Structural determinants for binding, activation, and functional selectivity of the angiotensin AT1 receptor

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Abstract
The renin–angiotensin system (RAS) plays an important role in the pathophysiology of cardiovascular disorders. Pharmacologic interventions targeting the RAS cascade have led to the discovery of renin inhibitors, angiotensin-converting enzyme inhibitors, and AT1 receptor blockers (ARBs) to treat hypertension and some cardiovascular and renal disorders. Mutagenesis and modeling studies have revealed that differential functional outcomes are the results of multiple active states conformed by the AT1 receptor upon interaction with angiotensin II (Ang II). The binding of agonist is dependent on both extracellular and intramembrane regions of the receptor molecule, and as a consequence occupies more extensive area of the receptor than a non-peptide antagonist. Both agonist and antagonist bind to the same intramembrane regions to interfere with each other’s binding to exhibit competitive, surmountable interaction. The nature of interactions with the amino acids in the receptor is different for each of the ARBs given the small differences in the molecular structure between drugs. AT1 receptors attain different conformation states after binding various Ang II analogues, resulting in variable responses through activation of multiple signaling pathways. These include both classical and non-classical pathways mediated through growth factor receptor transactivations, and provide cross-communication between downstream signaling molecules. The structural requirements for AT1 receptors to activate extracellular signal-regulated kinases 1 and 2 through G proteins, or G protein-independently through β-arrestin, are different. We review the structural and functional characteristics of Ang II and its analogs and antagonists, and their interaction with amino acid residues in the AT1 receptor.

Key Words
- angiotensin II
- AT1 receptor
- β-arrestin
- renin–angiotensin system

Introduction
Angiotensin II (Ang II), the principal component of the rennin–angiotensin system (RAS), plays an important role in the structural and functional regulation of the cardiovascular and renal system. Molecular biology and the development of Ang II receptor blockers have demonstrated the existence of two major subtypes of Ang II receptors, AT1 and AT2. Both receptors belong to the seven-transmembrane receptor or G protein-coupled receptor (GPCR) superfamily. Since the first description of the AT1 receptor sequence in 1991, two highly
homologous isoforms, AT1A and AT1B, have been identified in rodents. In humans, one AT1 receptor gene (AGTR1) is located on chromosome 3. AT1A and AT1B receptor subtypes are pharmacologically indistinguishable. The AT1A receptor accounts for 90% of the total binding, and is predominant in the kidney, vascular smooth muscle cells, heart, liver, and in some areas of the brain, while the AT1B receptor is found predominantly in the pituitary and adrenal glands, placenta, lung, and brain (reviewed in Jagadeesh (1998)). Both subtypes are selectively antagonized by losartan. A third subtype, AT1C, was isolated from rat placenta and was shown to be 90% homologous to AT1A and 82% homologous to AT1B (Hahn et al. 1993). The AT1 receptors of humans, rats, and mice contain a single polypeptide of 359 amino acids. The receptors are well conserved between species. The amino acid sequences show that the rat AT1 receptor is about 95% identical to the human AT1 receptor and share 32% sequence homology with rhodopsin (de Gasparo et al. 2000).

Site-directed mutagenesis and molecular-dynamics simulation studies have delineated specific amino acids on Ang II and the type of binding interactions with the receptor determinants for ligand-binding facilitating the conformational rearrangements leading to activation and signal transduction. The agonist receptor interactions play a crucial role both in determination of the functional selectivity and the expression level of the receptor. Thus, molecular determinants of receptor activation distinguish G protein-dependent signaling and G protein-independent signaling (reviewed in Balakumar & Jagadeesh (2014)). Phosphorylation by GPCR kinases terminates receptor activation and promotes β-arrestin recruitment. β-arrestin-scaffolded signaling mediates ‘secondary signaling’ that involves multiple kinases that link to cytoprotective downstream signaling molecules (Balakumar & Mahadevan 2011, Audet & Bouvier 2012, Shukla 2014). Selective G protein-independent signaling has led to the development of ‘biased agonists’ that preferentially activate G protein-independent signaling while blocking the often detrimental G protein activation, demonstrating their use in cardiovascular diseases. Thus, Ang II through AT1 receptors stimulates multiple signaling pathways, cross-talks with several tyrosine kinases, and transactivates growth factor receptors (Akazawa et al. 2013). Similarly, the molecular mechanisms of non-peptide AT1 receptor antagonists are different because of their different molecular structures (Miura et al. 2013). These drugs block the diverse effects of Ang II and provide multiple pharmacologic benefits, some of which may not be class effects, e.g. PPARγ activation by telmisartan (Balakumar et al. 2012, Kakuta et al. 2014) and irbesartan (Imaizumi et al. 2013). This review focuses on recent advances in the structural and functional characteristics of Ang II–AT1 receptor interactions that discern G protein-dependent signaling from G protein-independent signaling pathways. Furthermore, we describe recent progress in molecule-specific differential effects of non-peptide AT1 receptor antagonists.

Molecular determinants of Ang II for AT1 receptor binding, activation, inhibition, and signaling

Structural requirements of the AT1 receptor are apparently different for binding, activation, inhibition, coupling to G protein, receptor and non-receptor tyrosine kinase activation, receptor phosphorylation, and receptor internalization. Ang II binds the receptor at several sites in the extracellular loops (ECL) and transmembrane helices. The bound Ang II molecule adopts vertical binding mode with its amino terminus interacting across the ECL and its carboxy terminus interacting more deeply within the transmembrane domain (TMD) core (Fillion et al. 2013). The ligand-binding pocket for the hormone is created with amino acid residues of transmembrane helices 2, 3, 4, 5, 6, and 7 (Boucard et al. 2000, Baleanu-Gogonea & Karnik 2006). Structure-activity studies with several analogs of Ang II have shown the importance of Arg2, Tyr4, His6, and Phe8 and the negatively charged carboxy-terminal region in the biological activity of the deduced conformation for Ang II (Nikiforovich & Marshall 2001, Wilkes et al. 2002). Site-directed mutagenesis has led several investigators to suggest that amino acid residues 1–7 define receptor affinity, specificity, and initiation of signal transduction, whereas the amino acid 8 involves receptor agonism. Although non-Ang II peptide antagonists bind several binding sites similar to Ang II in the transmembrane helices, the biological activity of Ang II stems from unique binding sites on the receptor recognized by Ang II that occupies a more extensive area of the receptor than the non-peptide antagonist (Ohyama et al. 1995, Fillion et al. 2013).

Molecular specifics of Ang II binding and activation of the AT1 receptor

Multiple studies have examined the structure–function aspects of individual Ang II amino acid residue positions and AT1 receptor regions (summarized in Table 1 and...
The major contacts in Ang II that activate the receptor are aromatic amino acids, Phe$^8$ and Tyr$^4$, the first and second agonist switches respectively. The phenylalanine at position 8 of Ang II is critical for agonist activity, as des-Phe$^8$-heptapeptide is devoid of AT$_1$ receptor agonist activity. Substitution at position 8 weakens agonist activity and produces an agonist-to-antagonist transition (e.g. [Sar$^1$,Ile$^8$]Ang II, a partial agonist). Replacement of C-terminal phenylalanine in Ang II with an aliphatic amino acid such as Ile, Ala, or Thr results in AT$_1$ receptor antagonist (Wilkes et al. 2002). Substitution with Ile or Ala reduced IP$_3$ signaling.

### Table 1 Prominence of Ang II residues in the activation of the AT$_1$ receptor

<table>
<thead>
<tr>
<th>Ang II molecule</th>
<th>Residue in the rat AT$_1$ receptor</th>
<th>Effect</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp$^1$</td>
<td>His183, Leu112, Ser115, Met284, Asn294, and Asn298</td>
<td>Substitution with sarcosine enhances binding and is still a full agonist. Deletion still maintains full agonism but binds with reduced affinity</td>
<td>Wilkes &amp; Marshall. (2002) and Fillion et al. (2010)</td>
</tr>
<tr>
<td>Arg$^2$</td>
<td>Asp281 and Asp278</td>
<td>Essential for receptor desensitization. Substitution largely reduces affinity without weakening receptor activation. Forms salt bridge with Asp281 for initial ligand positioning, opening of ligand-binding pocket and agonism, stabilized by Asp278</td>
<td>Wilkes et al. (2002), Baleanu-Gogonea &amp; Karnik (2006) and Nikiforovich et al. (2006)</td>
</tr>
<tr>
<td>Val$^3$</td>
<td>Thr260, Asn294, and Asn298</td>
<td>The terminal $\alpha$-amino group makes weak H-bonds</td>
<td>Wilkes et al. (2002), Baleanu-Gogonea &amp; Karnik (2006) and Nikiforovich et al. (2006)</td>
</tr>
<tr>
<td>Tyr$^4$</td>
<td>Asn111</td>
<td>Second agonist switch for full-agonist activity</td>
<td>Miura et al. (1999) and Nikiforovich et al. (2005)</td>
</tr>
<tr>
<td>Ile$^5$</td>
<td>Leu112, Asn200, Phe249, and W253</td>
<td>Determinant of the receptor-bound conformation of Ang II</td>
<td>Tzakos et al. (2004) and Nikiforovich et al. (2006)</td>
</tr>
<tr>
<td>His$^6$</td>
<td>Phe259, Thr260, and Asp263</td>
<td>Substitution reduces affinity and efficacy; described as a pivoting-point for the adoption of Ang II conformations</td>
<td>Yamano et al. (1995), Nikiforovich &amp; Marshall (2001) and Wilkes et al. (2002)</td>
</tr>
<tr>
<td>Pro$^7$</td>
<td>Val108, Tyr113, His256, and Thr260</td>
<td>Substitution reduces affinity for the receptor, essential for the conformational geometry of Ang II</td>
<td>Wilkes et al. (2002) and Nikiforovich et al. (2006)</td>
</tr>
<tr>
<td>Phe$^8$</td>
<td>Phe259, Thr260, and Asp263</td>
<td>The first agonist switch. Substitution reduces ability to generate IP$_3$, receptor phosphorylation, and MAPK activation. Substitution with Gly or Ile generated analogs that lacked G protein-dependent signaling, but were more selective for engaging $\beta$-arrestin-dependent signaling</td>
<td>Miura et al. (1999), Holloway et al. (2002) and Zimmerman et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>Trp253, His256, and Phe289</td>
<td>Interaction is critical for Gq signaling</td>
<td>Noda et al. (1995a), Han et al. (1998) and Nikiforovich et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>His256 and Phe259</td>
<td>The $\alpha$-carboxylate group interacts with the $\alpha$-amino group of Lys199 to form a strong salt bridge that positions the Phe$^8$ side chain over Pro$^7$, thus stabilizing Pro$^7$–Phe$^8$ peptide bond</td>
<td>Hjorth et al. (1994), Monnot et al. (1996), Noda et al. (1996) and Nikiforovich et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>Lys199</td>
<td>Stabilizes the ionic bridge formed between the $\alpha$-carboxyl of Phe$^8$ and Lys199</td>
<td>Yamano et al. (1995)</td>
</tr>
<tr>
<td></td>
<td>Trp253 and His183</td>
<td>Stabilizes the ionic bridge formed between the $\alpha$-carboxyl of Phe$^8$ and Lys199</td>
<td>Nikiforovich et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>Phe77, Asn111, Asn294, and Asn295</td>
<td>Interacts with the $\alpha$-carboxyl of Phe$^8$</td>
<td>Nikiforovich et al. (2006)</td>
</tr>
</tbody>
</table>
phosphorylation, and MAPK activation (Holloway et al. 2002). In the study of Zimmerman et al. (2012), Phe8 was replaced with smaller non-aromatic residues such as Gly or Ile. The generated analogs lacked G protein-dependent signaling capabilities, but were more selective for engaging β-arrestin-dependent signaling. The first two of four main interactions of α-COOH of Phe8 and the benzyl-alanyl moiety of Phe8 are critical for docking of the hormone and receptor activation respectively (Miura et al. 1999). Docking the α-COOH group with the ε-amino group of Lys199 (TMD5; a strong salt bridge) positions the Phe8 side chain for an optimal interaction with His256 (TMD6; Noda et al. 1995a, Nikiforovich et al. 2006) and Phe259 within the pocket of the receptor.
(van der Waals contacts). This suggests that a certain level of opening or closure of the fissure between helices V and VI is required for the optimal conformation of the ligand-binding pocket and receptor conformation (Pignatari et al. 2006). The inactive state of the receptor suggests the presence of a salt bridge between His256 and Glu173 (ECL2), which could break upon Phe8 binding to His256 (Oliveira et al. 2007). Although interaction of ε-COOH with Lys199 is not critical for receptor activation, it is important for positioning the side chain of Phe8 over Pro7, thus stabilizing the Pro7–Phe8 peptide bond (Noda et al. 1996). The highly conserved aromatic residues His183 (ECL2) and Trp253 (TMD6) stabilize the ionic bridge formed between the carboxylate of Phe8 and Lys199 (Yamano et al. 1995). Non-peptide antagonists also interact with His256 and Lys199 residues (Noda et al. 1995b, Fierners et al. 2000). However, their interaction stabilizes an inactive conformation of the receptor that distinguishes agonist from antagonist (Wilkes et al. 2002).

The third interaction controls a conformation-dependent reconfiguration of the ground state of the Ang II-binding pocket. The second agonist switch Tyr4 and Asn111 (TMD3) side chains are critical for Ang II signaling. In the absence of receptor activation, an intramolecular hydrogen bond exists between Asn111 and Tyr292 (TMD7) (Groblewski et al. 1997) and/or Asn295 (Balmforth et al. 1997), which are in close spatial proximity. This interaction is considered a major determinant of the inactive conformation of the AT1 receptor (Hunyady et al. 1998). Upon binding, Tyr4 of Ang II disrupts this hydrogen bonding, allowing Asn295 and/or Tyr292 to interact with the conserved residue Asp74 (TMD2; Marie et al. 1994, Groblewski et al. 1997, Inoue et al. 1997). Release of constrained Asn111 from the agonist interaction produces a conformational change in the Ang II-binding pocket, which results in the active state of the receptor (R→R*). Asn111 mutation produces a partial and constitutively activated receptor (R'). This suggests a need for stabilization of the R' conformation for agonist-dependent full-receptor activation (R*) so that it can couple to G proteins (R*G) (Balmforth et al. 1997, Feng et al. 1998). Thus, activation of the AT1 receptor proceeds in two distinct Ang II-dependent steps. The initial rate-limiting step, R→R' (achieved through interaction between Tyr4:Asn111 and Phe8:His256), is immediately followed by the second step, R'→R* (achieved through proper aligning of groups in the ligand-binding pocket and stabilizing R*) for full activation (Karnik 2006). In addition to docking Asn111, Tyr4 interacts with several other amino acid residues in the ECL (Nikiforovich & Marshall 2001). As Kobilka (2000) noted, each of these binding interactions may induce a small but functionally important conformational change in the receptor, with full activation being attained only after all contact points between ligand and receptor are made.

Asp1 of Ang II is not critical because its substitution with sarcosine (N-methylglycine) enhances binding affinity to the receptor with full-agonist activity. Deletion (des-Asp1-Ang II/Ang III) still maintains full agonism but binds with reduced affinity. This indicates that Asp1 is important for binding affinity and duration of action, but is not essential for biological activity (Fillion et al. 2010). Site-directed mutagenesis studies suggest an electrostatic interaction between the β-COOH group of Asp1 with His183 of the receptor (Wilkes et al. 2002) contributing toward stabilization of the receptor-bound conformation of Ang II (Feng et al. 1995). The Ang II complex with the AT1 receptor modeling study predicts that the terminal ε-amino group of Asp1 makes weak H-bonds with Leu112 (TMD3) and Ser115 (TMD3) and strong H-bonds with Asn294 and Asn298 (Baleanu-Gogonea & Karnik 2006). The fourth interaction is a charge pair interaction between Arg2 of Ang II and Asp281 (ECL3). The docking model proposed by Santos et al. (2004) suggests an interaction of Asp1 of Ang II with Arg23 of the receptor to bring the guanidinium group of Arg2 into contact with Asp278 (ECL3) of the receptor. The lack of a β-carboxyl group in [Sar1]Ang II allows Arg2 to interact with Asp281 of the receptor. Noting the contrasting role of Asp278 and Asp281, Hjorth et al. (1994) found that Asp281 is more involved in Ang II binding than that of Asp281. They also suggested distinct modes of interactions for peptide agonists and non-peptide antagonists. In contrast, Feng et al. (1995) and Inoue et al. (1997) noted a direct charge pair through strong interaction between Arg2 side chain of Ang II and Asp281 of the receptor, and the loss of this interaction leads to partial agonism. Since arginine side chains can participate in as many as five hydrogen bonds, Feng et al. (1995) noted a potentially weak role for Asp278 in stabilization of the Arg2–Asp281 salt bridge because of its close proximity to Asp281. Mutation of Asp281 does not affect the action of the non-peptide agonist (L-162,313), therefore it does not interact with Asp281 (Feng et al. 1995). Unlike Arg2–Asp281 strong interaction, the Asp1–His183 interaction provides a weak docking site and is unlikely to be important for agonism (Feng et al. 1995). Interaction of Arg2 with residues in the third ECL is essential for initial ligand positioning, opening of the ligand-binding pocket, and full-agonistic activity, as these would improve the...
interactions of Ang II with TMDs (Feng et al. 1995, Baleanu-Gogonea & Karnik 2006).

A role for Arg² in receptor agonism was demonstrated in studies where the non-peptide partial agonist L-162,313 did not interact with Asp281 side chain (Perlman et al. 1995). Substitution of Arg² with lysine or ornithine remarkably decreased the ability of Ang II to induce tachyphylaxis, suggesting that Arg² is essential for receptor desensitization (Costa-Neto et al. 2000). In addition, Asp74 (TMD2), a highly conserved amino acid in the GPCR super family, which has been demonstrated to play a central role in the mediation of agonist binding-induced G protein activation (Bihoreau et al. 1993) is predicted to make a salt-bridge with Arg² (Baleanu-Gogonea & Karnik 2006). These observations suggest that the amino-terminal region of Asp¹ and Arg² of Ang II interacting with amino acid residues in ECL and the carboxy-terminal region of Phe⁸ enter almost one-third of the way into the membrane to interact with amino acid residues in TM helices (Boucard et al. 2000).

Several amino acid residues in ECL2 are important for ligand binding (the initial stage) and for specific interactions with the first, third, and sixth side chains of Ang II (Boucard et al. 2000, Nikiforovich et al. 2006, Unal et al. 2010). The fully extended structure of Ang II is deeply immersed in the TM region, with Val³ of Ang II contacting with Ile172, and Phe⁸ of Ang II contacting with Phe293 and Asn294 of the AT₁ receptor (Boucard et al. 2000, Nikiforovich & Marshall 2001). Another modeling study suggests the interaction of Val³ with Thr260 of TMD6 (Nikiforovich et al. 2006), as well as Asn294 and Asn298 of TMD7 (Baleanu-Gogonea & Karnik 2006). His⁶ in Ang II is important for the affinity of ligand binding and efficacy and is described as a ‘pivoting-point’ for the adoption of Arg II conformations formed by aromatic ring cluster involving Tyr⁴, His⁶, and Phe⁸ (Nikiforovich & Marshall 2001, Wilkes et al. 2002). His⁶ engages four residues Thr253, Phe259, Thr260, and Asp263 in TMD6 (Yamano et al. 1995, Nikiforovich & Marshall, Wilkes et al. 2002) and one residue Glu173 in ECL2 (Baleanu-Gogonea & Karnik 2006). Side chains of amino acids Val108 and Tyr113 (TMD3) and His256 and T260 (TMD6) interact with Pro⁷ of Ang II (Nikiforovich & Marshall 2001). Although the side chains of Arg², His⁶, and Pro⁷ do not contribute to the agonist properties of Ang II, they are essential for the conformational geometry of Ang II that places the side chains of two agonist switches Tyr⁴ and Phe⁸ in an agonist conformation state. Similarly, Val³, Ile⁵, and Pro⁷ of Ang II likely are determinants of the AT₁ receptor-bound conformation of Ang II (Tzakos et al. 2004).

Key residues in the AT₁ receptor implicated in receptor binding, activation, and functional selectivity

A multitude of studies has investigated the structural features of the ligand-binding and activation sites of the AT₁ receptor. Some of these studies are discussed below and summarized in Table 2 and illustrated in Fig. 1. Like other GPCRs, AT₁ receptors attain different conformational states with variable responses for various Ang II analogs activating multiple signaling pathways (PLCβ–PKC, adenylyl cyclase–cAMP, ERK1/2, transactivation of receptor tyrosine kinase, JAK–STAT, and nuclear transcription factors; Hunyady & Catt 2006). In addition, the functions of GPCRs are dynamically regulated by a variety of mechanisms such as receptor phosphorylation and internalization induced by various kinases. As structural requirements for different downstream effectors activated by the ligand-bound receptor are different, the receptor may adopt distinct conformations to activate its distinct effectors. Mutational data suggest that the receptor holds epitopes that may be required for agonist-induced activation of ERK1/2 signaling, but not Gq/PLCβ/PKC signaling (Hines et al. 2003). This partial activation of the receptor that dissects G protein-independent signaling from G protein-dependent signaling was demonstrated with two octapeptides by substituting residues at position 4 and/or 8 in Ang II, G protein signaling antagonist SII ([Sar¹,Ile⁴,Ile⁸]Ang II), and the Ang II analog TRV1200027 ([Sar¹,D-Ala⁸-OH]-AngII). This produces β-arrestin-selective signaling ligands (Violin et al. 2010, Wilson et al. 2013) of which activated states induced by partial agonists are different from that of the full-agonist Ang II. This implies that the conformational requirements for receptor activation and G protein coupling are different from those of G protein-independent coupling that initiate ERK1/2 activation (Haendeler et al. 2000, Doan et al. 2001). Like many GPCRs, AT₁ receptor can activate parallel signaling pathways that have different structural requirements.

The site-directed mutagenesis studies report that the aromatic cluster of Ang II Tyr⁴, His⁶, and Phe⁸, which is responsible for its agonist activity, interacts with many amino acids predominantly in helices 2, 3, 6, and 7 that are involved in signal transduction. A significant structural change occurs in the receptor during the process of ligand binding and receptor activation. Conformational adaptability is an essential feature of GPCRs. As in other GPCR families, binding of Ang II induces changes in the receptor conformation by inducing rigid body motion of TM helices and helix–helix interactions. These are essential for the determination of G protein specificity.

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<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Region in the receptor</th>
<th>Function</th>
<th>Other effects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe77</td>
<td>TMD2</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Trp94–Gly97</td>
<td>ECL1</td>
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<tr>
<td>Lys102</td>
<td>TMD3</td>
<td></td>
<td></td>
<td>Monnot <em>et al.</em> (1996)</td>
</tr>
<tr>
<td>Ser105–Val116</td>
<td>TMD3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys135, Ser136, Arg137, Arg139, and Arg140</td>
<td>ICL2</td>
<td>Substitution impaired G protein-coupling</td>
<td>Substitution did not affect internalization</td>
<td></td>
</tr>
<tr>
<td>Arg167</td>
<td>TMD4</td>
<td></td>
<td></td>
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<tr>
<td>Lys199</td>
<td>TMD5</td>
<td></td>
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<tr>
<td>Tyr215</td>
<td>TMD5</td>
<td>Essential for G protein coupling</td>
<td>Deletion interfered with receptor internalization</td>
<td></td>
</tr>
<tr>
<td>Ala221 and Leu222</td>
<td>ICL3</td>
<td>Deletion interfered with Gq-mediated signaling</td>
<td>Deletion interfered with ERK activation; retained cdc-42-mediated activation of JNK</td>
<td></td>
</tr>
<tr>
<td>Leu222</td>
<td>ICL3</td>
<td>Mutation markedly impaired IP$_3$ signal generation</td>
<td>Mutation impaired receptor internalization</td>
<td>Mutation markedly decreased the affinity for Ang II. Required for agonist activation. Required for optimal conformation of the receptor for phosphorylation. Mutation diminished PKC and GRK-mediated receptor phosphorylation</td>
</tr>
<tr>
<td>Asp236 and Asp237</td>
<td>ICL3</td>
<td>Mutation has no impact on IP$_3$ production</td>
<td>No effect on MAPK activation, receptor desensitization, and internalization</td>
<td></td>
</tr>
<tr>
<td>Amino acid</td>
<td>Region in the receptor</td>
<td>Function</td>
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<tr>
<td>Ile238 and Phe239</td>
<td>ICL3</td>
<td>Replacement caused impairment of G protein-coupling</td>
<td>Replacement caused parallel impairment of receptor internalization</td>
<td>Other effects</td>
</tr>
<tr>
<td>C-terminal part</td>
<td>ICL3</td>
<td>Substitution impaired G protein-coupling</td>
<td>Substitution did not affect internalization</td>
<td></td>
</tr>
<tr>
<td>His256</td>
<td>TMD6</td>
<td>Participates in signal transduction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu265</td>
<td>ECL3</td>
<td>Mutation decreased ligand binding and IP₃ formation</td>
<td></td>
<td></td>
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<tr>
<td>Asp278 and Asp281</td>
<td>ECL3</td>
<td></td>
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<tr>
<td>Tyr292</td>
<td>TMD7</td>
<td>Mutation impaired IP₃ response</td>
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<tr>
<td>Tyr292</td>
<td>TMD7</td>
<td>Required for G protein activation</td>
<td>Not required for MAPK signaling</td>
<td></td>
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<tr>
<td>Phe293</td>
<td>TMD7</td>
<td>Essential determinant of G protein-coupling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asn294</td>
<td>TMD7</td>
<td>Not required for IP₃ production</td>
<td>Required in orientation of helices for receptor activation</td>
<td></td>
</tr>
<tr>
<td>Asn295</td>
<td>TMD7</td>
<td></td>
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<tr>
<td>NPLFY motif (298–302)</td>
<td>TMD7</td>
<td>Essential for coupling to IP₃ production and interaction with G proteins</td>
<td>Not required in agonist-induced internalization</td>
<td></td>
</tr>
<tr>
<td>Phe301</td>
<td>TMD7</td>
<td></td>
<td></td>
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<tr>
<td>Tyrosines 292, 302, 312, 319, and 339</td>
<td>TMD 7 and C-terminus</td>
<td>Mutation is incapable of ligand-induced G protein activation, IP₃ production, and calcium signaling</td>
<td>Mutation retained Ang II-induced activation of the tyrosine kinase signaling (JAK2–STAT1)</td>
<td></td>
</tr>
<tr>
<td>Tyr312, Phe313, and Leu314</td>
<td>C-terminus</td>
<td>Essential determinants for G protein coupling and activation of Gq</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyr319</td>
<td>C-terminus</td>
<td>Mutation did not interfere with IP₃ signaling</td>
<td>High specificity for Ang II-induced transactivation of EGF. Implicated in the binding of JAK2</td>
<td></td>
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<tr>
<td>Phe309 truncation</td>
<td>C-terminus</td>
<td>Preserved Ang II-induced IP₃ response</td>
<td>Abolished Src activation</td>
<td></td>
</tr>
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References:
Zhang et al. (1998), Chaki et al. (1994), Han et al. (1998) and Miura et al. (1999), Correa et al. (2002), Hjorth et al. (1994), Feng et al. (1995) and Inoue et al. (1997), Nikiforovich et al. (2006), Hines et al. (2003), Perdin et al. (2002) and Yee et al. (2006), Hunyady et al. (1998) and Nikiforovich et al. (2006), Hunyady et al. (1998), Hunyady et al. (1995a), Laporte et al. (1996) and Nikiforovich et al. (2006), Hunyady et al. (1995a), Sano et al. (1997), Sano et al. (1997), Hunyady et al. (1994), Seta & Sadoshima (2003), Doan et al. (2001), Seta et al. (2002).
and stimulation of GDP/GTP exchange (Inoue et al. 1997, Miura et al. 2003). Each ligand (agonist, inverse agonist, or neutral antagonist) would induce different conformational changes and may stabilize the receptor conformation in different ways that play a role in receptor activation and signaling (Miura et al. 2012a). As noted earlier, agonist binding disrupts the network of intermolecular interactions between Asn111 and Asn295 and/or Tyr292 that stabilize the inactive state to favor an active conformation, and thereby influences the arrangement of TMDs (Kobilka & Deupi 2007). Movement of helices is critical for activation of GPCRs. Each ligand may induce rotation of helices but differs in the extent of rotation (Miura et al. 2012a). This involves transmembrane movement that takes the form of a slight clockwise rotation of TMD2 (Miura & Karnik 2002), counterclockwise rotation of TMD3 (Martin et al. 2004, Petrel & Clauser 2009) and TMD6 (Petrel & Clauser 2009), and lateral movement of TMD7 (Boucard et al. 2003) (Fig. 1). During this process, the receptor undergoes a series of conformational rearrangements involving rotation and movement of different mostly hydrophobic transmembrane helices, creating a water-accessible crevice that forms the binding pocket for water-soluble ligands (Martin et al. 2004). Movements of TM helices are a mechanism by which the AT1 receptor is activated, and inhibition of movements abolishes G protein activation. The movements of TMDs 3 and 6 at the cytoplasmic side of the membrane are crucial in the activation of GPCRs (Moukhametzianov et al. 2011). The following amino acids from TMDs 2, 3, 4, 5, 6, and 7 are critical in forming the ligand-binding pocket to nestle Ang II: Ser105, Leu112, and Tyr113 in TMD3; Phe249, Trp253, His256, and Thr260 in TMD6; and Phe293, Asn294, Asn295, Cys296, and Leu297 in TMD7 (Nikiforovich & Marshall 2001, Baleanu-Gogonea & Karnik 2006). In addition, the C-terminal region of the second and C- and N-terminal regions of the third cytoplasmic loops and the membrane-proximal region of the carboxy-terminal tail (residues 309–318) are implicated in G protein activation. The integrity of TMD4 is critical for specific G protein interaction (Feng & Karnik 1999). The floor of the seventh TMD provides residues where the binding sites of peptide and non-peptide ligands overlap. For several GPCRs, this domain is implicated in both binding and activation.

**TMD2 and TMD7 interaction**

The functional significance of TMD2 and TMD7 interactions is common to many GPCRs. We and others
have demonstrated the involvement of interactions between conserved motifs LALAD (residues 70–74), Phe77 in TMD2 and NPLFY (residues 298–302), Tyr292, Phe293, Asn294, and Asn295 in TMD7 in determining AT1 receptor functional selectivity and activation (Hunyady et al. 1995a, 1995b, 1997, Nikiforovich et al. 2006, Smit et al. 2001). The ability of TMD2 to interact dynamically with TMD7 in agonist binding to receptor activation is influenced by Phe77 in TMD2 (Miura et al. 2003). The polar conserved residue Asp74 deeply located in the TMD2 makes a salt bridge with Arg2 of Ang II, and its mutation abolishes the ability of Ang II to produce IP3 (Bihoreau et al. 1993, Nikiforovich et al. 2006). The carboxylate group of Asp74 interacts with the hydroxyl group of released non-conserved Tyr292 (after its breakaway from Asn111) by a hydrogen bond and is essential for receptor activation and coupling (Marie et al. 1994). Single or double mutations (Asp74 and Tyr292) abolished binding and impaired an IP3 response (Nikiforovich et al. 2006). The molecular modeling studies suggest that Asp74, Asn111, and Tyr292 lie in a plane that is three or four helical turns below the membrane surface and buried deep in the lipid bilayer (Monnot et al. 1996).

As many as ten different amino acids (Tyr292, Phe293, Asn294, Asn295, and NPLFY motifs) in TM7 helix interact with Ang II to receptor binding and G protein activation. The amino acid sequence NPXXX is a signature motif of the receptor–G protein coupling (Petrel & Clauser 2009). The amino acid sequence NPXXX is a signature motif of the GPCR that plays a key role in agonist-induced conformational change (Hunyady et al. 1995a). Mutagenesis and photolabeling studies have indicated the interaction of Phe8 side chain of Ang II with His256 and Phe293 for G protein coupling and receptor activation respectively (Noda et al. 1995a, Perodin et al. 2002). Mutation of H256 impaired IP3 production, suggesting its criticality for Gq protein activation (Noda et al. 1995a). In contrast, mutation of Phe293 did not block either Ang II-induced IP3 response or G protein-independent MAPK activation. [Sar1,Ile4,Ile8]Ang II acted as a partial agonist for both IP3 and MAPK signaling (Yee et al. 2006). This led Yee et al. (2006) to conclude that Phe293 may be involved in constraining the receptor to a Gq-inactive state, which is normally only overcome by Ang II. Structural determinants for IP3 signal generation and MAPK activation are different as evidenced in the mutational analysis of D74, Y292, and N295-mutant AT1 receptors. These epitopes are required for G protein-dependent signaling but not for G protein-independent p42/44 MAPK signals. With selective mutations, they still produced ERK1/2 activation similar to that of WT receptor but abolished IP3 release (Hines et al. 2003).

We reported that Asn294, located between Tyr292 and the NPLFY motif, is an essential determinant of G protein coupling, but not of peptide-ligand binding (Hunyady et al. 1998, Nikiforovich et al. 2006). Mutation of the next residue, Asp295, located deep in the transmembrane structure, decreased receptor-binding affinities of Ang II, non-peptide agonist (L-162,313 and L-163,491), and antagonist (losartan) alike without impairing IP3 generation, suggesting that this residue is required for normal binding (Hunyady et al. 1998). The complementary functions of these two adjacent asparagine residues might play a role in orientation of TM helices for receptor activation. The conserved NPLFY sequence (Asn298, Pro299, Leu300, Phe301, and Tyr302) is strategically placed to receive signal from agonist-induced conformational changes in the ligand-binding region as they lie in close proximity to the functionally important Asn46–Asp74 pair (Hunyady et al. 1995a). Mutation of Asn298, Pro299, and Tyr302 markedly reduced both coupling to IP3 production and interaction with G proteins (Hunyady et al. 1995a, Nikiforovich et al. 2006). The Phe301 in the motif is essential for normal binding of Ang II and the non-peptide antagonist losartan (Hunyady et al. 1995a). The amino acid sequence NPLFY does not participate in agonist-induced internalization of the receptor, suggesting that the endocytosis of AT1 receptors is regulated by an alternative mechanism (Hunyady et al. 1995a).

**TMD3 and TMD6 interaction**

Binding of Ang II to AT1 receptors induces cell signaling involving motion and conformational changes in transmembrane helices 3 and 6 to orient helices and loops (especially third intracellular loop (ICL)) for efficient receptor–G protein coupling (Petrel & Clauser 2009). Aromaticity of agonist switches Tyr4 and Phe8 is critical, in which amino-aromatic interaction (Asp111:Tyr4) is stronger than aromatic–aromatic stacking interaction (His256:Phe8). The release of constrained Asn111 interacts with the side chain of Tyr1 to initiate the process of receptor activation (Grobblewski et al. 1997). This suggests a complex regulatory role for Asn111 side chain in the AT1 receptor activation (Feng et al. 1998). Martin et al. (2004) have systematically analyzed all residues within the third TMD (Ile103–Tyr127) of the human AT1 receptor that contribute to the formation of the ligand-binding pocket, which extends from the extracellular surface of the receptor to the transmembrane portion. Side chains of several residues such as Lys102, and...
from Ser105 to Val116 of TMD3, are involved in interactions with Ang II as their mutation alters receptor affinity for Ang II and its analogs (Balmforth et al. 1997, GrobIEWSKI et al. 1997, FENG et al. 1998, Miura et al. 1999). Single-point mutation of Lys102 (TMD3) and Lys199 (TMD5) resulted in a loss of the structural integrity necessary for peptide and non-peptide antagonist binding (Monnot et al. 1996). The authors suggest that these two residues are likely to represent an overlapping point in the binding site for peptide and non-peptide ligands. In addition, they contemplate that substitution of Lys102 would provoke an overall alteration in receptor structure in view of its position at the neighboring disulfide linkage (Cys101 and Cys180). A slight counterclockwise rotation in view of its position at the neighboring disulfide linkage (Cys101 and Cys180). A slight counterclockwise rotation of TMD3 (Fig. 1) exposes residues Ala104, Ser109, Asn111, (Cys101 and Cys180). A slight counterclockwise rotation in view of its position at the neighboring disulfide linkage (Cys101 and Cys180). A slight counterclockwise rotation of TMD3 (Fig. 1) exposes residues Ala104, Ser109, Asn111, Leu112, and Val116 of TMD3 toward the ligand-binding pocket (Martin et al. 2004). Mutation of Asn111 or Ser115 does not alter the binding affinity for peptide analogs, but modifies the binding of non-peptide AT1 and AT2 receptor antagonists. However, mutation of Ser115 caused a marked reduction in the ability of the receptor to mediate Ang II-induced IP3 formation (Monnot et al. 1996). This (Ser115) and other three polar residues noted earlier (Asp74, Asn111, and Tyr292) lie in a plane and deeply located in their respective TMDs, and have been shown essential for receptor activation and coupling (Marie et al. 1994, Monnot et al. 1996). The conserved DRY (Asp125, Arg126, and Tyr127) motif in the cytoplasmic end of TMD3 is a major determinant of G protein activation and undergoes internalization on Ang II stimulation (Ohyama et al. 2002, Gaborik et al. 2003). In the ground state, DRY motif that lies close to the cytosolic extremity of TMD6 stabilizes the inactive conformation of the receptor. In the activated state model, a rotation of TMD3 (Fig. 1) modifies the environment of the DRY motif where it is oriented toward the center of the binding pocket (Martin et al. 2004). Mutation of both Asp125 and Arg126 caused substantial impairment of G protein-dependent IP3 production, but less severely affected internalization kinetics (Gaborik et al. 2003). However, it retained β-arrestin-dependent ERK1/2 signaling (Wei et al. 2003) and Src tyrosine kinases activation (Seta et al. 2002). Although the structural requirements for IP3 generation and agonist-induced internalization are different, the observation of parallel impairment of both pathways suggests that the DRY sequence has a structural role in the agonist-induced conformational change during receptor activation (Szalai et al. 2012).

As noted before, TMD6 and TMD2 lie opposite to each other, facing the receptor central cavity (Petrel & Clauser 2009). Several of the residues placed at the external third of helix 6 are crucial for Ang II-binding and activation of the receptor. Point mutations at residues Val254, His256, and Phe259 caused impairment of signal transduction without a remarkable loss of affinity of Ang II (Han et al. 1998). Yamano et al. (1995) showed a large reduction in Ang II binding with replacement of Trp253, Phe259, and Asp263 in the AT1 receptor. They postulate that the C-terminal carboxylate anion of Phe6 of Ang II and the ε-amino group of Lys199 (TMD5) form an ionic link and that Trp253 stabilizes the salt bridge. In addition, the triad Phe259, Thr260, and Asp263 could provide the docking site for imidazole of His6 and the C-terminal carboxylate of Phe6 of Ang II (Yamano et al. 1995).

Other TMDs

The integrity of TMD4 for specific G protein coupling has been demonstrated with the creation of chimeras of AT1 and AT2 receptors (Feng & Karnik 1999). In this study, replacement of amino acids 146–166 (TMD4) of the AT1 receptor by the topologically equivalent TMD4 segment (amino acids 162–180) of the AT2 receptor resulted in Gq–PLC uncoupling. The deletion of a single residue, Cys149, of the AT1 receptor did not affect agonist interaction or receptor activation, but affected the specificity of coupled G protein. This implies that the TMD4 provides a unique interaction surface for G proteins and a key regulatory factor in receptor-G protein selection (Feng & Karnik 1999). Arg167 (TMD4) is conserved throughout Ang II receptor isoforms. Molecular modeling from Yamano et al. (1995) suggests that the side chain of Arg167 extends toward the phenolic group of Tyr4 of Ang II and is important for Ang II binding. During ligand binding, the carboxyl group of Phe6 interacts via a salt bridge with Lys199 (TMD5) of the AT1 receptor. The side chain of Lys199 is long enough to place the ε-amino group on the indole ring of Trp253 (a conserved residue in TMD6) by the virtue of an amino–aromatic interaction (Yamano et al. 1995). Mutation of Lys199 caused marked loss-of-binding affinity for Ang II (Noda et al. 1995a, Yamano et al. 1995). The residue is not critical for receptor activation, but is important for the optimal positioning of the side chain of Phe6 as it stabilizes the position of this side chain over the Pro7–Phe8 peptide bond (Noda et al. 1996). As non-peptide ligands also interact with Lys199, the interaction distinguishes agonist from antagonists (Noda et al. 1995b, Monnot et al. 1996). The highly conserved Tyr215 of the TMD5 might cluster with the highly conserved DRY sequence of the TMD3 to play an essential role in the signal generation and...
internalization of several GPCRs (reviewed in Jagadeesh (1998)). Mutation of Tyr215 resulted in a parallel inhibitory effect on G protein coupling, signaling, and internalization kinetics of the AT1 receptor, suggesting that Tyr215 participates in propagation of the agonist-induced conformational changes from the binding site to the ICLs (Hunyady et al. 1995).

The integrity of helix V for optimal organization of ligand-binding pocket and the native structure of helix VI for correct folding, dimerization, and surface expression of the AT1 receptor was demonstrated in the studies of Pignatari et al. (2006). Although the overall TM architecture of AT1 receptor is similar to that of rhodopsin, the positions of the kinks and curvature in TMDs are different (Baleanu-Gogonea & Karnik 2006). These authors report Val40, Thr80, His132, Ala159, Leu202/Gly203, Ser252, and Pro299 in helices 1, 2, 3, 4, 5, 6, and 7, respectively, induce the kinks.

**Extracellular loops**

In the absence of ligand, the activity of TMDs is constrained by the extracellular domain. Ang II initially binds to ECL to induce a change in the ECL conformation to set a motion of ECLs to cause movements of the TM helices that in turn cause conformational changes in the cytoplasmic domain to initiate signal transduction (Karnik et al. 2003). The involvement of the amino terminal and all three ECLs of the AT1 receptor in the binding of Ang II suggest a relatively large surface area for the recognition of peptides (Fillion et al. 2013). Many residue side chains at the N-terminus (Arg23, His24, Tyr26, and Ile27) and at the ECL1 (Thr88, Tyr92, and Lys102) of the AT1 receptor have been reported to be involved in the binding of Ang II, but not, or to a limited extent, that of [Sar1]Ang II (Hjorth et al. 1994, Costa-Neto et al. 2000, Santos et al. 2004). Their results suggest the occurrence of different binding patterns for natural agonist and [Sar1]Ang II. Although ECL1 is short and not directly involved in the binding, it is suggested that the loop influences the shape of the ligand-binding pocket (Fillion et al. 2010). Klco et al. (2006) have identified a conserved structural motif, WPPG (Trp94, Pro95, Phe96, and Gly97) within the first ECL. Mutation in the motif has been shown to disrupt receptor activation but not ligand binding. The motif is important in translating the ligand-binding signal directly to movements within the TM bundle (Klco et al. 2006). Furthermore, Nicastro et al. (2003) have postulated that the motif to form a type II β-turn that is involved in the binding of Ang II.

All GPCR structures predict the ECL2 as part of the ligand-binding pocket. Crystallographic structures and structure–function studies reveal the canonical role for ECL2 in ligand-induced activation of GPCR (Unal et al. 2010, Peeters et al. 2011). Unal et al. (2010) used reporter-cysteine accessibility mapping to show that ECL2 is a pivotal structural element in generating different conformational states of AT1 receptor. Their study showed that ECL2 conformations are different in basal, Ang II-bound, and non-peptide antagonist-bound states. The end of the TM helix (Arg167) and ECL2 amino acid residues (from Ile172 to Thr190) form a hairpin that folds into the TMD (Baleanu-Gogonea & Karnik 2006). As noted before, the salt bridge between Glu173 (in the hairpin structure) and His256 (TMD6) and the disulfide bridge between ECL2 and top of helix 3 break upon receptor activation by agonist binding (Oliveira et al. 2007).

A disulfide bond linking ECL2 and TMD3 is conserved in most of the members of the GPCR superfamily, suggesting that ECL2 might be the core of EC domain structure. The four cysteine residues (Cys18, 101, 180, and 274) in extracellular regions form two thiol-sensitive disulfide bridges and stabilize the tertiary structure (Yamano et al. 1992, Bergsma et al. 1993). The thiol-reducing agent dithiothreitol reduces the affinity and the binding capacity of AT1 receptors, but increases the affinity of AT2 receptors for its ligands (reviewed in Jagadeesh (1998)). Disruption of the disulfide bonds by mutation impairs protein folding and cell-surface expression. Single- and double-amino acid mutation or substitution reduced agonist affinity by approximately ten- and 100-fold respectively (Bergsma et al. 1993, Ohyama et al. 1995). Disruption of both of the disulfide bonds nearly completely abolished Ang II binding to the AT1 receptor, suggesting the contribution of the disulfide bonds to the maintenance of the binding conformation (Ohyama et al. 1995). The extracellular disulfide bridges in conjunction with a β-hairpin fold in ECL2 appeared to shape the entrance of the ligand-binding site by maintaining in close vicinity the residues of the ECL2 that are important for ligand binding and functions (Fillion et al. 2013). ECL2 is the site for glycosylation. Post-translational modifications such as glycosylation enhance receptor stability, probably by protecting nascent receptors from proteolytic degradation. In our study, impaired glycosylation caused reduction in cell surface receptor expression, but had a little or no effect on ligand binding or signaling ability (Jayadev et al. 1999).

In all class-A GPCRs, ECL3 is small and has been proposed to be important in GPCR signaling (Klco et al.
The hydrophobic cluster in ECL3 of the δ-opioid receptor was suggested to form a hairpin-like structure essential in the early steps of receptor activation (Décaillot et al. 2003). In thyrotropin receptor, the hydrophobic cluster of amino acid residues in the center of the ECL3 interacts with ECL2 in the form of a hydrogen bond that strongly influences signal transduction and activation of G proteins (Claus et al. 2005). In the AT₁ receptor, a cysteine bridge links ECL3 and amino terminus. Mutation of Leu265 decreased ligand-binding affinity and ability to stimulate IP₃ formation. This led authors (Correa et al. 2002) to suggest that the aliphatic side chain of Leu265 may be involved in the formation of the ligand-binding sites through allosteric effects, which stabilizes the receptor structure around ligand-binding site for full-agonist activity. As noted previously, a charge pair interaction of Arg² of Ang II with residues in the ECL3 (278 and 281) is essential for initial ligand positioning, opening of the ligand-binding pocket, and full-agonistic activity.

**Intracellular loops**

The AT₁ receptor interacts with at least two different G proteins, G₁₁ and G₁₅, to regulate a series of second messengers-generating enzymes and ion-transporting systems. The main intracellular regions of GPCR that play a pivotal role in G protein coupling are the second and third ICLs (Kai et al. 1998, Hunyady et al. 2004). Discrete differences in structural rearrangements of ICLs 2 and 3 might exist between states that allow G protein coupling, β-arrestin recruitment, phosphorylation (PKC- or GRK-dependent), and internalization. The amino acids in the N-terminal part of the second ICL, just below the conserved DRY sequence of the TMD3, are a determinant of G protein activation (Gaborik et al. 2003, Hunyady et al. 2004). Marion et al. (2006) report that a stretch of ten amino acid residues in the proximal second ICL are directly involved in the activation of β-arrestins. Some of the bulky apolar amino acids (Leu, Ile, Val, Met, or Phe) in this region are highly conserved in many of the GPCRs. We (Gaborik et al. 2003, Hunyady et al. 2004) analyzed the role of amino acids distal to DRY sequence (Leu128, Ala129, Ile130, Val131, His132, Pro133, and Met134) in G protein activation and internalization. Our study (Gaborik et al. 2003) showed that both Ile130 and Met134 are strongly required for IP₃ signal generation and that Met134 is required for normal agonist-induced internalization. This suggests that Met134 may have a complex role during receptor activation. Besides these, several polar residues, such as Lys135, Ser136, Arg137, Arg139, and Arg140 in the C-terminal region of the second ICL, are also required for G protein activation (Ohyama et al. 1992, Chaki et al. 1994). Furthermore, substitution of second ICL impaired Ang II-induced Src–Ras–ERK activation (Seta et al. 2002, Hunyady et al. 2004).

In studies with chimeric AT₁A and AT₂ receptors, we (Hunyady et al. 2004) showed that the second ICL and the carboxy-terminal region of the third ICL have no major role in agonist-induced internalization of the AT₁A receptor, but are required for IP₃ signaling by the AT₁A receptor. However, the amino-terminal region of the third ICL has a dual role to play, which includes IP₃ signaling and the internalization of the AT₁A receptor (Hunyady et al. 2004). In this study, Ang II failed to generate both signaling and internalization of the AT₂ receptor, suggesting that Ang II induces substantially different conformational changes in AT₁ and AT₂ receptors. Franzoni et al. (1999) have characterized the proximal (amino acid residues 213–231) and distal (amino acid residues 227–242) halves of the third ICL for the purpose of identifying the domains involved in the complex molecular process of receptor activation, G protein selection, and G protein coupling. The sequence EIQKN (Glu227, Ile228, Gln229, Lys230, and Asn231) common to N- and C-terminal parts of the loop adopts a distinct structural organization. Their study suggests that the N-terminal part folds into an amphipathic α-helix, and this may be modulated by the flexible C-terminal part of the loop through Pro233. Receptor activation and G protein selection do not require interaction with G protein, but involves conformational modifications and exposition of specific receptor cytoplasmic domains (e.g. N-terminal part of third ICL selectively interacts with the proper G protein). The distal part of the loop regulates the conformational stabilization and proper exposition of the required loop region (Franzoni et al. 1999).

Among specific amino acids in the amino-terminal region of ICL3, mutation of Leu222 markedly decreased the affinity for Ang II, but showed normal binding for the peptide antagonist [Sar¹,Ile⁸]Ang II. In addition, we (Hunyady et al. 1996a,b) demonstrated in this mutant a parallel impairment of AT₁ receptor internalization and IP₃ signal generation, suggesting the participation of this region for the agonist-induced conformational change of the receptor. Deletion of Ala221 and Leu222 interfered with Gq-mediated signaling, receptor internalization, and ERK activation. However, it retained its ability to activate JNK (Hunyady et al. 1996a,b). These are key amino acid residues in AT₁ receptor for Ang II stimulation of p42/44 MAPK activation (Haendeler et al. 2000). The diacidic
motif (Asp236 and Asp237) in the C-terminal region of the third ICL is required for optimal conformation of the receptor for GRK-mediated phosphorylation of its COOH-terminal tail. Mutation of these residues diminished Ang II-induced receptor phosphorylation without significantly impairing IP₃ production, MAPK activation, and receptor desensitization and internalization (Olivares-Reyes et al. 2001). This suggests that the active signaling state of the receptor is not the same as that recognized by GRKs and targeted for phosphorylation, as discreet differences in structural rearrangements may exist for each of these states. Replacement of apolar residues Ile238 or Phe239 caused parallel impairment of receptor internalization and G protein coupling (Zhang et al. 1998). In the study of Chaki et al. (1994), mutation at the polar amino acids in the second and third ICLs did not affect internalization, while the G protein coupling was impaired. The authors concluded that both ICLs are not required for receptor internalization.

**COOH-terminal tail**

A ligand-activated conformation change in the AT₁ receptor releases the carboxy-terminal tail from fixation to allow presentation of 54 amino acids for various signaling molecules such as phosphorylation by GRKs and PKC, G protein-coupling, β-arrestin recruitment, JAK/STAT, and receptor and non-receptor tyrosine kinases, among others (Satou & Gonzalez-Villalobos 2012, Vukelic & Griendling 2014). Owing to ligand activation, receptors rapidly internalize via aggregation within the plasma membrane and are recruited into clathrin-coated pits and vesicles. Phosphorylation of serine and threonine residues by GRKs and PKC in the carboxy terminus regulates Ang II-stimulated endocytosis. There are 13 serine and threonine residues, most of them distributed in the middle and distal part of the carboxy terminus (Fig. 1), which are suggestive of multiple phosphorylation sites. While studying the differential behaviors of AT₁ and AT₂ receptors, we (Hunyady et al. 2004) analyzed the importance of the carboxy tail (Asn298–Glu359) in chimeric angiotensin receptors. Substitution of the carboxy-terminal part of the AT₁ receptor impaired the internalization of the AT₁ receptor (Hunyady et al. 2004). Deletion of the C-terminus sequence upstream of amino acid 309 did not considerably alter the expression and properties of the receptor (Ohyama et al. 1992). C-terminal truncation at Phe309 abolished Src activation but preserved the Ang II-induced IP₃ response (Seta et al. 2002). Following deletion of the AT₁ receptor that truncates into helix 8 in the regions 307–311, we showed a decrease in receptor expression at the cell surface (Gaborik et al. 1998). The highly conserved Phe309 and adjacent lysine residues were the major determinants of AT₁ receptor expression. Although the binding of the peptide antagonist ([Sar¹, Ile⁸]Ang II) was not affected, a decrease in Ang II-induced IP₃ production was observed (Gaborik et al. 1998).

Using a series of truncation and deletion mutants, Chaki et al. (1994) demonstrated the requirement of the region from 310 to 327, which contains several lysine residues (Fig. 1), to play a dominant role in the agonist-induced receptor internalization. Mutation of five tyrosine residues (292, 302, 312, 319, and 339) rendered the AT₁ receptor incapable of ligand-induced G protein activation, IP₃ production, and calcium signaling. However, the mutant receptor retained Ang II-induced activation of tyrosine kinase signaling (activation of JAK2 and its effector STAT1) and cell proliferation (Doan et al. 2001). Interestingly, the proximal region (residues 304–316) of the cytoplasmic tail is one of the critical regions that interact with G proteins (Kai et al. 1998). Amino acids Tyr312, Phe313, and Leu314 are essential determinants for G protein coupling and activation of Gq (Sano et al. 1997). Each of the amino acids in the YIPP motif (319–322) in the C-terminal tail of the receptor interacts with PLC-γ (Venema et al. 1998) and JAK2 (Ali et al. 1997). Tyr319 in the motif mediated Ang II-induced transactivation of EGFR and cell proliferation (Seta & Sadoshima 2003). Mutation abolished this effect without interfering with IP₃ signaling (Hunyady et al. 1994). In addition, Ang II-induced activation of other tyrosine kinases including Src, JAK2, and ERK was not abolished suggesting its high specificity for EGFR (Seta & Sadoshima 2003).

A truncation at residue Ala324 that removed all potential carboxyl-terminal phosphorylation sites showed no agonist-dependent phosphorylation. However, it still interacted with β-arrestin-2, although with much lower affinity than did the WT receptor, and robustly stimulated G protein-mediated IP₃ production (Wei et al. 2004). Similarly, C-terminus truncation at residue Ser329 severely impaired desensitization and internalization. The truncated receptor was hyperactive and mediated augmented cellular responses, such as increased IP₃ turnover. The study indicated that the sequence Ser329 through Ser335 (seven residues) is involved in both receptor desensitization and internalization (Conchon et al. 1998). In the studies by Thomas et al. (1998), ~80% of Ang II-stimulated phosphorylation was abolished by truncation after Lys333, suggesting that a preponderance of phosphorylation occurs in the region...
between Lys333 and Asp343. A number of studies (Hunyady et al. 1994, Thomas et al. 1998, Qian et al. 1999, Wei et al. 2004) have analyzed in detail the amino acids of the truncated region (Ser329, Thr332, Lys333, Met334, Ser335, Thr336, Leu337, Ser338, and Ser348) involved in G protein-dependent and G protein-independent signaling pathways by performing single-or multiple-amino acid mutations or deletions. These studies suggest that the carboxy-terminal tail is important for interaction with β-arrestins, and that it plays a minor role in G protein-dependent signaling.

Serine/threonine sites in the C-tail are important for high-affinity binding of β-arrestin-2 to AT1 receptors. Binding of arrestins mediates desensitization by physically preventing the interaction between G protein and receptor and by initiation of receptor internalization. Because the mutant receptor can still couple to G protein, G protein-mediated ERK1/2 activation is not compromised. AT1 receptors can stimulate ERK1/2 activation by two distinct pathways involving G protein and β-arrestin (Wei et al. 2004). The primary PKC phosphorylation sites mapped to Ser331, Ser338, and Ser348 do not appear to be involved in β-arrestin binding (Qian et al. 1999). A few studies (Oakley et al. 2001, Qian et al. 2001) have found residues Ser346–Ser348 to play a minimal role in β-arrestin binding. Subsequently, Qian et al. (2001) and Wei et al. (2004) have found the central portion of the AT1A receptor carboxy terminus (Ser332, Ser335, Thr336, and Ser338) as the major determinant for GRK-mediated phosphorylation, β-arrestin-1 binding, and internalization of the receptor.

Non-peptide antagonists binding of the AT1 receptor

As noted previously, individual ligands can induce different receptor conformations, but also stabilize the receptor conformation sufficiently to denote differences in agonism. Thus, both peptide and non-peptide antagonists occupy the same AT1 receptor ligand-binding pocket and each may induce differential changes in the cytoplasmic face of the receptor for stabilization of the bound ligands (Noda et al. 1995b). A small difference in the molecular structure of non-peptide antagonists (Fig. 2) may cause a difference in the binding affinity and in the interactions of various functional groups with amino acids in the AT1 receptor (Table 3). For example, irbesartan, which has a cyclopentyl group instead of the chloride in losartan, shows the highest affinity, whereas losartan shows the lowest affinity among angiotensin receptor blockers (ARBs; Imaizumi et al. 2013). The binding site pocket of non-peptide antagonists are formed by epitopes located within the extensive area of TMDs of 3, 4, 5, 6, and 7, with essentially no contribution from the extracellular domains (Perlman et al. 1995, Hunyady et al. 1996a,b). This is illustrated in the studies of Fong et al. (1992) in the neurokinin receptor systems, where point mutations of amino acids in the extracellular domains impaired the peptide and not non-peptide ligand binding. Most of the contact sites in the TMDs on the AT1 receptor identified with the antagonist state of the receptor were also identified following activation of the receptor by agonists (Noda et al. 1995b; Miura et al. 2012a). Thus, non-peptide antagonists and Ang II bind to the same or overlapping intramembrane regions to interfere with each other’s binding to exhibit competitive, surmountable interaction (Schambye et al. 1994, Imaizumi et al. 2013). On the other hand, insurmountable antagonists interact differently with the TMDs of the AT1 receptor. They bind tightly and dissociate slowly, causing the functional loss of the occluded receptors (de Gasparo et al. 2000). Losartan and eprosartan are surmountable, while other ARBs such as irbesartan, valsartan, olmesartan, telmisartan, and azilsartan are insurmountable antagonists (Tuccinardi et al. 2006).

The nature of interactions with the residues in the receptor is different for each of the ARBs given the small differences in the molecular structure between drugs in this class. This could lead to different degrees of inverse agonism (Fujino et al. 2010). The binding of non-peptide antagonists is critically dependent on Tyr113, Lys199, His256, Gln257, and Asn295 to induce inverse agonism toward IP3 production. The hydroxyl group of Tyr113 of the receptor forms a hydrogen bond with the nitrogen of the imidazole or imidazolone ring of ARBs. The ε-amino group of Lys199 that positions the side chain of Phe8 of Ang II is implicated in the binding of carboxylate, anionic tetrazole, phenyl, or benzimidazole moiety of all non-peptide ligands (reviewed in Jagadeesh (1998), Miura et al. 2012a) and Imaizumi et al. (2013)). The ε-nitrogen of His256 is involved in the interaction with the carboxyphenyl group of imidazole acrylic acid derivative eprosartan (Schambye et al. 1995) and carboxyimidazole group of other non-peptide antagonists (Hunyady et al. 1996a,b, Ohno et al. 2011, Imaizumi et al. 2013). Gln257, in addition to Lys199, is implicated in the inverse activity of azilsartan (Miura et al. 2012b). Azilsartan has a greater antihypertensive effect than other ARBs (Rakugi et al. 2012) and shows unique binding behavior to the AT1 receptor because of its 5-oxo-1,2,4-oxadiazole moiety (Miura et al. 2014). This study demonstrated that azilsartan interacts with Tyr113 and Gln257. These interactions were critical for blocking Ang II-induced IP3 production and ERK activation (Miura et al. 2014). The
inter-helical space characterized by narrow and compact groove lined by TM helices 3–6 accommodates biphenyl and tetrazol moieties of antagonists such as candesartan and irbesartan (Sokkar et al. 2011). A quantitatively similar reduction in binding affinity was noted for all non-peptide antagonists with the mutation or substitution of Asn295, because the imidazole and phenyl groups are common to all of the antagonists (Perlman et al. 1995, Schambye et al. 1995). Another key amino acid residue in the TMD7 that has a major role in binding of peptide agonist and the non-peptide antagonist losartan is Phe301 (Ji et al. 1994, Hunyady et al. 1995a). The floor of the seventh TMD has amino acids that are common for both peptide and non-peptide ligands and accounts for competition among ligands (Hunyady et al. 1996a, b).

Moderate-to-marked attenuation in the binding of several non-peptide antagonists have been reported in mutants Lys102, Asp111, Tyr113, Val108, Ala163, Arg167, Phe171, Phe182, Tyr184, Thr198, Phe204, Ser252, and Leu300. Some of the ARBs may not directly interact with

**Figure 2**

Chemical structures of clinically used AT1 receptor antagonists. Losartan (2-n-butyl-4-chloro-5-hydroxymethyl-1-[(2’-1H-tetrazol-5-yl)biphenyl-4-yl]imidazole); Valsartan ([5S]-N-valeryl-[(2’-1H-tetrazol-5-yl)biphenyl-4-yl]valine); Irbesartan (2-butyl-3-[(2’-1H-tetrazol-5-yl)biphenyl-4-yl]methyl]-1,3-diazaspiro[4,4]non-1-en-4-one); Telmisartan (4’-[(1,4-dimethyl-2’-propyl[2,6’-bi-1H-benzimidazo][1,1’-biphenyl]-2-carboxylic acid), the I, II, and III indicates the distal benzimidazole, the central benzimidazole, and the biphenyl carboxylic acid group, respectively, of the molecule; Candesartan (1-cyclopentylcarbonyloxethyl 2-ethoxy-1-[(2’-1H-tetrazol-5-yl)biphenyl-4-yl]methyl][1H-benzimidazole-7-carboxylate); Irbesartan (2-n-butyl-4-spirocyclopentane-1-[(2’-tetratozol-5-yl)biphenyl-4-yl]methyl]-2-imidazol-5-one); Olmesartan (5-methyl-2-oxo-1,3-dioxol-4-yl)methyl 4-(hydroxy-1-methyl[2’-1H-tetrazol-5-yl]-2-propyl-1-[(2’-1H-tetrazol-5-yl)-1,1’-biphenyl-4-yl]methyl]-1H-imidazole-5-carboxylate); Eprosartan (E-a-[2-butyl-1-[(4-carboxyphenoxy)methyl]-1H-imidazol-5-yl]methylene]-2-thiophenepropanoic acid); and Azilsartan (5-methyl-2-oxo-1,3-dioxol-4-yl)methyl 2-ethoxy-1-[(2’-5-oxo-5,4-dihydro-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]methyl]-1H-benzimidazole-7-carboxylate).
Table 3 Interactions between non-peptide antagonists and residues in the AT1 receptor

<table>
<thead>
<tr>
<th>ARBs</th>
<th>Residue in rat AT1 receptor</th>
<th>Remarks</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Losartan</td>
<td>van der Waals interaction between Val108 and the phenyl ring, hydrogen bonding with</td>
<td>Surmountable. Weak inverse agonist, lowest binding affinity for the AT1 receptor</td>
<td>Ji et al. (1994), Tuccinardi et al. (2006), Fujino et al. (2010) and Imaizumi et al. (2013)</td>
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<td></td>
<td>Tyr113 and imidazole ring, hydrogen bonding between Tyr184 and tetrazole ring, salt bridge</td>
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<td></td>
<td>between Lys199 and OH group in the imidazole ring, a tight binding between chloride and</td>
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<td></td>
<td>a hydrophobic pocket (formed by Leu112, Tyr113, Phe204, His256, and Gln257) in the</td>
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<td></td>
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<tr>
<td>Valsartan</td>
<td>Three strong interactions: Lys199 binds to the tetrazole group by an ionic bond and to the</td>
<td>Strong inverse agonism involves interaction with Lys199. Has</td>
<td>Miura et al. (2008) and Fujino et al. (2010)</td>
</tr>
<tr>
<td></td>
<td>phenyl group by cation–π interaction, Ser105 binds to the α-carboxyl group, Ser109 binds</td>
<td>moderate affinity and greater selectivity for the AT1 receptor</td>
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<tr>
<td></td>
<td>to the phenyl core by OH–π interaction</td>
<td></td>
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<tr>
<td>Irbesartan</td>
<td>van der Waals interaction between Val108 and the phenyl ring, hydrogen bonding with</td>
<td>A strong inverse agonist. Shows the highest binding affinity and slower</td>
<td>Tuccinardi et al. (2006), Fujino et al. (2010), Ohno et al. (2011) and</td>
</tr>
<tr>
<td></td>
<td>Tyr113 and imidazole ring, hydrogen bonding between Tyr184 and tetrazole ring, salt bridge</td>
<td>dissociation rate from the receptor. PPARγ activation induces significant</td>
<td>Imaizumi et al. (2013)</td>
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<tr>
<td></td>
<td>between Lys199 and imidazole ring, a tight binding between the cyclopentyl group and a</td>
<td>adiponectin production</td>
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<tr>
<td>Eprosartan</td>
<td>Carboxyphenyl group interacts with His256, carboxyl group binds to Lys199, phenyl/</td>
<td>Surmountable</td>
<td>Schambye et al. (1994, 1995)</td>
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<td></td>
<td>imidazole binds to Asn295</td>
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<tr>
<td>Telmisartan</td>
<td>The distal benzimidazole domain binds to lipophilic pocket comprised of Val116, Phe204,</td>
<td>Unique ‘delta lock structure’ supports strong binding affinity and</td>
<td>Ohno et al. (2011) and Imaizumi et al. (2013)</td>
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<td></td>
<td>Phe208, and Trp253. The central imidazole binds to Tyr113, Phe171, Phe182, and Lys199.</td>
<td>marked lowering of blood pressure. PPARγ activation induces significant</td>
<td></td>
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<tr>
<td></td>
<td>The b phenyl carboxylic acid interacts with Tyr184 and His256</td>
<td>adiponectin production</td>
<td></td>
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<tr>
<td>Candesartan</td>
<td>The carboxylate group forms a salt bridge with Lys199, the tetrazole ring forms H-bond</td>
<td>Inverse agonist, insurmountable</td>
<td>Tuccinardi et al. (2006), Miura et al. (2012b) and Imaizumi et al. (2013)</td>
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<td></td>
<td>with Gln257, the biphil group by van der Waals interaction with Tyr113. The ligand also</td>
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<td>binds to a lipophilic pocket delimited by Ser105, W253, His256, Ile288, and Tyr292.</td>
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<td>Olmesartan</td>
<td>The carboxyl and the hydroxyl groups in the imidazole moiety interact with Lys199/His256</td>
<td>Strong inverse agonist and has strong affinity for the AT1 receptor</td>
<td>Miura et al. (2008, 2012a)</td>
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<td></td>
<td>and Tyr113, respectively, and the tetrazole group interacts with Gln257</td>
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<td>Azilsartan</td>
<td>Three strong interactions: the oxadiazole forms hydrogen bonding with Gln257, the</td>
<td>Strong inverse agonist. Binds tightly to and dissociates slowly from the</td>
<td>Miura et al. (2012b, 2014)</td>
</tr>
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<td></td>
<td>biphilphenyl group by van der Waals interaction with Tyr113, the carboxyl group forms an</td>
<td>receptor</td>
<td></td>
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<td></td>
<td>ionic interaction with Lys199</td>
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These residues (Ji et al. 1994, Ohno et al. 2011, Imaizumi et al. 2013). The hydroxyl and carboxyl groups in the imidazole and the tetrazole group in the biphilphenyl moiety of olmesartan interact with Lys112, Tyr113, Lys199, His256, and Gln257 in the AT1 receptor (Imaizumi et al. 2013). The affinity of olmesartan was reduced by more than tenfold in Leu112Ala and Tyr113Phe mutants relative to WT receptor (Miura et al. 2012a). Telmisartan, unique among ARBs in its pharmacologic and pharmacokinetic properties (Wienen et al. 2000, Cianchetti et al. 2008, Zidek et al. 2013), binds firmly with the AT1 receptor through a unique ‘delta lock’ structure (Ohno et al. 2011). The molecular modeling by Ohno et al. (2011) demonstrates that the high affinity of telmisartan to the AT1 receptor involves three distinct moieties in the structure (Fig. 2). The distal benzimidazole core interacts with the lipophilic pocket formed by Val116, Phe204, Phe208, and Trp253; the central
benzimidazole core binds to Tyr113, Phe171, Phe182, and Lys199; and the biphenyl carboxylic acid core with Tyr184 and His256 residues. Table 3 summarizes the interaction between each ARBs and the amino acid residues in the AT1 receptor.

Conclusions and perspectives

Overactivation of RAS plays a pivotal role in the pathophysiology of the cardiovascular and renal system, and is largely implicated in hypertension, cardiac hypertrophy, heart failure, ischemic heart disease, and nephropathy. Ang II by activating the AT1 receptor is a key mediator of the RAS that accounts for many cardiovascular and renal disorders. This is evidenced by the increased therapeutic use of AT1 receptor blockers, angiotensin-converting enzyme inhibitors, and the renin inhibitor for the management of cardiovascular disorders. Similarly, the binding of Ang II to extracellular and intracellular domains of the AT1 receptor implicates the importance of critical changes in the conformation of several TMDs. In this process, AT1 receptor may attain different conformation states with ligand binding that activates multiple downstream signaling cascades such as G-protein-dependent and G protein-independent signaling pathways, which are incompletely understood in the context of Ang II-mediated cardiovascular contraction, endothelial dysfunction, cell migration, protein synthesis, hypertrophy, inflammation, and fibrosis. These pathophysiologic events implicate several and complex signaling proteins and transcription factors involving ERK1/2, P38 MAPK, Pyk2, c-Src family kinases, Tyk2, EGFR, PDGFR, NfκB, β-arrestin, and JAK/STAT. Understanding the structure and function of the AT1 receptor is a key to understand cellular signaling that would support in the development of improved and promising therapeutics for managing cardiovascular disorders. Similarly, the binding interaction of non-peptide antagonists inside the AT1 receptor might help in the rational design of specific ligands that enhance their clinical benefits, as not all ARBs have the same beneficial effects.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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