in ANG II desensitization in smooth muscle cells (6, 75, 76, 289). However, no results could be obtained from a series of studies supporting this correlation (23, 97, 102, 202, 280). Hence, sequestration does not appear to play a major role in desensitization but may instead be involved in the resensitization process (79).

III. AT₁ RECEPTOR STRUCTURE-ACTIVITY CORRELATIONS

A. Receptor Structure

1. Sequences of AGPCRs

Using the rhodopsin structure (151, 221) as a template, one may define the topography of the AGPCR sequence segments relative to the plasma membrane (Fig. 2) starting at the extracellular (N_t domain) and ending at the cytosolic side (C_t domain). Between these two extremes there is a seven-transmembrane (7TM) bundle (helices I–VII) intercalated by three intracellular loops (IC-1, IC-2, and IC-3) and three extracellular loops (EC-1, EC-2, and EC-3).

Multiple sequence alignment was performed for ~2,800 AGPCRs according to the rhodopsin structure and oriented by very conserved residues in the 7TM helices and adjoining domains which were found without deletion or insertion in many receptors of the family (see Table A in the supplemental material¹ and the consensual segments of sequences in Figs. 2 and 3) (13, 207, 209). Despite these conserved residues, true fingerprints which discriminate family A from other GPCR families, AGPCRs are heterogeneous because they belong to different classes of receptors, containing specific motifs inserted in the loops and terminal domains. However, within receptor types and subtypes, there is a remarkable similarity

regarding both residues and lengths of the various segments (see GPCRDB).

Therefore, AT₁ and AT₂ ANG II receptor types were included in the AGPCR family and are easily identified by multiple sequence alignment (see GPCRDB and Table B in the supplemental material) (Fig. 3). However, the efficiency of this procedure for characterizing the AT₁A, AT₁B, and other subtypes (114, 143) is subject to questions (see Table C and the text of the supplemental material for more details).

To allow a comparison of the different GPCR sequences, residue positions shall be identified using the general GPCRDB numbering system (99). In this system, the numbers for positions in the 7TM bundle have three digits, the first being the number of the helix (1–7) and the remaining two being the number of the position relative to the reference positions (Fig. 2).

The same method was applied to number positions of extramembrane domains that are conserved for some classes of receptors, as indicated in Figure 2 for the EC-1 and EC-2 loops of a large majority of nonolfactory AGPCRs and for the EC-3 loop of ANG II, bradykinin, endothelin, and other receptors (41; see GPCRDB). In receptor domains devoid of conserved motifs (for instance, the IC-1 and IC-3 loops), the respective sequences were divided into two halves (N and C) that were numbered according to the references used in the adjoining helices. As an example, Table 1 shows the segments of an AT₁ receptor sequence (AG1R_RAT; Refs. 115, 187) with the respective natural and general GPCRDB numbers. Residue positions used as references for numbering are

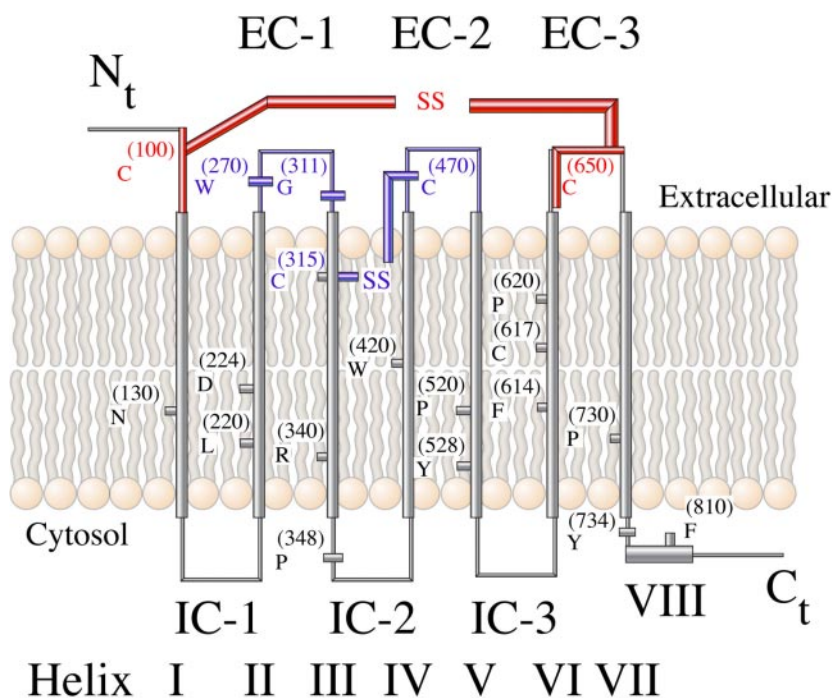


FIG. 2. Schematic representation of the AGPCR 7-transmembrane (7TM) bundle structure, adjacent loops, and domains. TM helices I–VII and cytosolic helix VIII are indicated; ECs and ICs are extracellular and cytosolic loops, respectively. Segments of sequences consensual to all AGPCRs are shown in black; EC-1 and EC-2 loops found in the large majority of nonolfactory AGPCRs are shown in blue; insertions, including a second extracellular disulfide bond found in ANG II, bradykinin, endothelin, and other receptors, are shown in red. Amino acid residues are represented by the one-letter code, and conserved positions used as references for alignment are indicated with the respective general numbers in parentheses.

¹The online version of this article has supplemental data.

indicated. In the text, for every position of the AT₁ receptor (or of other classes of AGPCRs), the numbering system is indicated as shown in Table 1: first the natural number (1–3 digits) followed by superscript A (AT₁ receptor), superscript R (rhodopsin), and superscript G (GPCRDB).

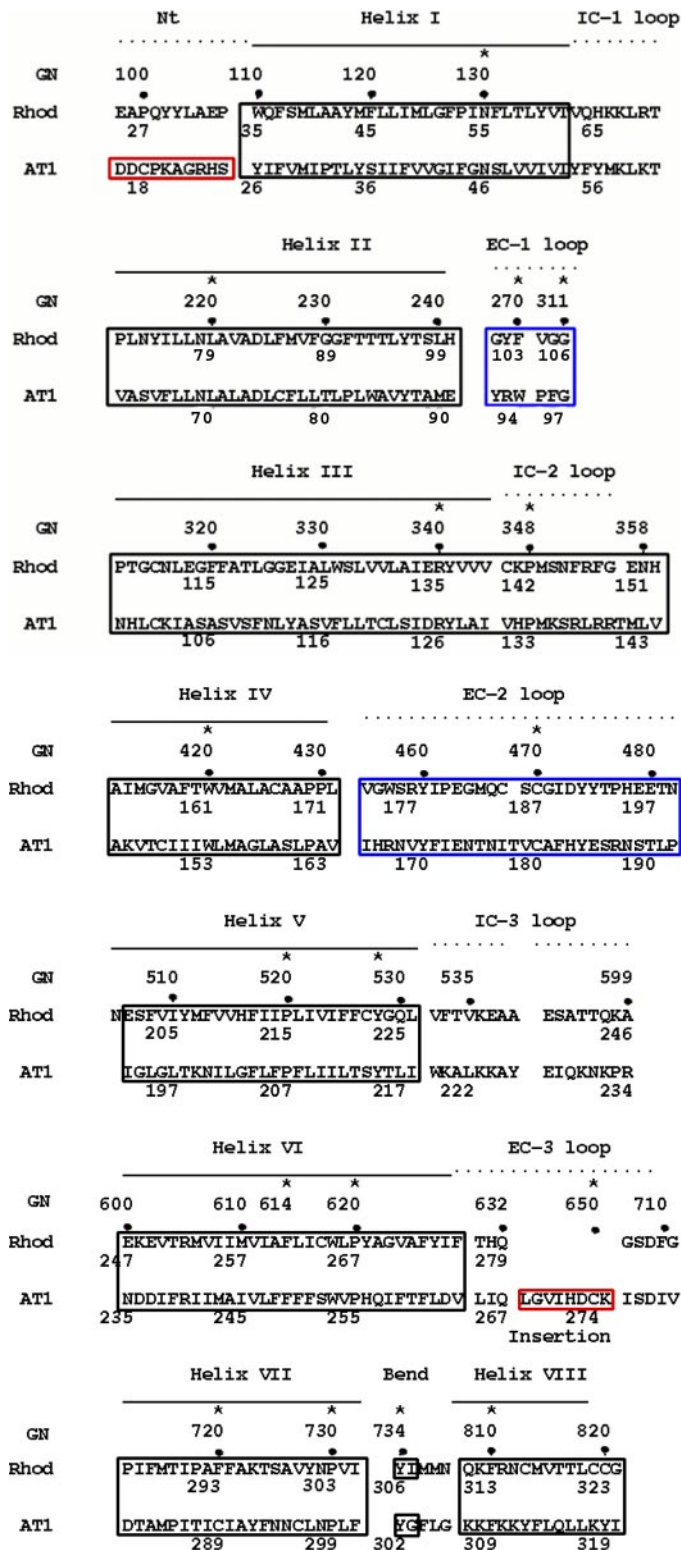


TABLE 1. *The GPCRDB numbering system applied to 7-transmembrane and extramembrane regions of AT₁ receptor sequences*

Region	References	Segment of Sequence	
		AG2_RAT	GPCRDB
N _t	Cys ^{18A(100)}	-25	-107
Helix I	Asn ^{46A(130)}	26-53	110-137
IC-1 loop (N)	Asn ^{46A(130)}	54-58	138-142
IC-1 loop (C)	Leu ^{70A(220)} Asp ^{74A(224)}	59-61	209-211
Helix II	Leu ^{70A(220)} Asp ^{74A(224)}	62-91	212-241
EC-1 loop (N)	Trp ^{94A(270)}	92-94	268-270
EC-1 loop (C)	Gly ^{97A(311)}	95-97	309-311
Helix III	Arg ^{126A(340)}	98-130	312-344
IC-2 loop	Pro ^{133A(348)}	131-134	346-359
Helix IV	Pro ^{153A(420)}	145-164	412-431
EC-2 loop	Cys ^{100A(470)}	165-192	455-482
Helix V	Pro ^{207A(520)} Tyr ^{215A(528)}	193-218	506-531
IC-3 loop (N)	Pro ^{207A(520)} Tyr ^{215A(528)}	219-226	532-539
IC-3 loop (C)	Pro ^{255A(620)} Trp ^{253A(618)}	227-234	592-599
Helix VI	Pro ^{255A(620)} Trp ^{253A(618)}	235-264	600-629
EC-3 loop (N)	Pro ^{255A(620)} Trp ^{253A(618)}	265-267	630-632
EC-3 loop (Ins)	Phe ^{249A(614)}		
EC-3 loop (C)	Cys ^{274A(650)}	268-275	644-651
Helix VII	Pro ^{299A(730)}	276-280	707-711
VII-VIII bend	Tyr ^{302A(734)}	281-301	712-732
Helix VIII	Phe ^{309A(810)}	302-306	734-738
C _t	Phe ^{309A(810)}	307-320	808-821
		321-	822-

See Figures 1 and 2 for identification and text for definitions.

(other AGPCRs), and then the respective general GPCRDB numbers (3 digits) in parentheses.

2. Rhodopsin structure

The ground or inactive structure of bovine rhodopsin (151, 221) consists of a 7TM bundle surrounding an ellipsoidal central cavity whose major axis is aligned with helices I-III and V-VII (Fig. 4). The broader side of this cavity is flanked by helices II-III and VI-VII, and the narrower sides are closed by helices I and V. The cavity is accessible from the intracellular side despite the existence of interactions between the cytosolic ends of helices II, III, and V-VII. The extracellular side is closed by the EC-2 loop hairpin that lies inside the central cavity parallel to the membrane surface, making interactions with

FIG. 3. Sequences of rhodopsin (Rhod) and AT₁ receptors. The segments are identified by the same colors as in Fig. 2. GN, general numbering for AGPCRs (shown in parentheses in the text); the numbers for sequences of rhodopsin and AT₁ receptor are in the lines below the respective sequences; *positions used as references to align the consensual segments of the sequences. The insertion of 8–10 residues observed in the EC-3 loops of AT₁ receptors and other AGPCRs (AT₂, bradykinin, endothelin, purine, Cys-leukotriene) contains a conserved Cys²⁷⁴⁽⁶⁵⁰⁾, supposedly making a second disulfide bond with an N_t conserved Cys^{18A(100)} (41).

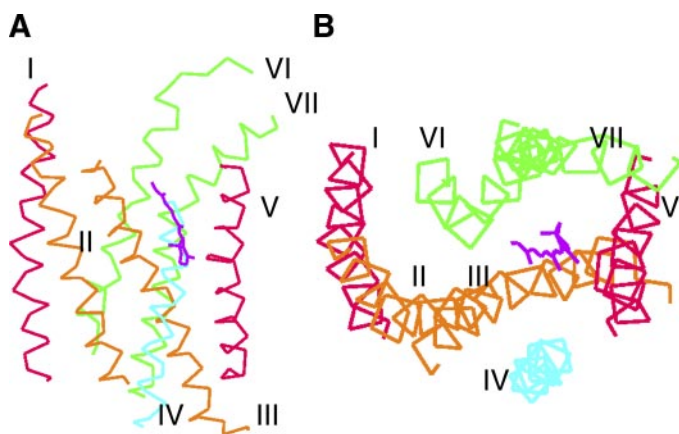


FIG. 4. Longitudinal (A) and cross-section (B) views of rhodopsin's 7TM bundle structure. The helix pairs II and III (gold) and VI and VII (green) form the broader walls of the ellipsoidal central cavity. Helices I and V (red) form the other sides of the cavity. Helix IV is shown in light blue, and retinal is shown in magenta.

side chains of helices. A remarkable interaction in rhodopsin is a disulfide bridge [Cys^{110R(315)}-Cys^{187R(470)}] between EC-2 and the top of helix III (Figs. 2 and 3), which is found in a majority of AGPCRs. Following the end of helix VII, the main chain forms a 90° bend and then a cytosolic helix (helix VIII) running parallel to the membrane. This helix, whose relative position was claimed to be due to crystal contacts, is tied to helix VII, to the VII-VIII bend, and to helices I and II by side chain interactions such as that involving Tyr^{306R(734)} and Phe^{313R(810)} (151) (Figs. 2 and 3).

3. Patterns of residue conservation

Patterns of residue conservation have previously been determined (209) on residue positions of AGPCR aligned sequences (see supplemental material Table A). For each position of the alignment, values of entropy (E) and variability (V) were determined and distributed along an E - V plot (Fig. 5; see legend of this figure for definitions of E and V). Over a broad range of values, it was possible to separate the positions of the sequence alignment according to levels of residue conservation by grouping them into boxes 11, 12, 22, 23, and 33, according to different ranges of E and V values.

When the residue positions of EV boxes were mapped in the rhodopsin structure, an oriented distribution was observed (Fig. 6): 1) the most conserved positions of boxes 11, 12, and 22 are at the cytosolic half of the 7TM bundle central cavity; 2) the positions of boxes 12, 22, and 23 with intermediate E and V values are at the middle of the 7TM bundle central cavity; 3) variable positions of box 23 are in the extracellular half of the central cavity and on the external wall of the 7TM bundle structure forming an interface with the membrane bilayer; and

4) the most variable positions of box 33 are at the extracellular and cytosolic limits of the 7TM bundle.

Applied to well-known protein families, such as globins, ras-like proteins, and serine-proteases (208), EV analysis allowed us to relate function to the structure of these molecules: 1) the more conserved positions are mostly at accessible regions of the structures forming a common main site, heme site in globins, nucleotide site in ras-like chains, and catalytic site in serine-proteases; 2) the more variable positions are mostly at the surface of the structures forming modulator sites, specific for each protein type and subtype; and 3) the positions of boxes 12, 22, and 23 are in the core of the proteins involved in structure-stabilizing or signal-transduction roles.

This structure-function map obtained from known proteins may be extrapolated to AGPCRs in general, what allowed us to assume that the conserved positions of these receptors (EV boxes 11 and 12 in Fig. 5) form a common main site and the variable positions (boxes 23 and 33 in Fig. 5) form modulator sites. The main site is at the cytosolic side of the 7TM bundle central cavity and may be related to a common cluster of conserved polar residues consisting of Asn⁽¹³⁰⁾, Asp⁽²²⁴⁾, Asp⁽³³⁹⁾, Arg⁽³⁴⁰⁾, Asn⁽⁷²⁹⁾, and Tyr⁽⁷³⁴⁾ (see Fig. 3) which includes a part of a sodium allosteric site for some AGPCR classes (191) (see sect. III D2). The most important modulator site (the

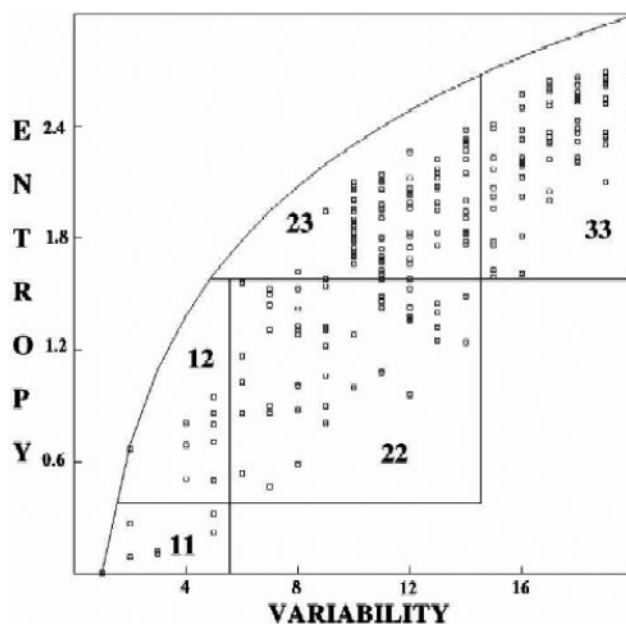


FIG. 5. Entropy/variability plot calculated for residue positions in multiple sequence alignment of ~2,800 AGPCRs. Variability (V) is the number of different residues (1-20) found at each position. Entropy (E) (0.0-3.0) is given by the equation $E = -\sum(i=1-20)f_i(\ln f_i)$, where f_i is the fraction of each amino acid found at each position. In the numbers designating boxes 11, 12, 22, 23, and 33, the first and second digits express increasing ranges of V and E values, respectively. Thus 11 and 33 are boxes containing highly conserved and highly variable residue positions, respectively.

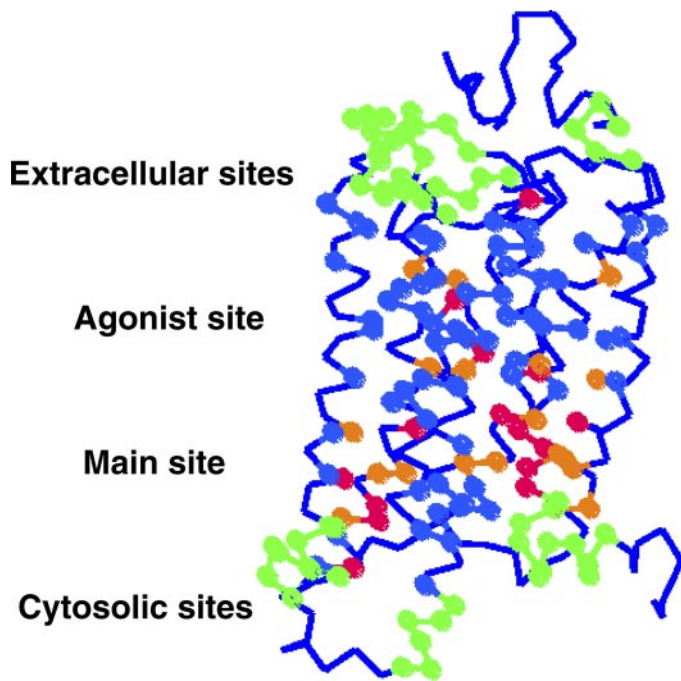


FIG. 6. Structure of rhodopsin 7TM bundle showing residue positions identified according to degree of residue conservation. Within the limits indicated for the membrane bilayer, only internal positions (pointing to the structure central cavity) are shown. Dark blue, variable internal positions (EV boxes 23 and 33); orange, internal positions with intermediate values of residue variation (EV boxes 12 and 22); red, very conserved internal positions (EV box 11); green, variable extramembrane positions; light blue, very variable extramembrane positions.

agonist site) is at the extracellular side of the 7TM bundle central cavity (29, 205) (Fig. 6) and corresponds to the retinal site in rhodopsin structure (221). Positions with intermediate *E* and *V* values in the alignment of all AGPCR sequences are in the core of the 7TM structure, between the two sites. Thus, in the course of receptor activation, a signal may be transmitted from one site to the other through positions with intermediate *EV* values (209).

Additional experimental evidence suggests the existence of other modulator sites (variable positions) in other regions of the AGPCR structure, such as the COOH-terminal end of helix III [including the Tyr⁽³³⁹⁾ residue of the DRY motif], the cytosolic ends of helices V and VI and adjacent sequences of the loop between these helices, helix VIII, and the VII-VIII bend (see references in supplemental material Table D).

4. The AT₁ receptor

Despite the low level of residue identity (~20% in relation to rhodopsin), AT₁ receptors can be modeled (203) in homology to rhodopsin structure (151, 221), at least at the level of sequence segments containing conserved residues (Fig. 7). Details and the rationale support-

ing this procedure are described in the supplemental material.

A model of inactive AT₁ receptor was built by homology to available inactive rhodopsin structures (Fig. 7A). A model of the activated receptor (Fig. 7B) was obtained by an expansion of the 7TM bundle, allowing the docking of one molecule of the peptide. For these modeling procedures, the alignment of the sequences in the 7TM bundle and extramembrane domains to the rhodopsin structure was made using the scheme shown in Figure 3 (249).

A) BINDING-RELATED RESIDUES. Binding assays using radiolabeled ANG II or peptide analogs, and cloned AT₁ receptors heterologously expressed in cultured cells, allowed identification of the following binding-related residues (Fig. 7, B and C): 1) the ANG II COOH-terminal carboxylate group is neutralized by the positive charge of the receptor's helix V Lys^{199A(512)} (94, 111, 183, 195, 302, 303); 2) the peptide's Tyr⁴ side chain binds at the receptor's Asn^{111A(325)} (55); 3) the receptor's Ser^{105A(319)} is required for ANG II binding (78); 4) ANG II's His⁶ and Phe⁸ side chains are in proximity to the receptor's His^{256A(621)} and Phe^{259A(624)} side chains (82, 195, 196); 5) the peptide's Arg² guanidinium group interacts either with the receptor's Asp^{281A(712)} (53) or Asp^{278A(709)} side chain (94) {this discrepancy seems to be due to the fact that different ligands (ANG II or [Sar¹,Leu⁸]-ANG II) were used in the two studies, as discussed in sect. mD1}; 6) the NH₂-terminal half of the EC-2 loop, including residues Val^{179A(469)} (94) and His^{183A(473)} (53, 129, 303), is involved in ANG II binding; 7) Lys^{101A(316)}, located at the extracellular end of helix III, is important for binding (94, 183); 8) other residues involved in agonist binding are the N_i domain Arg^{23A(105)} (249), His^{24A(106)}, Tyr^{26A(108)}, and Ile^{27A(109)} (94) as well as the EC-1 loop Thr^{88A(238)} (42) and Tyr^{92A(268)} (42, 94).

Besides site-mutation studies, structures of AGPCRs have been analyzed by two indirect procedures: the substituted-cysteine accessibility method (16, 120, 176, 181) and the methionine proximity assay (36, 154). In these methods, residues are replaced by Cys and Met, and their relative positions in the three-dimensional structure of the receptors are defined as a function of kinetic parameters determined with Cys-specific reactants and by means of photoaffinity labeling, using (in the case of the AT₁ receptor) ANG II analogs with benzophenone residues in position 8 (36).

The relative location (inside or outside the structure) of residues along the sequences of helices was estimated in many AGPCRs from cysteine accessibility results (16). Also, movements of helices upon AT₁ receptor activation, as denoted by change in accessibility of strategically placed Cys residues, was studied by Miura and Karnik (176) and Miura et al. (181). A drawback linked to the Cys accessibility method is that this residue is revealed by bulky reagents. Because rhodopsin structures (151, 221)

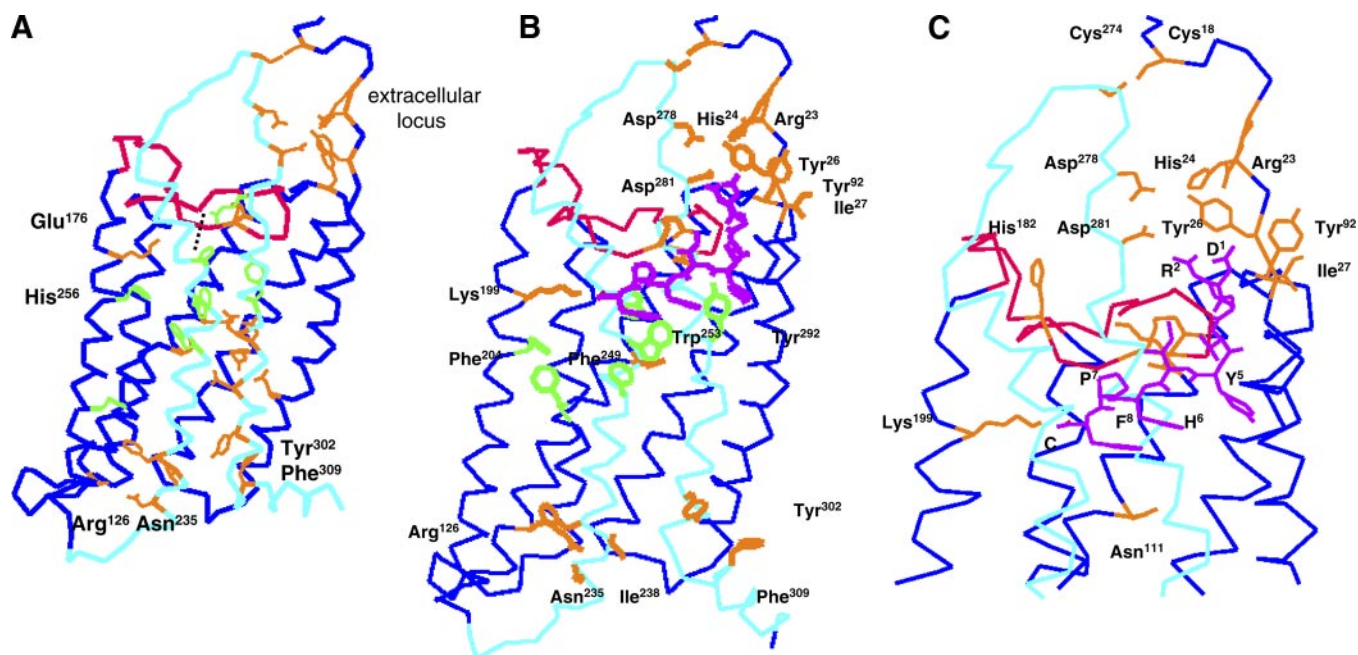


FIG. 7. *A*: model of the inactive AT₁ receptor. The residues closing the extracellular locus (*top right*) and making bonds at the cytosolic ends (*bottom*) are shown in orange. The EC-2 loop hairpin is shown in red. The salt bridge between the EC-2 loop's Glu^{176A(464)} and helix VI's His^{256A(621)} is shown in green. *B*: model of activated AT₁ receptor. The agonist site is at the top of the figure, with the ANG II molecule in magenta. Aromatic residues involved in the activation mechanism are shown in green in the middle of the structure. Bonds between the cytosolic ends (residues in orange) are dissociated. *C*: the agonist site. Peptide residues are identified by the one-letter code. See binding of helix V's Lys^{199A(512)} to the peptide's COOH-terminal carboxylate group and of helix VII's Asp^{281A(712)} to the peptide's R², at the *middle left* and *top right* (extracellular locus) of the figure, respectively.

have an EC-2 loop interacting with the extracellular halves of helices and closing the extracellular mouth of the 7TM bundle central cavity, even small conformational changes, such as those produced by mildly bulky reagents, might artificially change the relative position of that loop and thus the accessibility of helices.

The Met-proximity assay also has limitations. To apply this method to AT₁ receptors, the ANG II's Phe⁸ residue is replaced by a bulky moiety that is likely to interfere with the role of this agonist's aromatic residue to induce receptor activation (178) and with the formation of the salt bridge between the agonist's COOH-terminal carboxylate group and the receptor's helix V Lys^{199A(512)} side chain (53, 94, 111, 302, 303). As a consequence, the obtained results were contradictory. Against results from other laboratories (53, 94, 111, 302, 303), a different geometry for the ANG II binding site was described in which very conserved AGPCR residues [Phe^{249A(614)}, Trp^{253A(618)}, Asn^{294A(725)}, and Asn^{295A(726)}], but not the specific Lys^{199A(512)} and Asp^{281A(712)} residues, are claimed to be involved in peptide binding (36).

B) RESIDUES PARTICIPATING IN RECEPTOR ACTIVATION. ANG II structure-activity studies showed that the peptide's Phe⁸ aromatic side chain is essential for triggering the response by interacting with the receptor's His^{256A(621)} (178) (Fig. 7). Aromaticity and residue size in the ANG II position 4 side chain also appear to be important for activating the

receptor, possibly by interaction with the receptor's Asn^{111A(325)}. In addition, experiments with Lys^{199A(512)} receptor mutants show that this residue can modulate the effect of the His^{256A(621)} side chain on receptor binding. Whereas the H256^{A(621)}A mutation does not affect binding, the loss of affinity of the [K199^{A(512)}A;H256^{A(621)}A] double mutant is significantly larger than that of the K199^{A(512)}A mutant (196), suggesting that, in the AT₁ structure, the Lys^{199A(512)} ammonium group is able to keep the His^{256A(621)} imidazole ring at a specific position, perhaps by forming an intramolecular hydrogen bond. This interaction might not contribute to ligand-receptor affinity (303), but could be important to form a more productive receptor-agonist binding mode. This might involve a relay-like structure consisting of ANG II's COOH-terminal carboxylate, the receptor's Lys^{199A(512)} ammonium group, and the His^{256A(621)} imidazole ring (82, 195).

B. Receptor Activation

AGPCR activation has been associated with a transition of the receptor's structure from a constrained inactive to an expanded active state (146, 149), in contrast to inactivation by binding of nonpeptide antagonists which drive a receptor to a more constrained inactive form (253, 294).

Physiological receptor activation is triggered by agonist binding (297), whereas constitutive receptor activation occurs in the absence of agonistic stimulus by side chain mutations and other modifications of the AGPCR structure (225).

1. Activation of rhodopsin

Activation of rhodopsin is seen as an expansion of the structure. Figure 8A shows that the more likely site of the 7TM bundle structure which may allow this expansion, without the breakage of covalent bonds, is that between two blocks of structure: a (helices I-V) and b (helices VI and VII). Inside the block a helices, helix V also moves in relation to helix III during activation (21, 224). Functionally, ground state rhodopsin has a compressed 7TM bundle structure with an 11-*cis*-retinal moiety linked to helix VII's Lys^{296R(723)} by a Schiff bond (151, 221, 254) (Fig. 8B, 1). Light absorption causes retinal isomerization to an all-*trans*-form (296), followed by slight modifications of the rhodopsin structure, probably limited to the retinal pocket (150, 254). In a late stage, this effect can spread over the structure giving rise to an equilibrium between metarhodopsins I (MI) and II (MII), the latter being the fully active form (112) that triggers transducin coupling and other cytosolic events (168, 244). The activation of rhodopsin can be approached by following the structural features relative to three states.

1) The first is the inactive state, containing important interactions such as those involving the retinal Schiff base

and helix III's Glu^{113R(318)} (Fig. 8B, 1); the EC-2 loop's Glu^{181R(464)} and helix VI's Tyr^{268R(621)} (151) (Fig. 8B, 2); the helix III's Arg^{135R(340)} and helix VI's Glu^{247R(600)} (Fig. 8B, 3); and the VII-VIII bend's Tyr^{306R(734)} and helix VIII's Phe^{313R(810)} (Fig. 8B, 4).

2) The second state is the MI form, in which the bridge between the Schiff base and Glu^{113R(318)} (Fig. 8B, 1) is ruptured and the counterion function exerted by this residue is transferred to Glu^{181R(464)} (243) (Fig. 8C, 5). These modifications lead to the release of the Tyr^{268R(621)} residue and consequently of the extracellular third of helix VI.

3) The third state is the MII form, produced by all-*trans*-retinal contacts with aromatic side chains in the middle of the 7TM bundle: helix VI's Trp^{265R(618)} and Tyr^{268R(621)}, and helix III's Trp^{126R(331)} (137, 157) (Fig. 8C, 6). As a consequence of change in these residues' positions, bonds between cytosolic ends of rhodopsin are ruptured (Fig. 8C, 7 and 8) leading to expansion (170) and activation of the receptor.

2. Physiological activation of AGPCRs and AT₁ receptors

The high variability of residues in the AGPCR agonist binding sites (Fig. 6) reflects the heterogeneity of ligand specificities. In general, the locations and dimensions of the sites may differ according to size of the ligands. For small molecules with retinal-like dimensions, such as bioamines, prostanoids, phospholipids, purines, and small

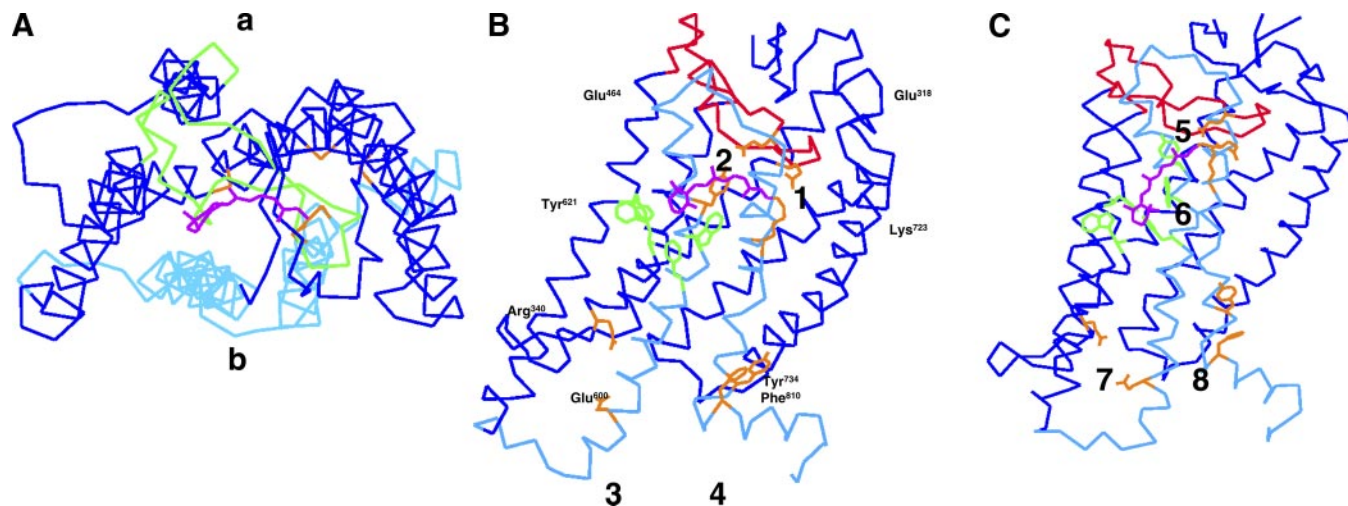


FIG. 8. The 7TM bundle of rhodopsin structure (151) is divided into two blocks: a (helices I-V) and b (TM helices VI and VII, and the cytosolic helix VIII), shown in blue and light blue, respectively. The retinal and the hairpin in EC-2 loop are shown in magenta and red, respectively, whereas residues involved in specific bonds [Lys^{R(723)}, Glu^{R(318)}, Glu^{R(464)}, Tyr^{R(621)}, Arg^{R(340)} and Glu^{R(600)}] are shown in orange. Aromatic residues placed at the middle of the 7TM bundle are shown in green. A: cross-section of the structure showing blocks a and b. B: inactive rhodopsin. Retinal-helix-VII's Lys⁽⁷²³⁾ Schiff base makes a bond with helix III's Glu⁽³¹⁸⁾ (1). Number 2 indicates hydrogen bond between EC-2 loop's Glu⁽⁴⁶⁴⁾ and helix VI's Tyr⁽⁶²¹⁾. At the bottom of the figure, two bonds are shown between helix III's Arg⁽³⁴⁰⁾ and helix VI's Glu⁽⁶⁰⁰⁾ (3) and between the aromatic rings of the VII-VIII bend's Tyr⁽⁷³⁴⁾ and helix VIII's Phe⁽⁸¹⁰⁾ (4). C: a model mimicking active rhodopsin (MII form) displaying the all-*trans*-retinal with the Schiff base making a bond (5) with the EC-2 loop's hairpin Glu⁽⁴⁶⁴⁾. The all-*trans*-retinal is in contact (6) with the cluster of aromatic residues. The bonds between cytosolic ends Arg⁽³⁴⁰⁾-Glu⁽⁶⁰⁰⁾ (7) and Tyr⁽⁷³⁴⁾-Phe⁽⁸¹⁰⁾ (8), present in the inactive structure, are now broken.

peptides, the binding sites overlap entirely the rhodopsin retinal pocket (29, 205). For larger ligands, e.g., peptides (43, 69, 94, 116), melanocortin (159, 210), and glycoprotein hormones (52, 153, 293), the receptor-agonist interaction also involves positions in the extracellular segments of the receptor structure.

A) AGONIST BINDING AND ACTIVATION. ANG II binding to the AT₁ receptor, leading to its activation, is likely to be a two-step process as suggested by studies of Le et al. (145) and Feng et al. (56). The first step involves binding of the peptide's COOH-terminal segment (Tyr⁴-Ile⁵-His⁶-Pro⁷-Phe⁸) at the retinal-like locus inside the 7TM bundle central cavity, oriented by a salt bridge between the peptide's COOH-terminal carboxylate and the receptor's Lys^{199A(512)} side chain (Fig. 7C, *left*). This binding, responsible for the basal agonistic activity, would lead to expansion of the 7TM bundle structure thus enlarging the space between the EC-2 loop's hairpin turn and helices I, II, VI, and VII (Fig. 7C, *right*). For the interaction of its NH₂ terminus (Asp¹-Arg²) with AT₁ receptor (second step of binding), the ANG II molecule should bend at the level of Val³-Tyr⁴ so that the Arg²'s guanidinium group would be at binding distance from the receptor's Asp^{278A(709)} and Asp^{281A(712)} side chains (Fig. 7B, *top*). By completing its interaction at the extracellular space between the EC-1, EC-3 loops, and the N_i domain, the peptide would open this space, favoring the separation of blocks a and b initiated at the 7TM bundle.

AGPCR activation due to agonist binding is to be compared with the mechanism triggered by retinal isomerization in rhodopsin, leading to MI formation (Fig. 8). Agonist binding could exert pressure on the receptor's retinal-like locus, pushing aside the 7TM bundle helices. The possibility of a role of aromatic residues on triggering AGPCR activation was raised by the higher density of these residues in the extracellular half of the receptor's 7TM bundle, flanking the limits of the agonist site (118, 209). As this site comprises variable positions (Fig. 6), activation in different AGPCRs may not be like that described above for rhodopsin, namely, breakage of the bond between EC-2 loop's Glu^{181R(464)} and helix VI's Tyr^{268R(621)} (Fig. 8), being specific for receptor classes and performed by different residues at different locations of that site.

In AT₁ receptor activation, a role of aromatic side chains at the receptor and at the agonist in pushing the structure towards an expanded form is observed. For instance, ANG II's Phe⁸ and Tyr⁴ (and His⁶) aromatic side chains are involved in receptor activation (70, 178, 247) through interactions with helix V's Phe^{204A(517)}, helix VI's Phe^{249A(614)}, Trp^{253A(618)}, His^{256A(621)} and Phe^{259A(624)}, and helix VII's Tyr^{292A(723)} side chains (82, 175, 178, 195, 196) (Fig. 7B).

Interestingly, the AT₁ receptor, like rhodopsin, has an EC-2 loop hairpin's residue, Glu^{173A(464)}, which in the

inactive receptor model (Fig. 7A) is supposed to make a salt bridge with helix VI's His^{256A(621)} side chain. This could be broken upon receptor activation by agonist binding, thus mimicking the mechanism for formation of the rhodopsin MI state. Also the disulfide bridge [Cys^{101A(315)}-Cys^{180A(470)}] between EC-2 and the top of helix III (Figs. 2, 3, and 8) could be broken in the course of this mechanism, thus supporting the hypothesis of a potential role which has been attributed to this bond in AT₁ receptors. Previously proposed by Pederson and Ross (228), Ohyama et al. (200), and Karnik et al. (128) to regulate the activation of these receptors, but mainly to be vital to keep the 7TM bundle structure, the Cys^{101A(315)}-Cys^{180A(470)} disulfide bridge has now been recognized, based on solid mutagenesis experiments, as an important factor to stabilize the inactive state of C5a receptors (136).

The signal started at the agonist site level (extracellular half of the structure) is transmitted along the AGPCR 7TM bundle by changes in receptor structure which in rhodopsin are related to the formation of the fully active MII state. In AGPCRs, this mechanism might follow a pathway along the central cavity of the 7TM bundle structure (agonist-site-interface-main-site) involving positions displaying a gradient of residue conservation (Fig. 6). Signaling would start at the agonist site by different receptor-class-specific (agonist-specific) mechanisms but should converge to a single receptor-family-specific pathway at the cytosolic side of the 7TM bundle.

Translation and rotation of 7TM helices have been proposed to occur during AGPCR activation. In AT₁ receptors, movements of helix II (176), helix VII (25), and helices II and VII (180, 181) have been observed. Nikiforovich et al. (193) have proposed a mechanism of extensive mobilization of the whole helix IV as a result of constitutive activation of AT₁ receptors elicited by mutation in the helix III residue Asn^{111A(325)} and adjacent residues in the three-dimensional structure. Applied to the physiological activation of AGPCRs, this mechanism would be an additional step in the scheme discussed in this and in the following sections.

B) EVENTS AT THE 7TM BUNDLE CYTOSOLIC ENDS. The activation of rhodopsin initiated at the 7TM bundle retinal site leads ultimately to the dissociation of bonds between the cytosolic ends of transmembrane helices, such as the Arg^{135R(340)}, Glu^{247R(600)}, and Tyr^{306R(734)}-Phe^{313R(810)} bonds shown in Figure 8B (7 and 8, respectively), which stabilize the receptors' inactive forms (151).

Many studies on the activation and inactivation of AGPCRs due to mutations on residue positions of 7TM cytosolic ends (see Table D in the supplemental material) reveal that these mechanisms are mainly controlled by interactions between conserved positions (340), (343) and (344) of helix III, the conserved position (810) of helix VIII, the conserved position (734) of the VII-VIII bend, and

the variable positions (600), (603), (604), and (606) of helix VI (see Figs. 2 and 3 for identification of positions).

In rhodopsin, the variable cytosolic half of helix VI seems to work like a stalk holding the inactive form of opsin. Thus Glu^{247R(600)}, Val^{250R(603)}, and Thr^{251R(604)} interact with helix III's Arg^{135R(340)}, Val^{138R(343)}, and Val^{139R(344)} (Fig. 9, green spheres), keeping together blocks a and b of the 7TM structure. In many AGPCRs, mutations at these positions lead to activation (see references in the supplemental material Table D). On the other hand, helix VI residues of Lys^{248R(601)}, Glu^{249R(602)}, and Ile^{253R(606)}, that in rhodopsin's structure are seen pointing outside or to helices VII and VIII (Fig. 9, blue spheres), can make interactions with these helices and thus favor receptor activation. Mutations at position (606) of AGPCRs lead to inactivation of receptors (see references in the supplemental material Table D).

A large residue variation observed in helix VI positions (600), (603), (604), and (606) of all AGPCRs suggests variable strength in the interactions between 7TM cyto-

solic ends, and thus of activation and inactivation patterns in the different classes of these receptors. Indeed, in opsins and a few other receptor classes (~25% of the AGPCRs) for which position (600) contains Glu, the inactive form is stabilized by a salt bridge (15) (Fig. 9). An electrostatic bond between helix III's Arg⁽³⁴⁰⁾ and Glu-(Asp) residues in other internal positions of helix VI, such as (603) and (604), might produce a similar effect. In fact, when Glu or Asp is introduced in positions (600) and (604) of serotonin 5-HT₆ (138) and mu-opioid (101) receptors, these proteins are driven from wild-type constitutively activated to inactive forms.

Some positions placed externally such as those of the EC-2 loop (red spheres in Fig. 9) are involved in AGPCR phosphorylation and G protein coupling mechanisms. The same role can be recognized for external positions of helix VI cytosolic end and helix VIII (not indicated in Fig. 9) (see references in the supplemental material Table D for the respective cytosolic ends).

In AT₁ receptors, position 235^{A(600)} is occupied by Asn, whereas positions 238^{A(603)} and 239^{A(604)} bear Ile and Phe, respectively (Fig. 7, A and B). N235^{A(600)}A mutation resulted in a slight increase of IP formation. Mutations carried out at positions 238^{A(603)} and, mainly, 239^{A(604)} revealed that replacement of Phe^{239A(604)} with polar residues had relatively minor effects on receptor responses, but its replacement by Asp or by positively charged residues reduced or significantly increased these responses, respectively (305). As receptor activation is linked to the breakage of bonds between helices III and VI, one may suppose that positive and negative charges at helix VI's position 239^{A(604)} are repelled and attracted, respectively, by the positively charged Arg^{126A(340)} side chain (Fig. 7, A and B). As helix VI Asn^{235A(600)} is able to form only a weaker hydrogen bond with helix III Arg¹²⁶⁽³⁴⁰⁾, the dissociation of helices III and VI upon N235^{A(600)}A mutation is less sensitive to produce constitutive activation than the breakage of a homologous strong Glu⁽⁶⁰⁰⁾[Asp⁽⁶⁰⁰⁾]-Arg⁽³⁴⁰⁾ salt bond of amine receptors and other AGPCRs. Similar mutations of Ile^{238A(603)} led to inconclusive results (305) due perhaps to the fact that this position is not so frontally located in relation to helix III as position 239^{A(604)}. In consonance with this interpretation, mutation on positions of Asp^{236A(601)} and Asp^{237A(602)} led to AT₁ receptor inactivation and G protein uncoupling (305) or reduction of phosphorylation (202), findings that are consistent with the accessibility of those two positions revealed in the modeled structure of Figures 7A and 9.

Besides the interactions between the cytosolic ends of helices III and VI, the inactive form of rhodopsin seems to be held by a bond between the conserved Tyr^{306R(734)} and Phe^{313R(810)} side chains (Fig. 9, right and bottom) (64), indicating that upon activation this bond is broken, releasing the cytosolic helix VIII from interactions with

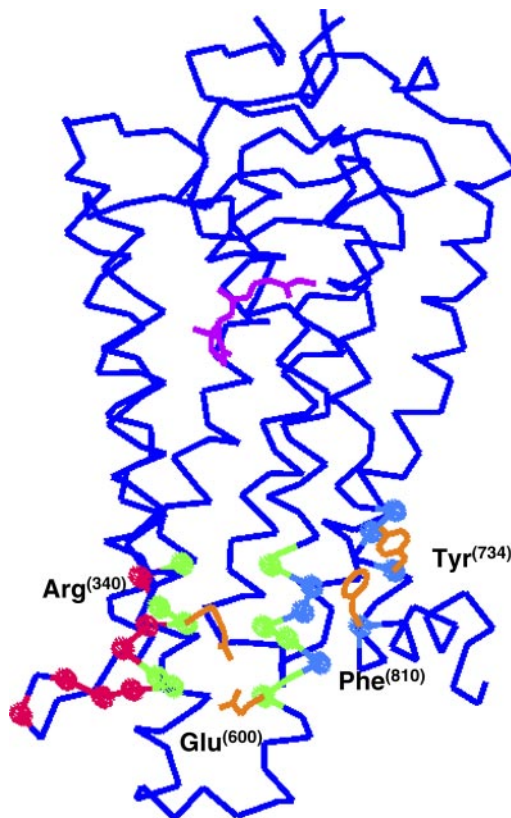


FIG. 9. The cytosolic ends of inactive rhodopsin showing: 1) helix III positions 340, 343, and 344 and helix VI positions 600, 603, and 604, facing each other (green spheres); 2) the external positions of helix III and IC-2 loop (red spheres); and 3) the positions of helices II, VI, VII, and VIII facing each other (blue spheres). A salt bridge between helix III's Arg^{135R(340)} and Glu^{247R(600)} and an aromatic interaction between the side chains of the VI-VII bend's Tyr^{306R(734)} and helix VIII's Phe^{313R(810)} are shown in orange. Blocks a and b (see Fig. 8) are shown in blue and light blue, respectively.

the 7TM bundle structure. A similar condition is found in other AGPCRs, such as the 5-HT(2C) receptor (242).

In short, two very conserved residues [Arg⁽³⁴⁰⁾ and Tyr⁽⁷³⁴⁾] in the cytosolic ends of AGPCR 7TM bundle structure can be used as referential positions to orient the interpretation of postactivation cytosolic events (Fig. 9). It is possible to speculate that activation may be uniform or not depending on whether the structure expansion attains these two spots simultaneously or separately, respectively. In the former event, the two bonds in the cytosolic ends of the rhodopsin [the bond between Tyr^{306R(734)} and Phe^{313R(810)} or the bond between Arg^{135R(340)} and Glu^{247R(600)} at the *right* or *left* of Fig. 9, respectively] are broken at the same time, and the structure is expanded uniformly. In the latter event, one of the two bonds of the rhodopsin structure could be broken first, thus contributing to change the relative orientation of the centrally positioned helix VI, and destabilize the interactions of this helix with the other side of the structure.

The ambivalence of this mechanism of signal transduction can have important consequences. The orientation of the signal towards one cytosolic end of the 7TM bundle could be dictated by different factors such as, for instance, specific effects due to the binding of agonist side chains to the agonist site (see Refs. 105, 198, 281 for AT₁ receptors and Ref. 20 for rhodopsin). This would control a mechanism of selective receptor activation to elicit preferentially either G protein coupling or another post-receptor activation event such as phosphorylation, arrestin binding, and internalization. Examples illustrating this point are shown and discussed in section III, *C1* and *D3*.

3. Constitutive activation

A) AGPCR IN GENERAL. We shall limit our analysis on constitutive activation of AGPCRs (225) to the mechanisms linked to residue mutations in the receptor's 7TM bundle and adjacent extramembrane segments. In these regions of the receptor structure, one can observe (Fig. 6) 1) a high residue variation in the agonist site (residue positions of Fig. 5's *EV* boxes 23 and 33) and in the central part of the bundle (residue positions of Fig. 5's *EV* box 22), and 2) a high residue conservation in the internal cytosolic half of the bundle central cavity (residue positions of Fig. 5's *EV* boxes 11, 12, and 22) except the helix VI (residue positions of Fig. 5's *EV* boxes 23 and 33). Thus the constitutive activation due to mutations in the extracellular and central part of the AGPCR structure is specific for types of receptors (or groups of a few different types), whereas in the 7TM bundle cytosolic ends, the same process has a common component site on positions of all helices but helix VI and a type-specific component sited in helix VI positions.

As examples to illustrate the problem, some constitutive activation events in AGPCRs, due to residue mutations in the extracellular side and middle of helices, are described below.

1) Rhodopsin is activated by mutation of the EC-2 loop hairpin Glu^{113R(318)} residue which stabilizes Schiff base positive charge, or of positions in helix II Gly^{90R(231)}, helix V Phe^{212R(517)}, helix VI Met^{258R(610)}, Phe^{262R(614)}, Trp^{265R(618)}, and Tyr^{269R(621)} which make bonds at other parts of the opsin-retinal interface (85, 137, 157, 261, 268) (Fig. 8). Due to these facts, the apoprotein form of rhodopsin is also constitutively activated (259).

2) AGPCRs with mutations of bulky and mainly aromatic residues are constitutively activated, for instance, Trp^{256G(618)}Phe mutation in bradykinin B2 receptors (160).

3) Besides AT₁ receptors, mutation of Asn residue in helix III position (325) can give rise to constitutive activation of bradykinin B2 (160) and cysteinyl-leukotriene (48) receptors.

4) Glycoprotein hormone (follicle stimulating hormone, thyroid stimulating hormone, and lutropin-choriogonadotropic hormone) receptors are constitutively activated by Asp⁽⁶¹⁴⁾ mutation and other mutations at neighbor residues in helix VI and helices II and VII [polar cluster positions (224), (725) and (726)] (7, 8, 171, 276, 290).

5) Mutations on helix V Ser^{204G(513)}, Ser^{207G(516)}, and probably Ser^{203G(512)} to Ala, all of them placed in the agonist pocket of β_2 -adrenergic receptors, also lead to constitutive activation (119).

Constitutive activation due to mutational events in cytosolic ends of AGPCRs are discussed in detail in section III, *B2a* and *D3*.

B) AT₁ RECEPTORS. Constitutive activation of AT₁ receptors has been elicited by single mutations on residues located along the receptor central cavity such as helix III's Asn^{111A(325)} (55, 77, 106, 165, 174, 195) and Asn^{111A(325)} surroundings in three-dimensional structure (193), helix VI's Ile^{245A(610)} (174), helix VII's Asn^{295A(726)} (17, 55, 181), and cytosolic helix VIII's Leu^{305A(806)} (55, 174, 223).

The N111^A(325)G mutation drives the AT₁ receptor to a constitutive activated state by overcoming a barrier that is normally imposed by the Asn^{111A(325)} side chain (55, 77, 165, 195). Substitution of this Asn residue appears to release the 7TM bundle structure from a steric effect, rather than from a polar interaction with receptor helix VII residues of Tyr^{292A(723)} (77) or Asn^{295A(726)} (17), which keeps the 7TM helices compacted and the receptor inactivated. This interpretation comes from the fact that Asn^{111A(325)} mutation to Gly was the most effective to elicit activation (55).

Based on modeling and mutagenesis procedures, Nikiforovich et al. (193) proposed a mechanism to elicit constitutive activation on AT₁ receptor by mutation in the

position of Asn^{111A(325)} and surroundings in both helices III and IV. This mechanism would consist of a chain of conformational changes along the transmembrane helix III from Leu^{112A(326)} to Tyr^{113A(327)} to Phe^{117A(331)}, which can be propagated to helix IV residues Ile^{152A(419)} and Met^{155A(422)} leading to an expressive movement of this helix as a whole and modifications in the structure of the IC-2 loop.

A screening of a randomly mutated cDNA library of AT₁ receptors (223) allowed the identification of the following 7TM mutations linked to constitutive activation of these receptors: F77^{A(227)}Y, N111^{A(325)}S, L112^{A(326)}H, L112^{A(326)}F, L118^{A(332)}H, I193^{A(506)}K, L195^{A(508)}P, and L305^{A(806)}Q. Except for positions (506), (508), and (806), the other positions are in or close to the interface between blocks a and b of the structure (Fig. 8A) and thus might contribute to the association of these receptor subdomains on the inactivated form of receptors.

Constitutive activation of AT₁ receptors has also been associated with mutations on residues Leu^{262A(627)} and Leu^{265A(630)} at the extracellular third of helix VI which increased the receptor-mediated adenylate cyclase activity (40, 41). Residue mutations in the cytosolic ends of AT₁ receptors have led to constitutive activation as described, for instance, by Gaborik et al. (66) for the DRY motif Asp^{125A(339)} (see the supplemental material Table D for many other occurrences).

4. AT₁ receptor inhibition

Specific mutagenesis studies (41, 53, 94) showed that competitive antagonistic peptides, such as [Sar¹,Leu⁸]-ANG II, bind AT₁ receptors in the same two-step fashion observed with the natural ligand. A minor difference is that [Sar¹]-ANG II requires both the EC-3 loop's Asp^{278A(709)} and Asp^{281A(712)} side chains for binding, whereas ANG II is more dependent on binding to Asp^{281A(712)} and to the N_t domain's Arg^{23A(105)}. The implication of this difference is further discussed with more details in section III D1.

AT₁ receptor inhibition by losartan is surmountable since it can be reversed by ANG II and follows a typical competitive mechanism in which only EC₅₀ values are increased. In contrast, inhibition by losartan's carboxylated metabolite EXP3174 is insurmountable and characterized by increased EC₅₀ values and decrease of maximum effect (291). Other carboxylate-containing derivatives, such as irbesartan (34) and candesartan (182), are also insurmountable inhibitors (274, 291). It has been proposed that this type of inhibition is dictated by a free carboxylate connected to the nonpeptide compound's imidazole ring that would interact with the receptor's Lys^{199A(512)} (Fig. 8) leading to insurmountable inhibition (61, 188, 197, 292, 294, 295). However, the insurmountable inhibition is also regulated by the receptor's Gln^{257A(622)}

residue at the extracellular third of helix VI (274), which allows for a different binding mode of inhibitors to the receptor agonist site.

Whereas AT₁ receptor residues such as Asp^{281A(712)}, at the EC-3 loop, are not required for losartan binding (129), mutagenesis screening showed that binding involves many residues along the 7TM bundle structure (17, 78, 107, 121, 129, 183, 195, 197, 230, 231, 253). After binding at positions in the extracellular halves of helices V and VI (retinal-like locus), losartan seems to follow a binding pathway different from that of ANG II (Fig. 7). Instead of bending to fit the receptor's extracellular locus, the antagonist molecule is extended along the 7TM bundle central cavity, between helices III, VI, and VII, probably contacting residues in the middle of the 7TM bundle position 111^{A(325)} in helix III, positions 294^{A(725)} and 295^{A(726)} in helix VII, and attaining the limits of the protein/cytosol interface [positions 300^{A(731)} and 301^{A(732)} in helix VII].

Interestingly, this type of nonpeptide binding mode to AT₁ receptors seems to correspond to the binding modes to the same receptors of ANG II-like molecules containing bulky side chains such as benzophenone residues instead of Phe at position 8 (36), or to the model of ANG II docking to the receptor obtained from molecular dynamics simulations monitored by energy-minimization criteria (14).

Perhaps due to its special binding mode, losartan can also block the constitutive activation produced by mutations in the middle of the AT₁ receptor's helices III, VI, and VII, and thus it may be considered an inverse agonist (174) which can prevent the installation of the receptor's tachyphylactic state (241). New findings, however, have shown that only insurmountable nonpeptide AT₁ receptor inhibitors such as EXP3174 and candesartan, but not losartan, are in fact strong inverse agonists that neutralize the constitutive activation caused by N111^{A(325)}G, N295^{A(726)}S, and L305^{A(806)}Q mutations (56).

C. Mechanisms Following Receptor Activation

1. Signal transduction

AGPCR activation propagates to the intracellular side of the receptor causing dissociation of interactions between the cytosolic ends of helices I-III and V-VIII. Considering the high residue conservation in the cytosolic half of the 7TM bundle central cavity (Fig. 6), this mechanism is likely to happen in many AGPCRs, including ANG II receptors. Due to the expansion of the structure, some cytosolic ends of the receptor become accessible for coupling to cytosolic proteins (170), triggering receptor postactivation events such as G protein coupling, phosphorylation, arrestin binding, internalization, and binding to other factors.

The various states assumed by AGPCRs to elicit different postactivation events seem to be interdependent and interconvertible (12, 54, 124, 140, 167, 229, 280, 298) and require the simultaneous participation of all receptor cytosolic ends (189, 304).

The cytosolic ends of AGPCR structure can then be coupled to G protein, phosphorylated by specific kinases, bound to arrestin or be internalized via dynamin- and clathrin-dependent coated-pit vesicles (80, 148). In AT₁ receptors, the pathway of G protein coupling is independent of that of arrestins and occurs only in response to ANG II aromatic (Tyr⁴ and Phe⁸) and Arg² side chains (45, 54, 97, 173). Arrestin pathway leads to autonomous cytosolic events (257, 258). In the case of rhodopsin, it has also been shown that the structure of the agonist can remotely regulate the selection of cytosolic events. In fact, modifications in the structure of retinal can turn this molecule into a partial agonist that is able to give rise to a shorter and less intense activated state of this opsin (20).

The two most known events following activation of AGPCRs, namely, G protein coupling and arrestin binding, are discussed in section III, C2 and C3. However, only molecular features of these events, which can be related to the mechanism of receptor activation, were selected for this discussion. In this context, oligomerization of AGPCRs shall not be the focus, since it is yet not clear whether this event is systematically due to previous agonist binding and is related to receptor activation (28, 117, 233, 277).

2. G protein activation

G protein activation mediated by activated GPCRs causes GDP release from the G α chain nucleotide site, GTP association, and consequent dissociation of G α chains from the G $\beta\gamma$ complex (37, 142, 272). Kinetic analyses in which rhodopsin's mutants were used to activate wild-type transducin heterotrimers showed at least two categories of results (50, 51, 162). First, mutations in the DRY motif of helix III and deletion in the EC-2 loop impaired initial coupling of rhodopsin to the G $\alpha\beta\gamma$ -GDP complex. Second, deletions in the IC-3 loop and in the cytosolic end of helix VI, replacement of the sequence in the IC-2 loop and mutations in NH₂-terminal positions of helix VIII, impaired GDP dissociation.

For nonopsin AGPCRs, the regions involved in G protein coupling are the same as those described for rhodopsin: Asp-Arg of the DRY motif of helix III, the IC-2 loop middle, the IC-3 loop and beginning of helix VI, and the cytosolic helix VIII (see the supplemental material Table D).

An important problem about receptor-G protein coupling is whether to analyze it by a chemical equilibrium or by a kinetic approach. According to chemical equilibrium

criteria, the interaction between biological molecules is driven by specific binding sites, and no G α -specific sites in the structure of receptors have been described. Nevertheless, G protein coupling to GPCRs occurs in practice, and two other approaches for this interaction should be considered.

1) No direct contact exists, and G protein coupling is driven by external environmental factors (88, 167) or by special adaptors providing specific connection between the different receptors and G α chain types. The existence of a long list of little characterized proteins with the ability to bind G protein chains and GPCRs (24, 65, 190, 235) favors this possibility.

2) G protein coupling to GPCRs can be interpreted by kinetic criteria according to mechanisms found in intrinsically unfolded protein structures (47). Being loose or dissociated from the main folds, these structures can adapt themselves to interactions with different structures and thus play roles such as that observed in the case of the activation of viral transcription factor (123). This mechanism is supported by the fact that the process is promiscuous (86, 88, 95, 139, 287), occurs at plasma membrane compartments, and can be associated with rafts (141, 265). Also, it has been linked to loosely folded peripheral regions of the G protein and GPCR structures which are not integrated in the basic folds, such as the cytosolic ends of AGPCRs and the NH₂-terminal and COOH-terminal tails of G α and G γ chains. All these structures have to be anchored to and clustered at a same region of the membrane, thus forming a compartment, to elicit function (37, 142, 272). G protein coupling is transitory, since it requires fast molecular contacts aiming at an easier dissociation of GDP from G α chain nucleotide sites followed by GTP association. Thus the receptor is immediately released and may be reutilized in a new cycle, and G $\beta\gamma$ chain complexes are released for specific functions.

Some models for rhodopsin coupling to transducin proposed along the last 10 years (11, 35, 62, 89, 133–135, 204, 206) are subject to questions since, by ignoring the promiscuity of the G protein-receptor interaction, they were proposed as if all criteria for chemical equilibrium were fully satisfied.

3. Arrestin binding

Arrestins are adaptors able to perform biphasic binding to activated AGPCRs and to clathrin (148), thus providing conditions for internalization of these receptors via the classical mechanism of coated-pit vesicles (60).

Among the four types of arrestins known, two (1 and 4) are in visual rod and cone cells and regulate opsins. Arrestins 2 and 3 (or β -arrestins 1 and 2) are present in many other cells and regulate other GPCRs (80, 148). Arrestin structures consist of two lobes with seven sandwiched β -strands connected by interactions of internal

polar or hydrophobic residue side chains. Interactions involving the arrestin COOH- and NH₂-terminal segments are the key elements that keep the protein structure closed under an inactive form (72, 83, 93, 220).

Due to this structural feature, binding of arrestins to AGPCRs is biphasic (73, 219, 234, 237). The first step follows protein kinase-mediated phosphorylation (155) of Ser and Thr residues at the ends of the receptor's COOH-terminal domain (172, 219). In interacting with the so incorporated phosphate groups, arrestins can have some of their internal interactions broken, thus allowing the expansion of their structures and high-affinity binding at receptor regions whose conformation was changed by agonist-mediated activation (44, 80, 132, 147, 219, 234, 237). Under special conditions, the first step of arrestin binding to AGPCR phosphate groups can be omitted, as for instance when a different splicing form of this protein (p44) is expressed (219, 220, 237).

In consonance with the previously discussed mechanism of AGPCR activation (see sect. III B), this arrestin binding mechanism can emphasize a point already discussed above for G protein coupling (see sect. III C2). Arrestin coupling to receptors has also to be analyzed by kinetic approaches. In fact, for G proteins, only a few arrestin types are known with the ability to couple many different classes of receptors. In addition, coupling of arrestins to receptor would have a main role of transiently exposing areas of these proteins for more stable binding to clathrin and formation of coated-pit vesicles (60).

D. Overview of the AT₁ Receptor

1. Activation in the 7TM bundle

Upon activation, disruption of bonds between helices releases the AGPCR's 7TM bundle from constraints, leading to expansion of the structure. Physiological activation, due to binding of full agonists, causes widespread receptor expansion, whereas constitutive activation, due to residue mutations at different sites of the 7TM bundle, leads to different degrees of expansion. This assumption is supported by the finding that ANG IV [ANG-(3–8)], which has a negligible effect on the wild-type AT₁ receptor, is also, as well as ANG II, a bad agonist for the D281^A(712)A mutant (145), indicating that the interaction of ANG II's Arg² with the receptor's Asp^{281A(712)} plays an important role in the general binding. Also, [Sar¹, Ile⁴, Ile⁸]-ANG II, which is inactive on the wild-type receptor, elicited a normal response by the constitutively active N111^A(325)G mutant (56, 181), indicating that ANG II's aromatic residues Tyr⁴ and Phe⁸ induce receptor activation mimicking the N^{111A(325)} mutation's effects (56).

The interaction between ANG II's Arg² and the EC-3 loop's Asp^{281A(712)} is important since, in the absence of

any of the two partners, the agonist-receptor complex can only be formed at higher EC₅₀ values. This finding could be explained by assuming that the extracellular locus of inactive AT₁ receptor is closed by bonds between residues of the EC-3 loop [Asp^{278A(709)} and Asp^{281A(712)}] and the N_t domain [Arg^{23A(105)} and His^{24A(106)}] (Fig. 8). The role of the Arg²-Asp^{281A(712)} bond would be to break these interactions, thus driving the opening of the extracellular locus, a mechanism that is absent when ANG IV is the ligand or the D281^A(712)A mutant is the receptor.

The expansion resulting from the N111^A(325)G mutation seems not to be restricted to the 7TM bundle but can attain the extracellular locus where it drives the separation of the EC-3 loop from the N_t domain. Under this condition, the activation of the N111^A(325)G mutant does not require the Arg²-Asp^{281A(712)} interaction between the peptide and receptor. This is confirmed by the fact that ANG IV can activate a double N111^A(325)G; D281^A(712)A mutant of AT₁ receptor (145).

Additional features of the AT₁ receptor extracellular locus, such as the specific roles of the Asp^{278A(709)} and Asp^{281A(712)} residues in agonist binding, can be recognized when ANG II analogs with modifications in position 1 are used as ligands. Both receptor's Asp residues are involved in the [Sar¹]-ANG II binding, but only Asp^{281A(712)} is important in the ANG II binding (53, 94). It was also reported that the receptor's Arg^{23A(105)} residue is involved in the binding of ANG II's NH₂-terminal end (probably of the Asp¹ side chain) but not when [Sar¹]-ANG II is the ligand (249). To explain these data, a scheme was built in which two states of the AT₁ receptor extracellular locus were considered (Fig. 10). In state 1, the ANG II's Asp¹ and Arg² bind to the receptor's Arg^{23A(105)} and Asp^{281A(512)}, respectively. In state 2, the peptide's NH₂-terminal segment is shifted towards the EC-3 loop so that the NH₂-terminal ammonium group now binds to the receptor's Asp^{278A(709)} while the Arg² side chain remains bound to the receptor's Asp^{281A(512)} (structure I in Fig. 10). When the ligand is [Sar¹]-ANG II, state 2 is favored by the positively charged Sar¹ moving away from the receptor's N_t domain and binding to the EC-3 loop's Asp^{278A(709)} (249). As [Sar¹]-ANG II is the most potent ligand to produce the tachyphylactic state in AT₁ receptors (211), it has been speculated that this functional event is associated with conformational state 2 of these receptors.

In summary, the first and second steps of ANG II activation (Fig. 8) may mimic the constitutive activation due to the N111^A(325)G mutation (55, 77, 106, 165, 195). The activation is propagated to the middle of the 7TM structure and to its cytosolic half, where it could change the arrangement of polar interactions, thus facilitating the separations of blocks a and b (Fig. 8A). This effect would be mimicked by mutations at these residues, especially the N295^A(726)S mutation in helix VII. In a final step, the activation reaches the cytosolic ends of the 7TM bundle,

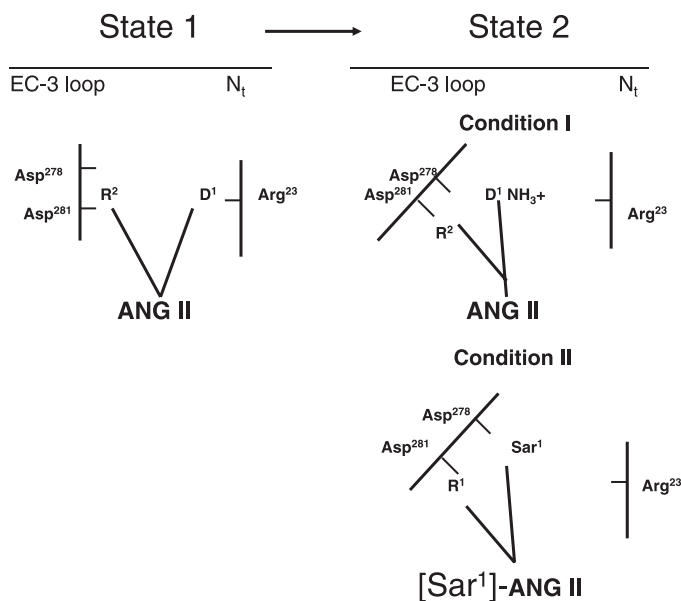


FIG. 10. Two states (*1* and *2*) of the AT₁ receptor extracellular locus (see Fig. 8, *top right*). Upon binding ANG II, the receptor first attains *state 1*, in which the N_t's Arg^{23A(105)} and the EC-3 loop's Asp^{281A(712)} residues contact the peptide's D¹ and R², respectively. In a second step, the complex relaxes to *state 2*, in which the receptor's N_t domain is dissociated from the peptide's D¹, which is left to contact the peptide's NH₂-terminal NH₃⁺ group or the receptor's EC-3 loop Asp^{278A(709)} residue (condition I of *state 2*). Upon binding [Sar¹]-ANG II, the receptor is supposed to go directly to *state 2* (condition II) as the Sar¹ group moves away from the receptor's N_t domain towards the EC-3 loop's Asp^{278A(709)} residue.

resulting in dissociation of these structures. The mutations at Tyr^{302A(734)} and Phe^{309A(810)}, disrupting the bond between them (see Ref. 242 for serotonin 2c receptors), and at Leu^{305A(806)}, reported to cause constitutive activation (56, 174, 223), may also be interpreted as facilitating the dissociation of the 7TM cytosolic ends.

Interestingly, AT₂ receptors that were previously described as constitutively activated under a wild-type form (179), were shown to have binding ability independent of ANG II's aromatic or Arg² side chains. It appears that these receptors could be compared with Asn^{111A(325)} mutants of the AT₁ receptor. In addition, AT₂ receptors have different residues at the extracellular locus's EC-3 loop and N_t domains so as to allow the hypothesis that this site could also be opened in agonist-free receptor forms.

2. The role of the cluster of polar residues: the sodium site

AGPCRs have a cluster of conserved polar residues (mostly belonging to EV boxes 11 and 12 of Fig. 5) in the cytosolic half of the 7TM bundle central cavity consisting of the following residues: Asn⁽¹³⁰⁾, Asp⁽²²⁴⁾, Ser⁽³²⁹⁾, Asp⁽³³⁹⁾, Asn(Ser)⁽⁷²⁵⁾, Asn(Ser)⁽⁷²⁶⁾, and Asn⁽⁷²⁹⁾. They are involved in signal transduction of practically all AGPCRs (see mutation data in GPCRDB and tiny GRAP).

Some AGPCRs, such as opioid (232), α₂-adrenergic (156), dopamine D₂ (269), neurotensin (166), and adenosine (18) receptors, are regulated by sodium whose action leads to decrease of receptor affinity for agonists without interfering with antagonist binding (156). These effects may be linked to the cation binding site located in the middle and in the cytosolic sides of the 7TM bundle central cavity shown in models built for dopamine D₂ receptor (191), but which can be applied to other AGPCRs, considering the conservation of the involved residues. These residues [Asn⁽¹³⁰⁾, Asp⁽²²⁴⁾, Ser⁽³²⁹⁾, Asn(Ser)⁽⁷²⁵⁾, Asn(Ser)⁽⁷²⁶⁾ and Asn⁽⁷²⁹⁾] are also found in the list of AGPCR polar cluster residues, which allows us to suggest that the effects due to sodium ions and the effects linked to AGPCR polar cluster are interrelated.

Sodium ions do not alter substantially the affinity of agonist binding to AT₁ receptors (23), but strong cation-mediated inhibitory effects on both constitutive and physiological activations of these receptors have been reported (56). As these receptors have all residues of the polar cluster and consequently of the sodium site, it is plausible to make an association between the cation-mediated inhibitory effects on AT₁ receptors and an AT₁ facsimile of the site of Neve et al. (191) built for dopamine D2 receptors.

The roles of the AGPCR polar cluster residues, including those of the sodium site, should be crucial as long as their replacements in many classes of receptors can lead to so extreme conditions as signal abolition. If this is so, how do you explain the fact that some natural replacements of these residues in other receptors are not accompanied by function impairment? Many examples showing these discrepancies are found in GPCRDB and tiny GRAP mutation data, and some of them deserve to be shown here for illustration.

1) The first experiments on the AT₁ receptor helix II showed that D74^{A(224)}N mutation produced drastic decay of IP formation (23), while D74^{A(224)}Y and D74^{A(224)}H mutations had little effect on phosphorylase activation but impaired largely the receptor internalization (102). Paradoxically, the position of Asp⁽²²⁴⁾ is occupied by Asn in many functional AGPCRs such as opsins (rhodopsins and visual blue or violet pigments) and receptors for chemokine, phospholipids, and other ligands.

2) N295^{A(726)}S mutation in AT₁ receptors usually leads to constitutive activation (56, 181), whereas N294^{A(725)}A mutation at the adjacent position of the same receptor impaired receptor activation (107). Asn and Ser are frequently replaced by each one in positions (725) and (726) of AGPCRs and are both absent in functionally active opsins and olfactory receptors. Ala is found in position (725) of many opsins and in position (726) of some purinergic receptors.

3) S115^{A(329)}A mutation in AT₁ receptors (183) and S(329)A mutation in α₁-adrenergic receptors (33) were

accompanied by IP formation decay without impairment of agonist binding. Paradoxically, Ser(329) which is found in many AGPCRs (amine and many peptide receptors) is replaced in many functionally active receptors by Gly or Glu (interleukin-8 and chemokine receptors), Thr (endothelin receptors), Ala (opsins), Asp or Glu (olfactory receptors).

A straightforward way to circumvent these conflicting events should go through some assumptions. The mechanism of activation of AGPCRs can basically be defined as an expansion of the 7TM bundle structure as described in section 3B (Fig. 8). There are all along the 7TM bundle structure many structural motifs that can modulate the extent of AGPCR activation. The cluster of polar residues in the middle and in the cytosolic sides of the structure is one of these motifs. In the cluster, the polar residues are interacting with each other giving rise to certain configurations that can favor the active or the inactive forms of AGPCRs. In this context, the modulation by sodium ions is a special condition for some AGPCRs in which the polar cluster acquired a configuration with appropriate residues located in a such manner that the formation of a cation site and modulation by specific binding were allowed. In this case, sodium ions have mostly been found to inhibit agonist binding in many AGPCRs as well as IP formation in AT₁ receptors (see above).

It was shown in AT₁ receptors that mutations in residues of the polar cluster, including residues of the sodium site, have effects on receptor function that are more inhibited by the cation. For instance, the effects due to the mutation in the position of Asn^{111A(325)} of AT₁ receptors is much less cation sensitive than mutations in positions 295^A(726) and 305^A(806) of the same receptors (56).

In the absence of sodium, and therefore under activating conditions, the organization of the polar residues forming the binding site and its neighborhood should be so as to facilitate the separation of blocks a and b and the expansion of the 7TM bundle structure (Fig. 8). This should be a special arrangement which, in the structure of the AT₁ receptor, depends on the Asn^{295A(726)} side chain. Sodium binding at this site might bring the polar residues together, thus inhibiting receptor activation. Mutations of Asn^{295A(726)} to other polar residues, such as Ser, should lead to other loose arrangements of the sodium site residues allowing easier expansion and larger activation when the cation is absent.

The existence of different configurations for the cluster of polar residues in AGPCRs is embodied by models describing the activation of different receptor classes. For instance, a special arrangement of hydrogen bonds has been considered in 5-HT_{2A} receptors involving internal polar residues of helix II Asp⁽²²⁴⁾ and helix VII Asn⁽⁷²⁹⁾. Whereas an Asp-Asn mutation in helix II eliminated cou-

pling, interchanging the residues at the two positions by a second mutation of Asn-Asp in helix VII restored receptor function. These data suggest that these residues are adjacent in space and interact with each other (255). As AT₁ receptor has both Asp^{74A(224)} and helix VII Asn^{298A(729)} residues, a same arrangement of polar residues may be postulated to exist in the cytosolic side of 7TM bundle central cavity of these receptors. Even the mechanism of Asn^{111A(325)} binding to Tyr^{292A(723)} postulated by Groblewski et al. (76) might be involved in this context.

In glycoprotein thyroid stimulating hormone receptors, the helix VI contains a conserved Asp instead of the prevalent residue of Phe in the position (614), which is supposed to make special arrangements in the AGPCR common cluster of polar residues (290).

3. Events at the cytosolic domains

The classic pathway of cytosolic events triggered by agonist-mediated activation of AGPCRs consists of G protein coupling and effector activation, followed by phosphorylation of the COOH-terminal Ser and Thr residues, arrestin binding, and internalization (80, 148). In AT₁ receptors, peptide binding leads to activation of G_{q/11} proteins and subsequently of phospholipase C, or to activation of protein G_i followed by regulation of cAMP levels (12, 173, 260).

Ohtsu et al. (199) provided evidence that Rho/ROCK activation through PYK2 and protein kinase C- δ is specifically required for ANG II-induced migration of vascular smooth muscle cells (VSMC) via JNK activation. Thus, in addition to the cell migration, activation of Rho/ROCK represents one of the key signal transduction pathways originating from the VSMC AT₁ receptor that mediates pathophysiological functions of ANG II such as contraction mediated by G₁₂/G₁₃ (71), atherogenic gene expression mediated by Rho-kinase, MEK/ERK pathways (273), and cardiovascular hypertrophy involving Rho-kinase and NAD(P)H oxidase system (90, 301).

A pathway leading to internalization is also observed (105, 198, 266, 279, 281). However, the assumption that the receptor state linked to G protein coupling is a prerequisite step for phosphorylation-arrestin-binding-internalization is subject to question (97). In fact, it appears that postactivation cytosolic events in AGPCRs do not follow a simple rule but are regulated by an intricate scheme, and the AT₁ receptor might be used as a model to analyze the events in AGPCR's 7TM bundle structure in view of the large number of available experimental data.

1) The pathway leading to AT₁ receptor endocytosis (38) seems to start with phosphorylation of C_t domain's Ser and Thr residues (104) and to be regulated by other receptor regions such as helix VIII's Leu³¹⁶⁽⁸¹⁷⁾ and Tyr³¹⁹⁽⁸²⁰⁾ (278) and the cytosolic end of helix VI [Asp²⁶³⁽⁶⁰¹⁾-Asp²⁶⁴⁽⁶⁰²⁾] (202).

2) To allow G protein coupling, many residues on the AT₁ receptor cytosolic end structure are required, such as Arg¹²⁶⁽³⁴⁰⁾ in helix III (54, 66, 201, 260, 299), Ile¹³⁰⁽³⁴⁴⁾ and Met¹³⁴⁽³⁴⁹⁾ in the IC-2 loop (66, 260), Tyr²¹⁵⁽⁵²⁸⁾ in helix V (103), Leu²²²⁽⁵³⁵⁾ in the IC-3 loop (109, 144, 201), Tyr²⁹²⁽⁷²³⁾ in helix VII (161), and Tyr³¹²⁽⁸¹³⁾, Phe³¹³⁽⁸¹⁴⁾, and Leu³¹⁴⁽⁸¹⁵⁾ in helix VIII (248).

3) The pathway leading from receptor activation to G protein coupling is initiated by ANG II binding, whereas analogs devoid of aromatic rings (such as [Sar¹,Ile⁴,Ile⁸]-ANG II) lead to the internalization pathway (56, 97, 105, 181, 280, 298).

4) Constitutively activated N111(325)A(G), I245(610)T, and L305(806)Q receptor mutants have been found located in intracellular vesicles, differing from wild-type receptors that are in the plasma membranes (174). Furthermore, the N111(325)G mutant induced basal IP production that was reverted by the inverse agonist EXP3174 but not by losartan (181).

5) When stimulated by ANG II or [Sar¹,Ile⁴,Ile⁸]-ANG II, the N111(325)G mutant induces maximum IP responses and is internalized without increase of phosphorylation (280).

The above results suggest a mechanism to select one of the two main AT₁ receptor postactivation pathways. G protein coupling would occur when a larger expansion of the AT₁ bundle is caused by agonist binding, exposing many residues in the cytosolic ends of this structure. This condition is likely to occur by binding of agonists containing aromatic side chains, and it could involve a large separation of the ends of helical columns due to simultaneous breaking of bonds linking helix III to helix VI and the VII-VIII bend to helix VIII as shown in Figure 9.

On the other hand, a phosphorylation-arrestin-internalization pathway may require only accessibility of the receptor's COOH-terminal domain for protein kinase action and consequent initial binding of arrestins or other factors. Nevertheless, this explanation contradicts findings that constitutively activated AT₁ receptors [such as N111^A(325)G mutants] can induce both pathways, G protein coupling and internalization, by a mechanism that is independent of phosphorylation (280).

4. Conclusions

Irrespective of the amino acid residues found in the middle of the 7TM central cavity (conserved positions in Fig. 6) or even in the agonist site (variable positions in Fig. 6), practically all AGPCRs can be physiologically activated by a general mechanism compatible with that of AT₁ receptors proposed by Feng and co-workers (54, 56): an initial two-step ANG II binding to the inactive receptor retinal-like locus and adjacent extracellular loops leading to a large structural rearrangement consistent with the Asn^{111A(325)} mutation effect, followed by breaking of in-

terhelix bonds leading to expansion of the 7TM bundle and separation of its cytosolic ends.

The existence of a cluster of polar residues in the middle of the 7TM bundle, forming or not a sodium binding site, could have been randomly developed during evolution in some receptors that made early contact with ions, and it should not be recognized as a rule in the basic general mechanism of AGPCR signal transduction.

Two main pathways of cytosolic events have been commonly observed. The first one involves an obligatory G protein coupling step and is selected by the binding of ANG II aromatic and/or NH₂-terminal residues. A second pathway that may be selected by binding of ANG analogs devoid of aromatic or NH₂-terminal residues leads directly to phosphorylation, arrestin binding, coated pit formation, and internalization steps without a previous G protein coupling event. The NH₂-terminal residues of the agonists ANG II and [Sar¹]-ANG II seem to bind by two distinct modes to the AT₁ receptor extracellular site (Fig. 7). As the [Sar¹]-ligand is the most potent molecule to trigger tachyphylaxis in AT₁ receptors, it was suggested that its corresponding binding mode might be associated with this special condition of receptors.

IV. FINAL REMARKS

Among other aspects of AGPCR function, the following themes are or may be supposed to become targets for current or future investigations.

A. Active Rhodopsin (AGPCR) Structures as a Result of Probabilistic Analyses

Following the determination of the inactive rhodopsin structure there was an expectation that the structure of activated rhodopsin and the entire functioning cycle of this protein would also be determined. However, high-resolution structures of activated rhodopsin could not be obtained by crystallographic procedures, perhaps because this form of protein is not sufficiently stable (254). This suggests that the study of rhodopsin and other GPCRs requires a probabilistic approach in which activated receptors behave as unstable or fluctuating structures. Thus activation should not enable them to strongly bind but rather to destabilize stable cytosolic proteins such as GDP-Gαβγ chain complexes and inactive forms of arrestins. This would allow the onset of other mechanisms such as GTP binding at an empty Gα nucleotide site, kinase-mediated phosphorylation of receptor and arrestin binding to the receptor and clathrin, first steps of known series of chain reactions occurring at the cytosol. The nonexistence of specific binding sites to mediate coupling of each GPCR to cytosolic proteins such as Gα

chains and arrestins provides corroboration for the probabilistic assumption.

B. The Extracellular Locus as a Site for Cross-Talking Mechanisms

An extracellular locus in AT₁ receptors (Fig. 7) was postulated as a result of the finding that the receptor's Arg^{23A(105)} residue contacts ANG II Asp¹ (249), thus raising the possibility of an agonist-mediated mechanism directed to the extracellular medium. The second disulfide bond linking two walls of the extracellular locus of the AT₁ receptor (the distal segments of EC-3 loop and N_t domain) also reinforces the same hypothesis, since in AT₂ receptors the homologous disulfide bond was found to be a regulator of dimerization (179). Interestingly, in AT₁ receptors, the same bond was suggested to be involved in a mechanism leading to stabilization of the extracellular site structure (39).

C. The AT₂ Receptor Is a Natural Constitutively Activated Form of AGPCR

Although AT₂ receptor sequences are similar to those of the AT₁ receptor (see the supplementary material Table B), the functions of these types of ANG II receptors are quite different. The AT₂ receptor seems to be constitutively activated to produce apoptosis (177) and, in this respect, may be considered as an Asn^{111A(325)} mutant of the AT₁ receptor (175). An inspection of the sequences of the AT₁ and AT₂ receptors shows some subtle differences that might be responsible for the dissimilar behavior of these proteins.

1) The His^{256A(621)} residue present at the extracellular third of the AT₁ receptor's helix VI, which might be bound to the EC-2 loop favoring the inactive form of the receptor, is displaced to position (622) in the AT₂ receptor, where it cannot play a similar role.

2) An Arg residue is found at the cytosolic COOH-terminal tail [position (600)] of the AT₂ receptor's helix VI which, as discussed above, would favor constitutive activation.

3) Asp⁽⁷⁰⁹⁾ is absent in the EC-3 loop of the AT₂ receptor, and an Asp⁽¹⁰⁶⁾ is found instead of the His^{24A(106)} at this position of the AT₁ receptor N_t domain. These differences suggest that the extracellular locus in the AT₂ receptor is likely to be opened in agonist-free forms, favoring constitutive activation.

D. The Cluster of Polar Residues: The Sodium Site

The fact is that all the mechanisms related to the cluster of polar residues and sodium site are still nebu-

lous. Systematic and exhaustive research is needed. All polar residues of the internal polar cluster of AGPCRs (AT₁ receptors included) have to be mutated and assayed at different concentrations of sodium to test the regulation of these ions (or of other ions) on effects produced by the mutations. The first steps in this direction were already given by Feng et al. (56) who revealed that AT₁ receptor N111^A(325)G and N295^A(726)S or L305^A(806)Q mutations were less and more sensitive to sodium ions, respectively, a condition which may a priori be interpreted as a function of the proximity of these residues to the ion binding site.

V. SUMMARY

The most prevalent physiological effects of ANG II, the main product of the renin-angiotensin system, are mediated by the AT₁ receptor, a rhodopsin-like AGPCR. Numerous studies of the cardiovascular effects of synthetic peptide analogs allowed a detailed mapping of ANG II's structural requirements for receptor binding and activation, which were complemented by site-directed mutagenesis studies on the AT₁ receptor to investigate the role of its structure in ligand binding, signal transduction, phosphorylation, binding to arrestins, internalization, desensitization, tachyphylaxis, and other properties. The knowledge of the high-resolution structure of rhodopsin allowed homology modeling of the AT₁ receptor. The models thus built and mutagenesis data indicate that physiological (agonist binding) or constitutive (mutated receptor) activation may involve different degrees of expansion of the receptor's central cavity. Residues in ANG II structure seem to control these conformational changes and to dictate the type of cytosolic event elicited during the activation: 1) agonist aromatic residues (Phe⁸ and Tyr⁴) favor the coupling to G protein, and 2) absence of these residues can favor a mechanism leading directly to receptor internalization via phosphorylation by specific kinases of the receptor's COOH-terminal Ser and Thr residues, arrestin binding, and clathrin-dependent coated-pit vesicles. On the other hand, the NH₂-terminal residues of the agonists ANG II and [Sar¹]-ANG II were found to bind by two distinct modes to the AT₁ receptor extracellular site flanked by the COOH-terminal segments of the EC-3 loop and the NH₂-terminal domain. As the [Sar¹]-ligand is the most potent molecule to trigger tachyphylaxis in AT₁ receptors, it was suggested that its corresponding binding mode might be associated with this special condition of receptors.

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For additional information, see the following databanks: 1) GPCRDB: <http://www.gpcr.org/7tm> (99); 2) tinyGRAP: <http://tinygrap.uit.no> (22); and 3) GenBank: <http://www.ncbi.nlm.nih.gov/entrez/>.

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