Identification of the region of AT2 receptor needed for inhibition of the AT1 receptor-mediated inositol 1,4,5-triphosphate generation

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Abstract Increase in the intracellular inositol triphosphate (IP_3) levels in *Xenopus* oocytes in response to expression and activation of rat angiotensin II (Ang II) receptor AT1 was inhibited by co-expression of rat AT2 receptor. To identify which region of the AT2 was involved in this inhibition, ability of three AT2 mutants to abolish this inhibition was analyzed. Deletion of the C-terminus of the AT2 did not abolish this inhibition. Replacing Ile249 in the third intracellular loop (3rd ICL) of the AT2 with proline, corresponding amino acid in the AT1, in the mutant M6, resulted in slightly reduced affinity to [^{125}I]Ang II (K_d = 0.259 nM), however, did not abolish the inhibition. In contrast, replacing eight more amino acids in the 3rd ICL of the AT2 (at positions 241–244, 250–251 and 255–256) with that of the AT1 in the mutant M8, not only increased the affinity of the AT2 receptor to [^{125}I]Ang II (K_d = 0.038 nM) but also abolished AT2-mediated inhibition. Interestingly, activation of the M8 by Ang II binding also resulted in increase in the intracellular IP_3 levels in oocytes. These results imply that the region of the 3rd ICL of AT2 spanning amino acids 241–256 is sufficient for the AT2-mediated inhibition of AT1-stimulated IP_3 generation. Moreover, these nine mutations are also sufficient to render the AT2 with the ability to activate phospholipase C.

Key words: Angiotensin II; AT2 receptor; Inositol triphosphate; *Xenopus* oocytes; Intracellular domains

1. Introduction

The renin-angiotensin system plays an important role in the regulation of blood pressure and hydromineral balance through the main effector peptide angiotensin II (Ang II). Ang II elicits its biologic actions by binding to specific membrane-bound receptors on target cells to activate multiple intracellular signal transduction pathways [1–5]. 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, mesentric 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 [6–8]. To date, two pharmacologically different Ang II receptor subtypes designated type 1 (AT1) and type 2 (AT2) have been cloned and characterized [9–15]. The AT1 receptor subtype is responsible for mediating the major physiological responses attributable to Ang II such as vasoconstriction, secretion of aldosterone, dipsogenic effect and cell growth [2,5]. Two highly homologous subtypes of AT1 receptor (94% amino acid identity), termed the AT1A and AT1B respectively were cloned from rodents [16,17]. The AT1, a 359-amino acid protein, which belongs to the superfamily of G proteins coupled receptors (GPCRs) activates phospholipase C (PLC) via Gq protein and inhibits adenylyl cyclase via Gi protein [18–20]. The third intracellular loop (3rd ICL) of the AT1 receptor is shown to interact with Gq and activate PLCβ [20–22]. Specific regions on the C-terminal cytoplasmic tail of the AT1 are involved in its coupling to Gi protein, and activating members of the Jak/STAT family and PLCγ [19,23–25]. The AT1 receptor is also known to activate protein kinase C, growth-related immediate transcription factors, and several mitogen-activated protein kinases [26–30].

The AT2 receptor gene encodes a 363-amino acid protein and shares structural similarity (both are proteins with seven transmembrane topology) and 34% homology at the amino acid level with the AT1 [14,15]. The exact physiological role of the AT2 is still emerging. Recently Vervoort et al. showed that lack of expression of the AT2 in brain, or mutations in the AT2 gene causes mental retardation in humans, and suggested that the AT2 has an important role in brain development and cognitive function [31]. AT2 is also known to play important roles in the development of kidney and urinary tract, and in the functions of ovary, uterus and pancreas [32–35]. The AT2 seems to function as a negative regulator of cell growth [36,37]. In many cell lines the AT2 is known to induce apoptosis and this effect is mediated by the 3rd ICL of the AT2 [38–41]. The AT2 also seems to exert negative regulation on many AT1-mediated signal pathways [42–45]. For example, the AT1-mediated cell growth is inhibited by the AT2 in many cell lines [46,47]. The AT2 also inhibits the AT1-mediated inositol 1,4,5-triphosphate (IP_3) production [48,49]. Direct protein–protein interaction is one of the mechanisms by which the AT2 exerts its regulatory effects. For example, we have shown previously that direct protein–protein interaction exists between the domain spanning the 3rd ICL and the C-terminal cytoplasmic tail of the AT2 receptor, and the ATP-binding domain of ErbB3 receptor, and proposed that this interaction could lead to AT2 receptor-mediated inhibition of the cell growth [50]. It was shown recently that heterodimerization of the AT2 and the AT1 receptors in

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Abbreviations: Ang II, angiotensin II; AT1, Ang II type 1 receptor; AT2, Ang II type 2 receptor; 3rd ICL, third intracellular loop; IP_3, inositol triphosphate

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rat pheochromocytoma PC-12 cells results in inhibition of the AT1-mediated IP_3 production by the AT2 [49]. Thus, the antagonistic effect of the AT2 on the AT1-mediated IP_3 production in these cells is caused by direct binding of the AT2 to the AT1. Expression of the split AT2 receptors ($AT2^{1-240}$ and $AT2^{241-363}$) prevented the formation of the heterodimers and abolished the AT2 receptor-mediated inhibition of AT1 receptor-mediated IP_3 production [49]. Since we have observed that the 3rd ICL and the C-terminal cytoplasmic tail of the AT2 are needed for the direct interaction between the AT2 and the growth-promoting ErbB3 receptor, we tested whether the same regions are also involved in the direct interaction between the AT2 and the AT1. We used *Xenopus* oocyte expression system to test this idea. Initially, we analyzed whether co-expression of the rat AT1 and AT2 receptors in *Xenopus* oocytes could result in the inhibition of the AT1-mediated IP_3 production. Then we generated three AT2 mutants and analyzed their ability to inhibit the AT1-mediated IP_3 production when co-expressed with the AT1 in these cells. This analysis helped us identify the region of the 3rd ICL of AT2 involved in the AT2-mediated inhibition of the AT1-mediated IP_3 production.

2. Materials and methods

2.1. Materials

Radiolabeled materials for sequencing and binding studies ([^{35}S]dATP, [^{125}I]Ang II, [^{125}I-Sar^1-Ile^8]Ang II, [^{125}I]CGP42112A) and 'Inositol-1,4,5-triphosphate [^{3}H] Radioreceptor Kit' were obtained from NEN Life Science Products (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. (Gaithersburg, MD, USA). The AT1-specific antagonist losartan (DuP 753) and the AT2-specific antagonist PD123319 were obtained from Merck and Co., Inc. (Whitehouse Station, NJ, USA) and Sigma (St. Louis, MO, USA) respectively. The other kits used in this study were DTth DNA Polymerase sequencing kit (Clontech, 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). Female *Xenopus laevis* were obtained from Nasco (Fort Atkinson, WI, USA).

2.2. Construction of the AT2 mutants

The C-terminal deleted AT2 receptor was generated as described previously [51]. The construction of the AT2 mutants designated M6 and M8 were carried out by PCR-mutagenesis using 'QuikChange™ Site Directed Mutagenesis Kit' purchased from Stratagene Products, La Jolla, CA, USA. A pSP64 poly(A) plasmid derivative harboring a DNA fragment encoding the open reading frame of the AT2 receptor described previously was used as the template [52]. To construct M6 in which the Ile249 of the AT2 was replaced with proline, the oligonucleotide primers used were as follows: the forward primer was 5′-TAT CAG AAG AAC AGA CCT ACC AGT GAC CAA GTC-3′ (+strand) and the reverse primer was 5′-GAC TG GTC ACG GGT AGG TCT GTT CTT GTC ATA-3′ (−strand). The position of the mutation was confirmed by nucleotide sequencing with dideoxy chain termination method [53]. The mutant M8 carried the following mutations: T241A, N242Y, S243E, Y244I, T250R, R251N, L255F and K256R along with I249P. This was constructed by sequentially replacing the amino acids of the 3rd ICL of the AT2 with that of the AT1 at positions 241–244, 250–251 and 255–256 along with 249 by PCR mutagenesis. The oligonucleotide primers used to introduce the mutations at positions 250–251 and 255–256 were as follows: the forward primer was 5′-GGG AAG AAC AGA ATT AGA AAT GAC CAA GTC TTC AGG-3′ (+strand) and the reverse primer was 5′-CCT GAA GAC TTG GTC ATT TCT AAT TCT GTT CTT CCC-3′ (−strand). The oligonucleotide primers used to introduce the mutations at positions 241–244 were as follows: the forward primer was 5′-CAT CTG CTG AAG GCC TAT GAG ATT GGG AAG AAC AGA ATT-3′ (+strand) and the reverse primer was 5′-AAT TCT GTT CTT CCC AAT CTC ATA GGC CTT CAG CAG ATG-3′ (−strand). The positions of the mutations were confirmed by nucleotide sequence analysis.

2.3. In vitro transcription, expression in Xenopus oocyte, ligand binding studies and measurement of IP_3 levels

To generate cRNAs corresponding to the wild type AT1, wild type AT2, C-terminal deleted AT2, and AT2 mutants M6 and M8, in vitro transcription was carried out. The templates used were the pSP64 poly(A) vectors carrying cDNAs corresponding to the wild type AT2 [52], C-terminal deleted AT2 [51], M6 and M8 mutants of AT2 (this study) and wild type AT1B (this study). The rat AT1B was cloned in pSP64 poly(A) vector by RT-PCR amplifying the DNA fragment corresponding to the open reading frame of the AT1B using the forward primer 5′-ATG ACC CTT AAC TCC TCT AC-3′ (+strand) and the reverse primer 5′-TCA CTC CAC TTC AAA AAA AGA TGC-3′ (−strand) and mRNA isolated from 17-day old rat fetus as template. In vitro transcription was carried out using 'Riboprobe Gemini in vitro transcription system'. Techniques for microinjection of cRNA and ligand binding experiments also were performed as described previously [52]. For IP_3 measurements, groups of five oocytes were used per sample as described previously [54]. The cells were exposed to 100 nM Ang II in pre-incubation buffer for 5 min. To achieve blocking of Ang II binding with PD123319 (for the oocytes expressing AT2 and AT2 mutants) or DuP 753 (for the oocytes expressing AT1) a 15 min pre-incubation in the presence of appropriate antagonist was performed before the addition of Ang II. At the end of a 5 min treatment with Ang II, the fluid was removed and the cells were subjected to flash-freezing in liquid nitrogen. Cells were stored at −70°C until the assay was performed according to the protocol of the 'Inositol-1,4,5-triphosphate [^{3}H] Radioreceptor Kit' purchased from NEN Life Science Products.

3. Results

3.1. AT2 receptor inhibits AT1 receptor-stimulated IP_3 generation in Xenopus oocytes

The antagonistic effect of the AT2 on the AT1-mediated signal mechanisms when both receptors are co-expressed in the same cells has been demonstrated in many situations [42–47]. Moreover, activation of the AT2 by Ang II results in vasodilation whereas activation of the AT1 by Ang II results in vasoconstriction [55–57]. AT1-mediated cell growth is inhibited by the co-expression of the AT2 [46,47]. The inhibition of the AT1-mediated activation of PLC by co-expression of the AT2 is also demonstrated previously [49]. The observation that heterodimerization of the AT2 and the AT1 receptors is responsible for the negative regulation of the AT1-mediated signaling by the AT2 is very interesting [49]. This observation unravels a new mechanism for cross-talk between GPCRs, which is a direct protein–protein interaction. Therefore, it is interesting to identify which specific domain of the AT2 is involved in this interaction with the AT1. Since interaction between the AT1 and the AT2 has a growth-inhibitory effect in many cell lines and we have shown previously that the 3rd ICL and the C-terminal cytoplasmic tail of the AT2 are involved in direct protein–protein interaction between the AT2 and the growth-promoting ErbB3 receptor, we assumed that these two regions could also be involved in direct protein–protein interaction between the AT2 and the AT1. Moreover, we have shown previously that expression of the rat AT1 receptor in *Xenopus* oocytes and activation by Ang II results in the increase of intracellular IP_3 levels [54]. We have also shown that replacing the 3rd ICL of the AT2 with that of the AT1 results in a chimeric receptor that is capable of increasing the intracellular IP_3 levels in oocytes [54]. These re-

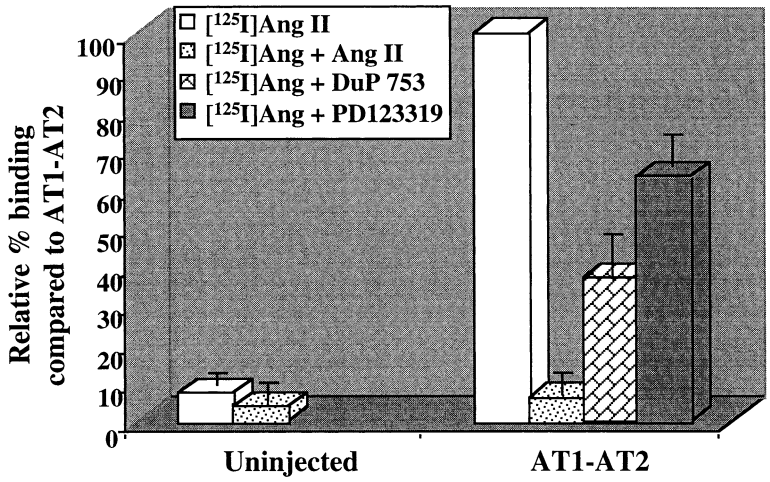

Fig. 1. Ligand binding properties of the *Xenopus* oocytes co-expressing the wild type AT1 and AT2 receptors from rat. The specific binding of the [^{125}I]Ang II (0.5 nM) to the oocytes co-expressing wild type AT1 and AT2 and uninjected oocytes are compared. Binding experiments were conducted for a period of 1 h. Results are shown as relative percent of the binding of the [^{125}I]Ang II to the oocytes co-expressing wild type AT1 and AT2 receptor. Receptor expression was quantitated by binding studies using six to eight oocytes from at least five donors (a total of at least 30 oocytes). Five different cRNA preparations were used for each sample. The standard errors are marked in black lines. The AT2-specific antagonist PD123319 (1 μM) or the AT1-specific antagonist losartan (DuP 753) (1 μM) were used for the partial blocking of the [^{125}I]Ang II binding. For complete blocking of the [^{125}I]Ang II binding to these oocytes, the cells were pre-incubated with non-radioactive Ang II (1 μM).

sults implied that *Xenopus* oocytes could be used as a convenient expression system to test the effect of co-expression of the AT2 on the AT1-mediated increase of intracellular IP_3 levels and to identify which domain of the AT2 is involved in the AT1–AT2 interaction.

To determine the effect of co-expression and activation of the AT2 on AT1-mediated increase of intracellular IP_3 levels, we co-injected equal amounts (25 ng) of cRNA corresponding to the AT1 and the AT2 into the oocytes. Ligand binding experiments using [^{125}I]Ang II (0.5 nM) showed that both the AT1 and the AT2 receptor subtypes were expressed in these cells (Fig. 1). Partial blocking of the [^{125}I]Ang II binding was achieved when the cells were pre-incubated with the AT2-specific antagonist PD123319 (1 μM) or the AT1-specific antagonist losartan (1 μM) (Fig. 1). Complete blocking of the [^{125}I]Ang II binding to these oocytes was achieved when the cells were pre-incubated with non-radioactive Ang II (1 μM) (Fig. 1). Uninjected oocytes did not show any significant binding to [^{125}I]Ang II (Fig. 1). A comparison of the intracellular IP_3 levels of the oocytes co-expressing the AT1 and the AT2 receptors to that of the oocytes expressing only the AT1 receptor is shown in Fig. 2. The activation of the AT1 receptor

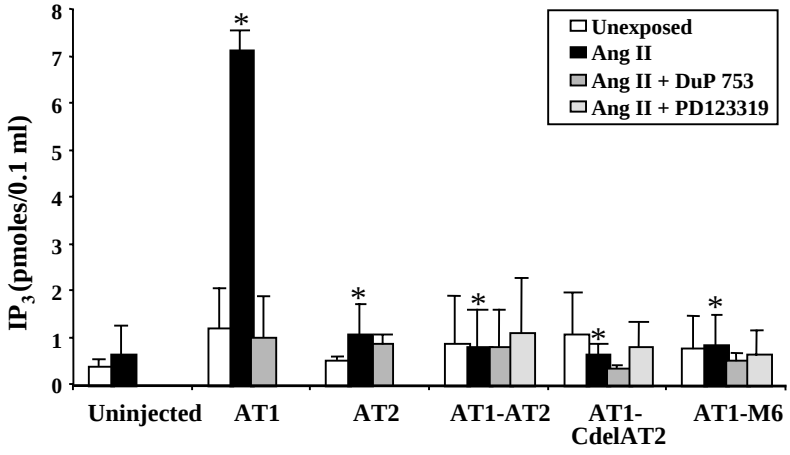

Fig. 2. AT2-mediated inhibition of the AT1-mediated increase in the intracellular IP_3 levels of *Xenopus* oocytes. The IP_3 levels of oocytes expressing the wild type AT1 receptor, or oocytes co-expressing different combinations of AT2 or its mutants with the AT1 (AT1 and AT2, AT1 and C-terminal deleted AT2 and AT1 and M6) are shown. The cells were exposed to 100 nM of Ang II for 5 min. When Ang II binding was blocked with PD123319 (AT2-specific ligand) or DuP 753 (AT1-specific ligand), a pre-incubation in the presence of 1 μM of the appropriate antagonist was performed before exposure to Ang II. IP_3 measurements were carried out according to the protocol of the 'Inositol-1,4,5-triphosphate [^{3}H] Radioreceptor Kit' purchased from NEN Life Science Products. 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 measurements and from each sample duplicates (100 μl each) were used to measure IP_3 levels according to the RIA kit. Oocytes from four different frogs were used and four different cRNA preparations were used. The standard errors for each data set are marked in black lines. *Significantly different when compared with the oocytes expressing the AT1 alone (Student's *t*-test, $P < 0.001$).

AT2:	235	RKHLLKTNSY GKNRITRDQV LK	256
AT1:	219	WKALKKAYEI QKNKPRNDDI FR	240
M6:	235	RKHLLKTNSY GKNRPTRDQV LK	256
M8:	235	RKHLLKAYEI GKNRPRNDQV FR	256

Fig. 3. A comparison of the amino acid sequences in the 3rd ICLs of the wild type AT1, AT2 receptors and the AT2 mutants M6 and M8 is shown. The numbers in the figure indicate the position of the amino acids in the AT1, AT2 and the mutants M6 and M8. The amino acid replacements introduced into the 3rd ICL of the AT2 to generate the AT2 mutants M6 and M8 are boxed.

by Ang II binding in the oocytes expressing the AT1 alone resulted in an increase in the intracellular IP_3 levels by about seven-fold when compared to the intracellular IP_3 levels of the uninjected oocytes (Fig. 2). This increase in the intracellular IP_3 levels was abolished when these cells were pre-incubated for 15 min in the presence of the AT1 antagonist losartan (DuP 753) (1 μM) (Fig. 2). The intracellular IP_3 levels of the oocytes expressing the AT2 alone and exposed to Ang II, were comparable to that of the uninjected cells which were exposed to Ang II (Fig. 2). Co-expression of the AT2 with the AT1 resulted in abolishing the AT1-mediated increase in the intracellular IP_3 levels (Fig. 2). Interestingly, pre-incubation with AT2-specific antagonist PD123319 did not reverse this effect. This observation is consistent with that found in the case of PC-12 cells co-expressing the AT1 and the AT2 receptors [49]. In those cells, the AT2-mediated inhibition of the AT1-mediated IP_3 production was independent of AT2 activation by the Ang II. The PD123319 could block the binding of [^{125}I]Ang II to the AT2 receptor present in these cells (Fig. 1). Therefore, the observation that PD123319 could not reverse the antagonistic effect of the AT2 on the AT1-mediated IP_3 production suggests that even in *Xenopus* oocytes this antagonistic effect of the AT2 is independent of the AT2 activation. Taken together, these results show that the signaling induced by Ang II binding to *Xenopus* oocytes co-expressing the rat AT1 and AT2 receptors mimic the signaling induced by Ang II binding to the PC-12 cells co-expressing the AT1 and the AT2 receptors.

3.2. C-terminal cytoplasmic tail of the AT2 receptor is not involved in the AT2 receptor- mediated inhibition of AT1 receptor-stimulated IP_3 generation

The C-terminal cytoplasmic tail of the AT2 receptor plays an important role in the ligand binding and signaling as shown previously [51]. This region of the AT2 was shown to negatively regulate the AT2-mediated reduction of cyclic guanosine monophosphate levels in *Xenopus* oocytes [51]. Deleting this domain of the AT2 also inhibited the protein–protein interaction between the AT2 and the ErbB3 receptor [51]. To determine the role of C-terminal cytoplasmic tail of the AT2 receptor in the AT2-mediated inhibition of the AT1-mediated IP_3 production in *Xenopus* oocytes, we co-expressed the wild type AT1 receptor with the C-terminal deleted AT2 and measured the levels of the intracellular IP_3. As shown in Fig. 2, the C-terminal deleted AT2 inhibited the AT1-mediated IP_3 generation in a similar manner to that of the wild type AT2. This observation implied that the C-terminal cytoplasmic tail of the AT2 receptor is not involved in the AT2-mediated inhibition of the AT1-mediated IP_3 production in *Xenopus* oocytes. Inhibition with PD123319 also did not reverse the ability of the C-terminal deleted AT2 to inhibit AT1-mediated IP_3 generation. This result further confirmed that both AT2 and C-terminal deleted AT2 inhibited AT1-mediated IP_3 generation by similar mechanism.

3.3. Mutational analysis of the 3rd ICL of the AT2 receptor

Since the C-terminal cytoplasmic tail of the AT2 did not

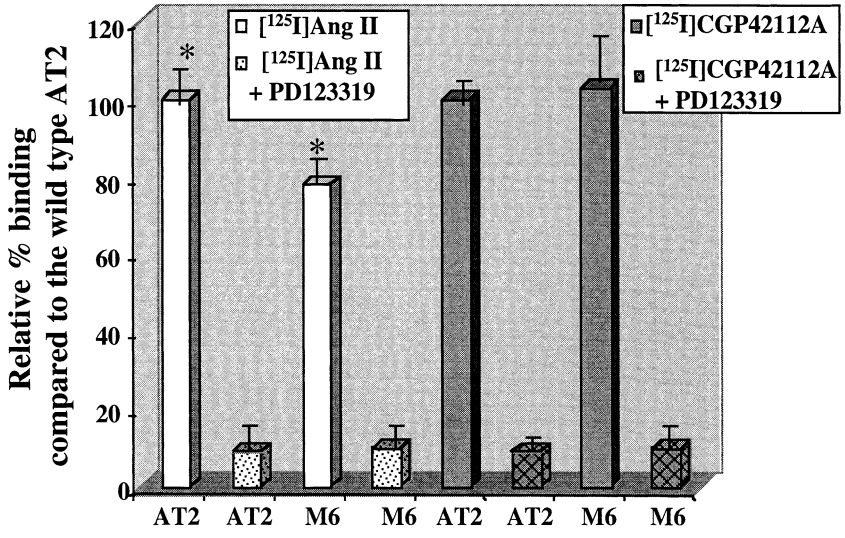

Fig. 4. Ligand binding properties of the *Xenopus* oocytes expressing the wild type AT2 receptor and the M6 mutant (Ile249Pro). The specific binding of the [^{125}I]Ang II (0.5 nM) and [^{125}I]CGP42112A (0.5 nM) to the oocytes expressing wild type and mutated receptors are compared. Binding experiments were conducted for a period of 1 h. Results are shown as relative percent 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 six oocytes from at least four donors (a total of at least 16 oocytes). Four different cRNA preparations were used for each sample. The value of 100% binding of a given ligand to the oocytes expressing the AT2 was determined from the mean of four different data sets and the standard errors were calculated accordingly. The standard errors are marked in black lines. *Significantly different when compared with the oocytes expressing the AT2 (Student's *t*-test, $P < 0.001$). The extent of binding of oocytes expressing M6 mutant or the AT2 to [^{125}I]CGP42112A was similar.

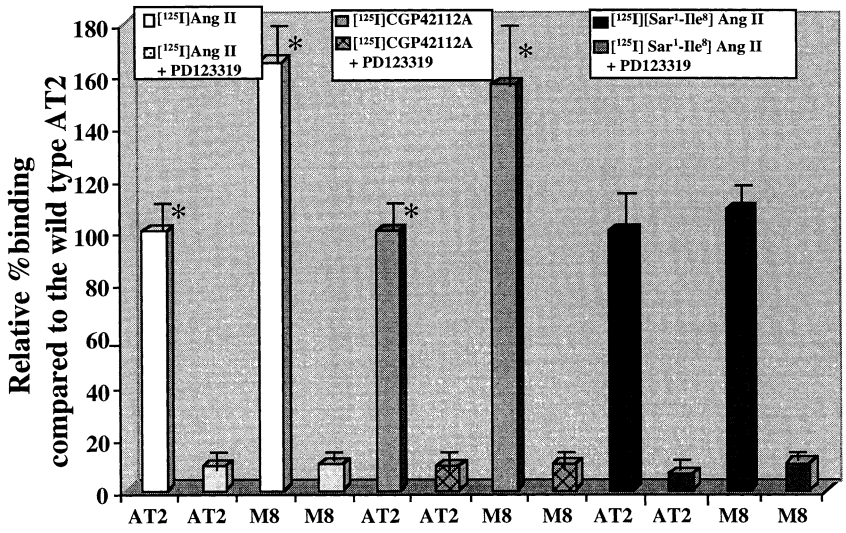

Fig. 5. Ligand binding properties of the *Xenopus* oocytes expressing the wild type AT2 receptor and the M8 mutant. The specific binding of the [^{125}I]Ang II (0.5 nM), [^{125}I]CGP42112A (0.5 nM), and [^{125}I-Sar^{1}-Ile^{8}]Ang II (0.5 nM) to the oocytes expressing wild type and mutated receptors are compared. Binding experiments were conducted using methods similar to those described in Figs. 1 and 4. Receptor expression was quantitated by binding studies using five oocytes from at least three donors (a total of at least 15 oocytes). Three different cRNA preparations were used for each sample. The value for 100% binding of a given ligand to the oocytes expressing the AT2 was determined from the mean of three different data sets and the standard errors were calculated accordingly. The standard errors are marked in black lines. *Significantly different when compared with the oocytes expressing the AT2 (Student's *t*-test, $P < 0.001$). The extent of binding of [^{125}I-Sar^{1}-Ile^{8}]Ang II to the oocytes expressing AT2 or M8 was similar.

seem to be involved in the AT2-mediated inhibition of the AT1-mediated IP_3 production in *Xenopus* oocytes, we then proceeded to analyze the role of the 3rd ICL of the AT2 in this function. We generated two mutants for this purpose. The first mutant had a proline at position 249 instead of isoleucine. A comparison of the amino acids in the 3rd ICL of the AT2 receptor and in the 3rd ICL of the AT1 is shown in Fig. 3. One striking difference between these two amino acid sequences is the presence of a proline (Pro233) in the AT1 which is absent in the corresponding position in the AT2. AT2 has an isoleucine (Ile249) in this position (Fig. 3). We assumed that introducing a proline in the 3rd ICL of the AT2 could cause a major conformational change that would lead to altering its functions. Therefore, we generated an AT2 mutant designated M6 in which Ile249 was replaced with a proline (Fig. 3). To determine whether this mutation influenced the ligand binding properties of AT2, we microinjected equal amounts of cRNA corresponding to wild type AT2 or M6 mutant into *Xenopus* oocytes. The ability of these receptors to bind peptidic ligands [^{125}I]Ang II and [^{125}I]CGP42112A (AT2-specific ligand) and the non-peptidic AT2-specific ligand PD123319 was evaluated by binding assays. As shown in Fig. 4, the oocytes expressing the M6 showed slightly decreased binding to [^{125}I]Ang II, when compared to the binding observed in the case of oocytes expressing the wild type AT2. However, the binding of [^{125}I]CGP42112A was comparable for both groups of oocytes (Fig. 4). The K_d values of the AT2 receptor and the M6 for the [^{125}I]Ang II were found to be 0.153 nM and 0.259 nM respectively. Similarly, for [^{125}I]CGP42112A, the K_d values of the AT2 receptor and the M6 were 0.103 nM and 0.104 nM respectively. To test the effect of replacing the Ile249 by proline on the ability of the AT2 to inhibit the AT1-mediated increase in the intracellular levels of the IP_3 in *Xenopus* oocytes, we co-expressed both AT1 and M6 mutant in these cells. As shown in Fig. 2, the Ile249Pro mutation did not affect the ability of the AT2 to inhibit AT1-mediated increase in the intracellular levels of the IP_3 in *Xenopus* oocytes. As seen in the case of AT2, inhibition by PD123319 did not affect the ability of M6 to inhibit AT1-mediated IP_3 generation. Therefore, introduction of proline at position 249 in the AT2 did not affect the mechanism by which AT2 inhibited AT1-mediated IP_3 generation.

Since converting Ile249 to proline did not alter either the ligand binding properties or the signaling properties of the AT2 considerably, we then introduced a series of amino acid replacements in the 3rd ICL of the AT2. Studies have shown that carboxy terminus (232–240) of the 3rd ICL of the AT1 receptor is an important determinant of G protein coupling [22,58]. The corresponding region of the AT2 is spanned by the amino acids 249–256. Since we already tested the effect of the replacement of the Ile249 with proline, we continued to make further changes in this region by replacing the amino acids in the AT2 with that of the AT1. We introduced four more mutations in this region and they were T250R, R251N, L255F and K256R. The diacidic motif (Asp236–Asp237) in the 3rd ICL of the AT1 receptor is not considered essential for the Ang II-stimulated IP_3 production, so we did not replace the Q253 of the AT2 with the corresponding Asp residue of the AT1 [59]. Moreover, we did not alter V254 since isoleucine in the corresponding position in the AT1 has similar properties. However, the residues 240–244 of the AT2 were shown to be essential for the AT2-mediated apoptosis [60]. The amino acid at position 240 in the 3rd ICL of AT2 is a lysine. However, the corresponding amino acid in the AT1 is also lysine, Lys223. Therefore, we replaced the amino acids 241–244 of the AT2 with the corresponding amino acids of the AT1. The mutations introduced in this region were T241A, N242Y, S243E and Y244I. The mutant that carried all the nine mutations was designated M8 (Fig. 3). The binding affinity of this mutant was studied in the similar manner

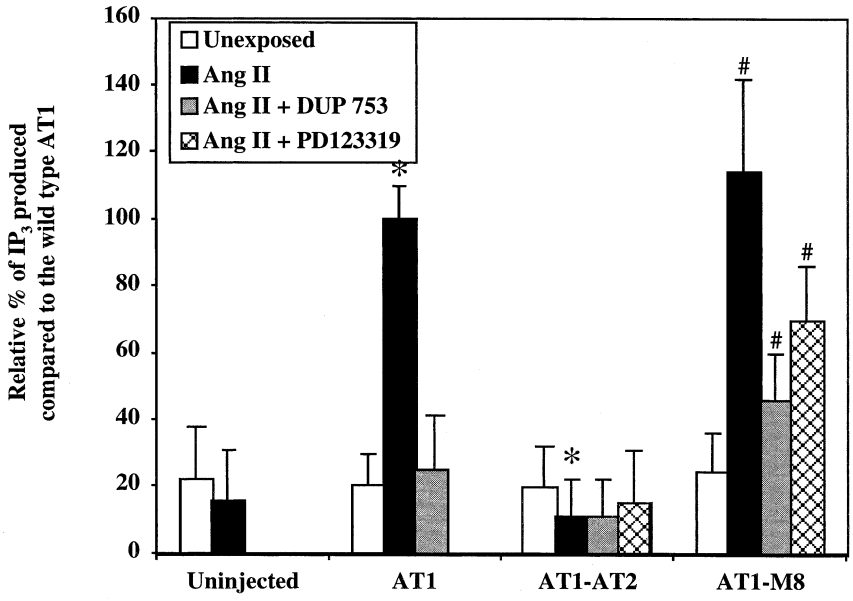

Fig. 6. Co-expression of the M8 mutant did not inhibit the AT1-mediated increase in intracellular IP_3 levels of *Xenopus* oocytes. The IP_3 levels of the oocytes solely expressing the wild type AT1 receptor are compared to that of the oocytes co-expressing the AT1 and AT2, or AT1 and M8 mutant. Exposure of each group of oocytes to Ang II or AT1-specific and AT2-specific antagonists was carried as described for Fig. 2. IP_3 measurements were carried out according to the protocol of the 'Inositol-1,4,5-triphosphate [^{3}H] Radioreceptor Kit' purchased from NEN Life Science Products and the techniques used were similar to those described for Fig. 2. Oocytes from three different frogs were used and three different cRNA preparations were used. The value for the IP_3 levels of oocytes expressing the AT1 and exposed to Ang II from three different data sets were compared, and the mean was taken as the 100%. The standard errors were calculated accordingly. The standard errors for each data set are marked in black lines. *Significantly different when compared with the oocytes expressing the AT1 alone (Student's *t*-test, $P < 0.001$). However, P is 0.4353 for the differences observed between the IP_3 levels of oocytes expressing wild type AT1 versus the oocytes expressing both wild type AT1 and M8 mutant and exposed to Ang II. #Significantly different when compared with the oocytes co-expressing the AT1 and the M8 and exposed to Ang II (Student's *t*-test, $P < 0.001$).

as described for Ile249Pro mutant. Oocytes expressing the mutant M8 demonstrated increased affinity to both [^{125}I]Ang II and [^{125}I]CGP42112A as shown in Fig. 5. The K_d values of the AT2 receptor and the M8 for the [^{125}I]Ang II were found to be 0.153 nM and 0.038 nM respectively. Similarly, for [^{125}I]CGP42112A K_d values of the AT2 receptor and the M8 were calculated to be 0.103 nM and 0.068 nM respectively. Thus, the effect caused by the Ile249Pro mutation on the wild type AT2 (a small reduction in the affinity to [^{125}I]Ang II) was not seen when the same mutation was introduced in the presence of other mutations.

We also measured the intracellular IP_3 levels of the oocytes co-expressing the AT1 receptor and the M8. Interestingly, co-expression of the M8 and activation by Ang II did not inhibit the intracellular IP_3 generated by Ang II-stimulated AT1 receptor (Fig. 6). Moreover, both losartan (DuP 753) and the AT2-specific antagonist PD123319 were able to reduce partially the IP_3 levels of oocytes co-expressing the AT1 and M8 and exposed to Ang II. Therefore, the M8 responded to the inhibition by the AT2-specific antagonist PD123319. Overall, these observations indicated that the region of the 3rd ICL of AT2 spanning the amino acids 241–256 is essential for the AT2-mediated inhibition of the AT1-mediated increase in intracellular IP_3 levels in *Xenopus* oocytes.

However, these observations regarding the ligand binding and signaling properties of the M8 raised some interesting questions. Previously we had shown that a chimeric receptor, in which the entire 3rd ICL of the AT2 was replaced with the 3rd ICL of the AT1, did not bind either [^{125}I]CGP42112A or [^{125}I-Sar^1-Ile^8]Ang II with high affinity [54]. This chimeric receptor carried 22 continuous amino acid replacements where the AT2 specific amino acids were replaced with that of the AT1. The AT2 mutant M8 carried only nine of these 22 amino acids replacements. Interestingly, this receptor did not loose affinity to [^{125}I]CGP42112A as the chimeric receptor, instead, showed increased affinity when compared to that of the AT2 (Fig. 5). Therefore, the question was whether the M8 could also bind [^{125}I-Sar^1-Ile^8]Ang II with high affinity like the AT2. To explore whether the M8 differed from the chimeric receptor in its ability to bind [^{125}I-Sar^1-Ile^8]Ang II, we performed ligand binding experiments on oocytes expressing the M8 using [^{125}I-Sar^1-Ile^8]Ang II. As shown in Fig. 5, our results showed that the oocytes expressing the M8 could bind the [^{125}I-Sar^1-Ile^8]Ang II with high affinity. The K_d values of the AT2 receptor and the M8 for [^{125}I-Sar^1-Ile^8]Ang II were calculated to be 0.172 nM and 0.170 nM respectively. Thus, the ligand binding properties of the chimeric receptor and the AT2 mutant M8 were not similar. The AT2 mutant M8 seemed to retain the ability to bind all the three peptidic ligands ([^{125}I] Ang II, [^{125}I]CGP42112A or [^{125}I-Sar^1-Ile^8]Ang II) with high affinity.

The second interesting question was how the AT2 antagonist PD123319 inhibited the IP_3 generation of oocytes co-expressing the M8 and the AT1. Since the mutations in the M8 abolished the AT2-mediated inhibition of the AT1-mediated IP_3 generation, the observation that the overall IP_3 generation in oocytes co-expressing the M8 and the AT1 was partially reduced in response to PD123319, was surprising. However, in creating M8, what we have actually done is to mimic the region of the AT2 spanning the amino acids 241–256 to the

corresponding region of the AT1. Since many amino acids in this region of the AT1 are implicated in its interaction with the Gq and consequent activation of PLC [22], it was possible that the M8 that mimics the AT1 in this region could also have acquired the ability to activate Gq and subsequently activate PLC. Indeed, we have seen that the chimeric receptor that carried the 3rd ICL of the AT1 in place of the 3rd ICL of the AT2 was capable of activating PLC [54]. Therefore, we tested whether the M8 has acquired the ability to activate PLC, like the chimeric receptor. Exposure of the oocytes expressing the M8 to Ang II (100 nM) resulted in increased levels of the intracellular IP_3, and this increase corresponded to 98% of the intracellular IP_3 levels of the oocytes expressing the AT1 receptor. This M8-mediated elevation of intracellular IP_3 levels in *Xenopus* oocytes was inhibited when the oocytes were pre-exposed to the AT2-antagonist PD123319 (1 μM). Taken together these results suggested that the M8 is a mutant that retains the ligand binding properties of the AT2, but possesses at least one of the signaling properties of the AT1, namely its ability to activate PLC. Since the M8 carries only nine of the amino acid substitutions found in the chimeric receptor, these results also showed that these substitutions are sufficient to render the AT2 receptor with the ability to activate PLC. Moreover, since these nine amino acid substitutions did not abolish the ability of the M8 to bind [^{125}I]CGP42112A or [^{125}I-Sar^1-Ile^8]Ang II, it is possible that the remaining 13 amino acid substitutions made in the AT2 to construct the chimeric receptor may have contributed to its inability to bind the above two ligands. The ability of PD123319 to inhibit the elevation of intracellular IP_3 levels in *Xenopus* oocytes co-expressing the AT1 and M8 could also be due to the fact that the M8 was capable of activating PLC on its own. It should be noted that we did not see a significant increase in the overall IP_3 levels of the oocytes co-expressing the AT1 and the M8 as compared to the oocytes expressing AT1 alone (Fig. 6). If both the AT1 and the M8 were activating the same Gq proteins or same PLC molecules, either of these molecules could act as rate-limiting factors in the overall IP_3 generation. Therefore, although both the AT1 and the M8 could activate PLC and elevate IP_3 levels, the overall increase in the IP_3 levels would depend upon the number of Gq proteins and PLC molecules that were available for activation by the M8 or the AT1 in *Xenopus* oocytes. Since, in oocytes co-expressing both the AT1 and the M8, both receptors would contribute to the activation of PLC, inhibiting the action of one receptor could result in partial inhibition of PLC activation. This could be the reason why the AT2 antagonist PD123319 was able to exert partial inhibition of the elevation of intracellular IP_3 levels in oocytes co-expressing both the M8 and the AT1.

In summary, the antagonistic effect of the rat AT2 expression on the PLC activation and concomitant IP_3 generation by rat AT1 receptor could be observed in *Xenopus* oocyte expression system. The observation that the antagonistic effect of the AT2 on the AT1-mediated signaling is ligand independent, further supports the idea that the nature of interaction between the AT1 and the AT2 in *Xenopus* oocytes is comparable to that seen in the PC-12 cells. Studies on the PC-12 cells have established that heterodimerization between the AT1 and the AT2 is involved in the antagonistic effect of the AT2 on the AT1 [49]. However, the specific domain of the AT2 that is needed for this interaction was not identified. Since the AT1 has to couple to Gq protein for PLC activation and the region of the AT1 needed for coupling to Gq protein is its 3rd ICL [18–22], any interaction between the AT1 and the AT2 that would result in inhibition of the ability of the AT1 to activate Gq (and thereby PLC) must occur in the cytoplasmic regions of the AT2. Since the region of the AT2 that was needed for its direct interaction with the ATP binding domain of the ErbB3 receptor (which also is cytoplasmic region of the ErbB3) included the C-terminal cytoplasmic tail and the 3rd ICL of the AT2 [50], we hypothesized that these regions could also be involved in the interaction between the AT2 and the AT1. Our results show that the C-terminal cytoplasmic tail of the AT2 does not play a significant role in this AT1–AT2 interaction. A single mutation, Ile249Pro in the 3rd ICL was also not enough to hamper the AT1–AT2 interaction that would abolish the ability of the AT1 to activate PLC. Therefore, we introduced eight more selective mutations based on what is known regarding the involvement of the amino acids in the 3rd ICLs of the AT1 and the AT2 in their functions from previous mutagenesis studies [58–60]. All the mutations were replacements of the AT2 specific amino acids by corresponding AT1 specific amino acids. This resulted in increasing the affinity of the mutant receptor, M8, to its peptidic ligands, suggesting that the 3rd ICL of the AT2 has an important role in determining its affinity to its ligands. Moreover, the slight decrease in affinity to [^{125}I]Ang II observed in the case of M6 that carries the single mutation, Ile249Pro was masked when eight more selective mutations were introduced to make the M8. Our results also show that these nine mutations are sufficient to render the AT2 receptor with the ability to activate PLC in *Xenopus* oocytes. Thus, the M8 seems to be a very interesting mutant that retains the ligand binding properties of the AT2, but has acquired at least one signaling property of the AT1, the ability to activate PLC. Moreover, these same mutations are also sufficient to abolish the AT2-mediated inhibition of the AT1-mediated IP_3 production. Since it is established that the 3rd ICL of the rat AT1 is involved in the activation of Gq that leads to the activation of PLC [21,22], it is possible that the interaction to abolish the AT2-mediated inhibition of the AT1-mediated IP_3 production could occur between the 3rd ICLs of both receptors. Finally, our results show that the region of the 3rd ICL of the AT2 that is spanned by the amino acids 241–256 is specifically involved in this AT1–AT2 interaction.

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