Role of the third intracellular loop of the Angiotensin II receptor subtype AT2 in ligand-receptor interaction

Jason Dittus, Shannon Cooper, Gerald Obermeir, Lakshmidevi Pulakat*

Department of Biological Sciences, Bowling Green State University, Bowling Green, OH 43403, USA

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Abstract Angiotensin II (Ang II) receptor subtypes AT1 and AT2 share 34% overall homology, but the least homology is in their third intracellular loop (3rd ICL). In an attempt to elucidate the role of the 3rd ICL in determining the similarities and differences in the functions of the AT1 and the AT2 receptors, we generated a chimeric receptor in which the 3rd ICL of the AT2 receptor was replaced with that of the AT1 receptor. Ligand-binding properties and signaling properties of this receptor were assayed by expressing this receptor in *Xenopus* oocytes. Ligand-binding studies using [^{125}I-Sar^{1}-Ile^{8}] Ang II, a peptidic ligand that binds both the AT1 and the AT2 receptor subtypes, and ^{125}I-CGP42112A, a peptidic ligand that is specific for the AT2 receptor, showed that the chimeric receptor has lost affinity to both ligands. However, IP_3 levels of the oocytes expressing the chimeric receptor were comparable to the IP_3 levels of the oocytes expressing the AT1 receptor, suggesting that the chimeric receptors could couple to phospholipase C pathway in response to Ang II. We have shown previously that the nature of the amino acid present in the position 215 located in the fifth transmembrane domain (TMD) of the AT2 receptor plays an important role in determining its affinity to different ligands. Our results from the ligand-binding studies of the chimeric receptor further support the idea that the structural organization of the region spanning the 5th TMD and the 3rd ICL of the AT2 receptor has an important role in determining the ligand-binding properties of this receptor.

Key words: Angiotensin II; AT2 receptor; Phospholipase A_2; *Xenopus* oocyte; Intracellular loop; Transmembrane domain

1. Introduction

Angiotensin II (Ang II), a multifunctional peptide, can act as a hormone as well as a neurotransmitter; it plays an important role in the regulation of blood pressure and body fluid homeostasis [1–5]. As a potent vasoconstrictor hormone, Ang II has been implicated in playing a crucial role in the pathogenesis of hypertension, congestive heart failure and a number of other related clinical conditions. The use of ACE inhibitors such as captopril and enlapril (which prevent the formation of Ang II) in the effective treatment of many cardiovascular diseases demonstrates the significance of the pathophysiological role of Ang II in cardiovascular diseases [6,7]. Recently, it was also demonstrated that Ang II increases the cerebral blood flow (CBF) during hypoxia in rabbits and that the ACE inhibitor captopril and the Ang II receptor antagonist saralasin could inhibit this increase of CBF [8]. Ang II exerts its effect on a cell by binding to the Ang II specific high affinity receptors present on the cell membrane, which initiates a specific signal transduction pathway.

High affinity receptors implicated in mediating the effects of Ang II have been identified in a number of peripheral tissues, such as those of the heart, mesenteric artery, aorta, adrenal cortex, liver, uterus, bladder and pituitary. In the ovary, locally produced Ang II is suggested to play a role in oocyte maturation [9–11]. In the brain, Ang II receptors are found both inside and outside of the blood-brain barrier [12]. The development of non-peptide receptor antagonists for Angiotensin II receptors has resulted in the discovery of Ang II receptors with different pharmacological properties [13–16]. The Ang II receptor subtype having a high affinity for losartan has been designated AT1, and the other receptor subtype with high affinity for WL-19, EXP655, PD123319 and CGP4212A has been designated AT2. The AT1 receptor is a 359 amino acid long protein and has two subtypes, AT1A and AT1B (two subtypes are found in rat and mouse whereas only one type is found in bovine, rabbit, pig, dog and human) [17–24]. The AT2 receptor is a 363 amino acid long protein that shares only 34% homology with the AT1 receptor [25,26]. These two receptor subtypes, AT1 and AT2, share structural similarity in that both are proteins with seven transmembrane topology. Upon activation by Ang II, these two receptor subtypes are shown to exert synergistic effects on certain cell types and opposing effects on other cell types. For example, both AT1 and AT2 receptors induce activation of phospholipase A_2 and generation of arachidonic acid in cardiac myocytes [27]. In contrast, while AT1 receptors induce cell proliferation, AT2 receptors induce apoptosis or growth arrest [28,29]. The responses induced by the cloned AT2 receptor indicate sensitivity to pertussis toxin, suggesting that this receptor is a G_i-protein-coupled receptor. However, this receptor does not demonstrate the GTPγS-induced shift to a low affinity form that is characteristic of the G-protein-linked receptors [25,26].

A comparison of the three intracellular loops of the AT1 and AT2 receptors had shown that homology is lowest in the third intracellular loop of the AT2 receptor [25,26]. Recent studies have also shown that introducing the synthetic intracellular third loop peptide of the AT2 receptor into rat aortic vascular smooth muscle cells resulted in partial inhibition of the serum-induced DNA synthesis and proliferation in these cells [30,31]. On the other hand, the third intracellular domain of the AT1 receptor is shown to be essential for coupling of this receptor to the Gq-protein and subsequent activation of Phospholipase C [32]. To further elucidate the role of the third intracellular loop in determining similarities and differences between the functions of the AT1 and AT2 receptors, we generated a chimeric receptor in which the 3rd intracellular loop (3rd ICL) of the AT2 was replaced with the amino acid

*Corresponding author. Fax: +1 (419) 372 2024.
E-mail: pulakat@bgnet.bgsu.edu

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sequence corresponding to the 3rd ICL of AT1. Here we report the ligand-binding properties of this chimeric receptor when it was expressed in *Xenopus* oocyte expression system.

2. Materials and methods

2.1. Materials

Radiolabeled material for sequencing and binding studies ([^{35}S]-dATP, [^{125}I-Sar^{1}-Ile^{8}]Ang II, ^{125}I-CGP42112A) were obtained from Dupont NEN (Boston, MA, USA). Restriction enzymes were purchased either from Boehringer Mannheim (Indianapolis, IN, USA) or from Promega (Madison, WI, USA). Oligonucleotides used for sequencing and mutagenesis were purchased from GIBCO BRL Life Technologies Inc. (Gaithersburey, MD, USA). The kits that were used in this study were, DTth DNA Polymerase Sequencing Kit (Clon-Tech, Palo Alto, CA, USA), Quick Change site-directed mutagenesis kit (Stratagene Products, La Jolla, CA, USA), and Riboprobe Gemini in vitro transcription system (Promega, Madison, WI, USA). *Xenopus laevis* were obtained from Xenopus One (Ann Arbor, MI, USA).

2.2. Construction of the chimeric receptor

This was done by using the 'sticky-feet PCR mutagenesis' method [33]. The DNA fragments encoding the open reading frames of the AT1 and the AT2 receptor genes were cloned in the plasmid pSP64-polyA previously [34]. Oligonucleotide primers carrying combinations of the AT2 and AT1 receptor sequences were used for this purpose. The first primer was 5′-AGCAACGTGTTACTTTGGAATCTGGA-AAGCTCTAAAGAAGGCT-3′; in this primer the first 22 nucleotides corresponded to that of the DNA sequence at the 3′ side of the fifth transmembrane domain of the AT2 receptor. The next 21 nucleotides corresponded to the 5′ side of the third intracellular loop of the AT1 receptor. The second primer was 5′-GCCAACACAACAGCA-GCTGCCATCCTAAAGATGTCATCGTTTCT-3′. In this primer the first 23 nucleotides were complimentary to the 5′ side of the sixth transmembrane domain of the AT2 receptor. The next 21 nucleotides were complimentary to the DNA sequence at the 3′ side of the third intracellular loop of the AT1 receptor. The first step was to PCR amplify the third intracellular loop of the AT1 receptor using these primers and the AT1 receptor gene as template by PCR amplification. The amplified DNA fragments were then used as 'sticky-feet primers' (since they carried the entire third intracellular loop of the AT1 receptor and sequences corresponding to the fifth and sixth transmembrane domains of the AT2 receptor) to perform PCR mutagenesis of the AT2 receptor gene clone in the plasmid pSP64polyA. PCR mutagenesis was carried out using Quick Change site-directed mutagenesis kit (Stratagene Products, La Jolla, CA, USA). The sequence of the mutant was verified by nucleotide sequencing with dideoxy chain termination method [35]. The plasmid carrying the DNA fragment encoding the chimeric receptor was designated pBG899. In vitro transcription to generate cRNAs encoding the wild-type and chimeric receptors were carried out as described previously [34]. Microinjection of *Xenopus* oocytes and ligand-binding experiments also were performed as described previously [34].

2.3. Measurement of IP_3 levels

IP_3 measurements were carried out according to the protocols of the 'Inositol-1-4-5-trisphosphate [^{3}H] radio receptor kit' purchased from DuPont NEN. Groups of five oocytes were exposed to 100 nM Ang II for 5 min and were frozen immediately in liquid nitrogen. Experiments were done in triplicates for the oocytes isolated from each frog. Thus, five oocytes were used to generate one sample (about 200 μl of cytosol is obtained after extraction according to the procedure of RIA kit) for IP_3 measurement and from each sample, duplicates (100 μl each) were used to measure IP_3 levels according to the RIA kit.

3. Results and discussion

3.1. Ligand-binding properties of the chimeric receptor

Amino acid sequence of the chimeric receptor is shown in Fig. 1. In an attempt to understand the contribution of the third intracellular loop (3rd ICL) in determining the similarities and differences in the functions of the AT1 and AT2 receptors, we compared the ligand-binding properties of the *Xenopus* oocytes expressing the cRNAs encoding the wild-type AT2 receptor and the chimeric AT2 receptor. The ligands used in this study were [^{125}I-Sar^{1}-Ile^{8}] Ang II, a peptidic ligand that binds both the AT1 and the AT2 receptor subtypes, and ^{125}I-CGP42112A, a peptidic ligand that is specific for the AT2 receptor. It was observed that the oocytes expressing the chimeric receptor showed highly reduced binding to both peptidic ligands [^{125}I-Sar^{1}-Ile^{8}] Ang II and ^{125}I-labeled CGP42112A (Fig. 2). It was reported that the amino acids located at the amino-terminus of the AT2 receptor and the 3rd transmembrane domain of the AT2 receptor may directly interact with Angiotensin II during its binding [36]. Therefore,

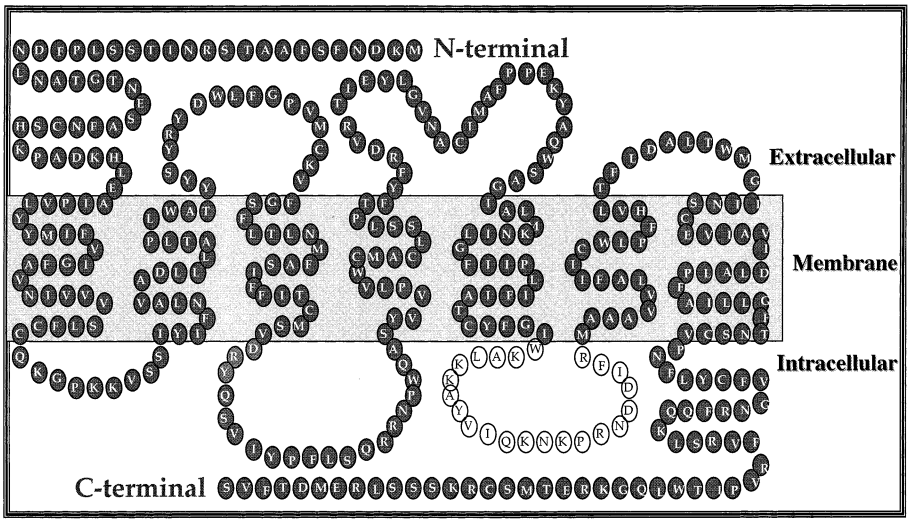

Fig. 1. Amino acid sequence and the predicted organization of the chimeric receptor generated by replacing the 3rd intracellular loop (ICL) of the AT2 receptor with that of the AT1 receptor. The amino acid sequence of the third ICL corresponds to that of the AT1 receptor and is marked by circles with no filling. PCR mutagenesis resulted in replacing the 9th amino acid from the amino-terminal region of the 3rd ICL, Glutamic acid, with Valine. However, this mutation did not seem to affect the ability of the receptor to activate phospholipase C pathway as determined by measuring the intracellular IP_3 levels.

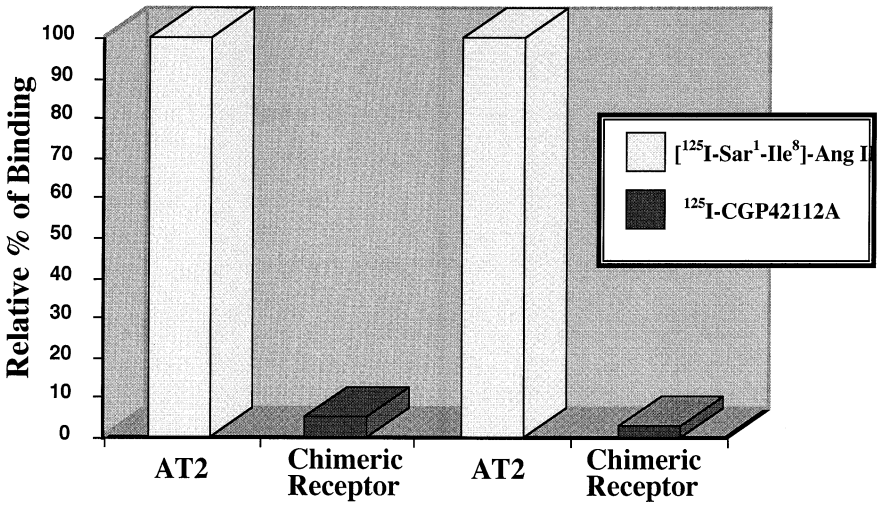

Fig. 2. Ligand-binding properties of the chimeric receptor. Comparison of the specific binding of the [^{125}I-Sar^1-Ile^8] Ang II (0.5 nM) and ^{125}I-CGP42112A (0.25 nM) to the oocytes expressing wild-type and chimeric receptor. Binding experiments were conducted for a period of 1 h according to the procedures described previously [34]. Results are shown as relative % of the binding of the appropriate ligand to the oocytes expressing wild-type AT2 receptor. Receptor expression was quantitated by binding studies using four to seven oocytes from at least three donors (a total of at least 12 oocytes). Three different cRNA preparations were used for each sample. Standard errors were within 5% of each value.

we did not anticipate that replacing the 3rd ICL of the AT2 receptor with that of the AT1 would affect the ligand-binding properties of the chimeric receptor considerably. However, results of our ligand-binding experiments using ^{125}I-labeled peptidic ligands indicated that structural organization of the region spanning the 5th transmembrane domain and the 3rd ICL of the AT2 receptor may play an important role in determining the ligand-binding properties of the AT2 receptor. Moreover, previously we have shown that the high affinity form of the AT2 receptor that binds the [^{125}I-Sar^1-Ile^8] Ang II is different from that which is needed to bind the ^{125}I-CGP42112A [34]. This was based on the observation that replacing the Lys^{215} located on the fifth transmembrane domain of the AT2 receptor with Glu or Gln resulted in generating AT2 receptors that could bind ^{125}I-CGP42112A with high affinity, but could not bind [^{125}I-Sar^1-Ile^8] Ang II. However, replacing the 3rd ICL of the AT2 receptor resulted in reducing the high affinity of the AT2 receptor to both ligands. Thus it seems that the 3rd ICL of the AT2 receptor is not only essential for coupling the receptor to its signal transducers, but also is necessary for maintaining the high affinity ligand-binding form of this receptor.

3.2. Coupling of the chimeric receptor to Phospholipase C pathway

Since the *Xenopus* oocytes expressing the chimeric receptor were unable to bind ligands [^{125}I-Sar^1-Ile^8] Ang II and ^{125}I-CGP42112A, it was necessary to ensure that the oocytes were capable of expressing the mutated receptor. It was shown previously that the 3rd ICL of the AT1 receptor is essential for the coupling of this receptor to Phospholipase C pathway upon Ang II binding. Therefore, in analyzing to see if the *Xenopus* oocytes microinjected with the chimeric receptor were indeed expressing this receptor, we compared the intracellular IP_3 levels of the oocytes microinjected with equal amounts of cRNAs encoding either the AT1 receptor or the chimeric receptor after exposing them to Ang II. Groups of five oocytes were exposed to Ang II and the intracellular IP_3 levels were measured using the 'Inositol-1-4-5-trisphosphate [^{3}H] radio receptor kit' purchased from DuPont NEN. As shown in Fig. 3, the intracellular IP_3 levels of oocytes expressing the chimeric receptor and exposed to Ang II were elevated to similar extents as seen in the oocytes expressing the AT1A receptor. This result implied that the chimeric receptor was expressed by the oocytes and it was indeed functional, although this expression could not be detected in radiolabeled ligand-binding experiments. Thus, the chimeric receptor mim-

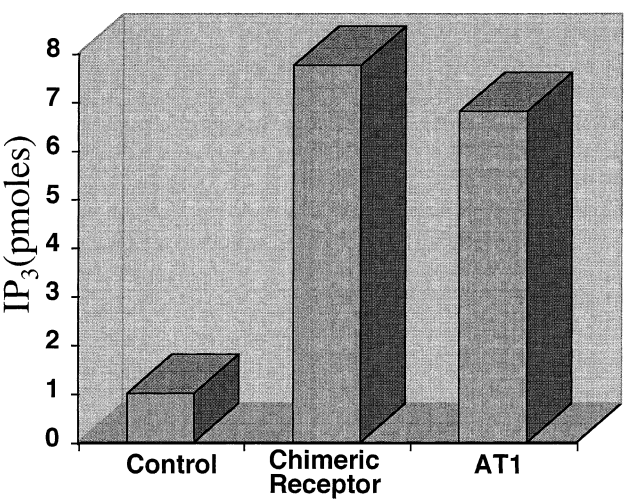

Fig. 3. Intracellular IP_3 levels of the oocytes expressing the chimeric receptor and exposed to 1 mM of Ang II. IP_3 measurements were carried out according to the protocols of the 'Inositol-1-4-5-trisphosphate [^{3}H] radio receptor kit' purchased from DuPont NEN. Five oocytes were used to generate one sample (about 200 μl of cytosole is obtained after extraction according to the procedure of RIA kit) for IP_3 measurement and from each sample duplicates (100 μl each) were used to measure IP_3 levels according to the RIA kit. Oocytes from three different frogs were used and two different cRNA preparations were used. Standard deviation for the IP_3 levels (in picomoles) of the oocytes expressing rAT1A was ±1.8, for chimeric receptor was ±1.6 and for uninjected was ±0.76.

icked the rAT1 receptor in its ability to activate the phospholipase C pathway. The inability of the chimeric receptor to bind peptidic ligands [^{125}I-Sar^{1}-Ile^{8}] Ang II and ^{125}I-CGP42112A was particularly interesting, since a mutation at the intracellular domain has resulted in changing the binding properties of the receptor significantly.

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