Identification of Angiotensin II Type 2 (AT2) Receptor Domains Mediating High-Affinity CGP 42112A Binding and Receptor Activation

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ABSTRACT

Chimeric angiotensin II (AngII) receptors constructed of portions of the AT2 receptor substituted into the AT1 receptor revealed the AT2 third extracellular loop and seventh transmembrane-spanning domain as major determinants for the ability to bind and activate in response to the AT2 receptor-selective agonist CGP 42112A. Radioligand binding experiments showed that chimeric AngII receptors possessing the AT2 third extracellular loop and seventh transmembrane-spanning domain bound CGP 42112A with high affinity approaching that of the wild-type AT2 receptor. The presence of the AT2 third extracellular loop appeared sufficient for high-affinity CGP 42112A binding, which was further enhanced by the additional presence of the AT2 seventh transmembrane-spanning domain. Experiments with PD 123319, losartan, and [Sar1,Ile8]-AngII showed that increases in binding affinity associated with these domains were specific for CGP 42112A. Use of phosphoinositide hydrolysis as a functional index to measure activation of these chimeric AngII receptors further demonstrated that the AT2 seventh transmembrane-spanning domain was especially critical for CGP 42112A to act as an agonist. The absence of the AT2 seventh transmembrane-spanning domain prohibited CGP 42112A-induced activation of these receptors, even in the presence of high concentrations of CGP 42112A sufficient to saturate the binding sites. This study is the first to identify binding determinants of the AT2 receptor that are selective for CGP 42112A, and indicates that these determinants are at least partially distinct from those for the AT2-selective antagonist PD 123319. These differences may be a factor in the pharmacodynamic difference between these two ligands.

Angiotensin II (AngII) is an octapeptide that serves as a key regulator of blood pressure, body fluid osmolarity, and ingestive behavior. In the periphery, AngII acts as a hormone that stimulates constriction of vascular smooth muscle (Griendling et al., 1997) and causes aldosterone secretion from the adrenal cortex and fluid reabsorption from the kidneys (Vallotton, 1987). In the central nervous system, AngII acts as a neuromodulator/neurotransmitter to increase thirst and sodium appetite (Fitzsimons, 1998). The net effect of the biological actions of AngII is to increase blood pressure and/or body fluid volume.

AngII exerts its biological effects by binding to cell surface receptors in the plasma membrane of its target cells. These membrane-bound AngII receptors belong to the superfAMILY of heterotrimeric G protein-coupled receptors (GPCRs) and are divided into two subtypes, designated the AT1 (angiotensin II type 1) receptor and AT2 (angiotensin II type 2) receptor. These subtypes were initially distinguished through the use of subtype-specific ligands (Chiu et al., 1989): the antagonist losartan selectively recognizes and binds to the AT1 receptor (Chiu et al., 1991), while the agonist CGP 42112A (Whitebread et al., 1991) and the antagonist PD 123319 (Blankley et al., 1991) both bind specifically to the AT2 receptor. Both receptor subtypes have since been cloned (Murphy et al., 1991; Kambayashi et al., 1993) and found to possess considerably different amino acid sequences (approximately 34% homology). It is not surprising, therefore, that they possess distinct pharmacological profiles and signaling properties.

The AT1 receptor has been shown in many systems to couple to Gq and signal through activation of phospholipase C and the subsequent release of inositol trisphosphate (IP3) and diacylglycerol (Peach and Dostal, 1990). Virtually all of

ABBREVIATIONS: AngII, angiotensin II; GPCR, G protein-coupled receptor; AT1, receptor, angiotensin II type 1 receptor; AT2, receptor, angiotensin II type 2 receptor; CGP 42112A, Nα-nicotinoyl-Tyr-(Nα-CBZ-Arg)-Lys-His-Pro-Ile-OH; IP3, inositol trisphosphate; SARILE, [Sar1,Ile8]-angiotensin II; DMEM, Dulbecco’s modified Eagle’s medium; SOE, splicing by overlap extension; PCR, polymerase chain reaction; ANOVA, analysis of variance; TM7, transmembrane 7.
the major biological actions classically associated with AngII are mediated via the AT$_1$ receptor subtype. Conversely, the AT$_2$ receptor is less well characterized: although its amino acid sequence conforms to the seven transmembrane-spanning domain topology observed in GPCRs (Kambayashi et al., 1993), its behavior is atypical compared with most GPCRs, and its signaling pathways have been historically difficult to characterize (Nahmias and Strosberg, 1995). While the ability of the AT$_2$ receptor to bind agonists with high affinity is unimpaired by high concentrations of guanine nucleotides (Kambayashi et al., 1993; Yee et al., 1997a), the AT$_2$ receptor has been shown to couple to the pertussis toxin-sensitive G protein G$_i$ (Kang et al., 1994). Although in some neuronal cell types the AT$_2$ receptor is capable of signaling through activation of the delayed rectifier potassium current (Kang et al., 1994) or inactivation of mitogen-activated protein kinase (Huang et al., 1996), the observation of an AT$_2$ receptor-mediated signaling pathway remains problematic for most investigators. The AT$_2$ receptor is widely expressed in fetal tissues (Grady et al., 1991; Reagan et al., 1996) and inhibits coronary epithelial cell proliferation (Stoll et al., 1995) as well as mediates apoptosis in cultured PC-12 cells (Yamada et al., 1996). While its biological purpose has yet to be established, the AT$_2$ receptor appears to play an antagonistic role to the AT$_1$ receptor, both at a cellular level (K$_+$ currents) (Gelband et al., 1997) as well as in a larger physiological context (AT$_1$ receptor-mediated cell proliferation versus AT$_2$ receptor-mediated apoptosis/antiproliferation). This has led to the proposal that the AT$_2$ receptor plays an important role in controlling tissue development and remodeling.

While there still exists a shortage of information on structure/function relationships of the AT$_2$ subtype (especially relative to that which exists for the AT$_1$ subtype) some progress has been made in the last few years. Reports from this group and others have identified elements of the AT$_2$ receptor involved in binding the endogenous ligand AngII. Deletion of the amino terminus of the AT$_2$ receptor drastically reduces affinity of the receptor for AngII (Yee et al., 1998), as does mutation of Lys$_{815}$ in the fifth transmembrane-spanning domain (Yee et al., 1997b), His$_{273}$ in the sixth transmembrane-spanning domain (Turner et al., 1999), and Arg$_{182}$ and Asp$_{297}$ in the extracellular loops (Heerding et al., 1997). Many of these same mutations reduced the affinity of the AT$_2$ receptor for the nonselective antagonist [Sar$_3$,Ile$_8$]-angiotensin II (SARILE) as well. There are, however, very little data describing domains involved in binding the subtype-selective ligands for the AT$_2$ receptor. Likewise, while other published reports have identified portions of the third intracellular loop as essential for coupling to intracellular effectors (Kang et al., 1995; Hayashida et al., 1996), there is a lack of information regarding the elements of the AT$_2$ receptor that are important in mediating the conformational change from inactive receptor to activated receptor following agonist binding. In the current study, we used chimeric AngII receptors composed of domains of both subtypes and a gain-of-function strategy to identify domains of the AT$_2$ receptor that are crucial for the ability to bind and activate in response to the AT$_2$-selective agonist CGP 42112A. Our results clearly demonstrate that both the third extracellular loop and the seventh transmembrane-spanning domain are important for high-affinity binding of CGP 42112A and activation in response to this AT$_2$-selective ligand.

### Experimental Procedures

**Materials.** Tissue culture medium and supplements, including LipofectAMINE reagent were obtained from Life Technologies (Gaithersburg, MD). [H]$^3$H]Inositols was obtained from American Radiolabeled Chemicals (St. Louis, MO) and [125]I-AngII was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Losartan was a gift from Dr. Ronald Smith (DuPont, Wilmington, DE) and PD 123319 was a gift from Dr. David Dudley (Parke-Davis, Ann Arbor, MI). COP 42112A was purchased from Sigma/RBI (Natick, MA) and all other peptide ligands were obtained from Peninsula Laboratories (Belmont, CA). All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted.

**Cell Culture and Transfections.** COS-1 cells were grown in polystyrene tissue culture flasks in medium consisting of DMEM (high glucose) supplemented with 10% fetal calf serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin in a humidified atmosphere of 5% CO$_2$ and 95% O$_2$ at 37°C. Wild-type AT$_1$, AT$_2$, and chimeric receptor cDNAs were later introduced into the COS cells by transfection with LipofectAMINE. Briefly, the growth medium was removed from the COS cells upon reaching approximately 80% confluence and replaced with transfection medium (un-supplemented DMEM containing 1.3 μg/ml of the selected cDNA and 5.5 μl/μl LipofectAMINE) for 5 h. Following the 5-h transfection interval, the transfection medium was removed and replaced with normal growth medium. Radioligand binding or IP$_3$ release assays were then performed 48 h following the transfection interval.

**Mutagenesis.** A modified version of the splicing by overlap extension (SOE) technique was used to generate the AngII receptor chimeras. A single round of SOE involved two steps: 1) amplification of individual fragments encoding the desired portions of each receptor using specifically designed complementary and overlapping primers (see below), followed by 2) purification of the fragments using the polymerase chain reaction (PCR). As a refinement to enhance the fidelity of SOE, a small amount of Pfu DNA polymerase (1:100 Pfu: Taq) was added. For the following chimeras, the joining of AT$_2$ and AT$_1$ cDNA sequences was performed in a single SOE round using the primers specified: AT$_2$[AT$_1$ TM6-CT]: 5’-ATCTTCCAGATGCATGCCCATAACCATC-3’ (forward sense primer) and 5’-GGAAGTGCAGTGTTG-3’ (reverse sense primer) and 5’-GCAAGTGCATGTGTTG-3’ (reverse sense primer) and 5’-GGAAGTGCAGTGTTG-3’ (reverse sense primer). For the following chimeras, the joining of AT$_2$ and AT$_1$ receptor cDNAs was performed by using two successive SOE rounds. For AT$_2$[AT$_1$ ECL3], the AT$_2$ receptor sequence from the third extracellular loop to its cytoplasmic tail was first added to an AT$_1$ receptor using 5’-CACATCTGGAGTTCGCT-3’ (forward sense primer) and 5’-GCAAGTGCATGTGTTG-3’ (reverse sense primer) and 5’-GCAAGTGCATGTGTTG-3’ (reverse sense primer). For the remaining chimeras, the joining of AT$_2$ and AT$_1$ receptor cDNAs was performed by using two successive SOE rounds. For AT$_2$[AT$_1$ ECL3], the AT$_2$ receptor sequence from the third extracellular loop to its cytoplasmic tail was first added to an AT$_1$ receptor using 5’-CACATCTGGAGTTCGCT-3’ (forward sense primer) and 5’-GCAAGTGCATGTGTTG-3’ (reverse sense primer) and 5’-GCAAGTGCATGTGTTG-3’ (reverse sense primer) and 5’-GCAAGTGCATGTGTTG-3’ (reverse sense primer). For AT$_2$[AT$_1$ ECL3], the AT$_2$ receptor sequence from the third extracellular loop to its cytoplasmic tail was first added to an AT$_1$ receptor using 5’-CACATCTGGAGTTCGCT-3’ (forward sense primer) and 5’-GCAAGTGCATGTGTTG-3’ (reverse sense primer) and 5’-GCAAGTGCATGTGTTG-3’ (reverse sense primer) and 5’-GCAAGTGCATGTGTTG-3’ (reverse sense primer).
using the T7 primer and the reverse antisense primer, while the second fragment was produced using the SP6 primer and the forward sense primer. Wild-type AT1 and AT2 cDNAs, which have previously isolated from the murine neuroblastoma N1E-115 cell line (Yee et al., 1997a), served as the template in these PCRs. Reaction conditions were 30 cycles of 94°C (1 min), 55°C (1 min), and 72°C (1 min). Following purification using the Wizard PCR Prep DNA Purification system (Promega, Madison, WI), the two fragments were combined in the overlap extension reaction using the same PCR conditions as described. Following production of the full-length chimeric receptor using SOE, the chimera was subcloned into the expression vector pCR3 (Invitrogen, Carlsbad, CA) and sequenced to confirm its validity.

**Radioligand Binding Assay.** Transfected COS cells were harvested, resuspended in phosphate-buffered saline and pelleting the cells by centrifugation at 23,000g for 10 min. The cells were then resuspended in assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl2, 0.3 TIU/ml aprotinin, and 100 μg/ml 1,10-phenanthroline) and lysed by polytron homogenization. Following a second centrifugation at 40,000g for 20 min to pellet the cell membranes, the final membrane pellet was resuspended in assay buffer and protein content was determined spectrophotometrically using the bicinchoninic acid protein assay (Pierce, Rockford, IL). The binding assays were initiated by addition of the desired amount of membrane protein (5–10 μg for the wild-type AT1 and AT2 receptors; 50–250 μg for the chimeric receptors) to assay mixture containing various concentrations of [125I]-AngII and unlabeled competitors. Nonspecific binding was defined as the amount of radioligand binding remaining in the presence of 1 μM SARILE. The binding assays proceeded for 60 min and were terminated by rapid filtration using a Brandell harvester (Brandell, Gaithersburg, MD). Radioligand binding was quantitated by gamma counting of the filters.

**Inositol Trisphosphate Assay.** Transfected COS cells were loaded with [3H]inositol (4.5 μCi/ml DMEM) for 18 h prior to assay. Transfected cells were then drug treated at the concentrations specified for 30 s, rinsed once with ice-cold phosphate-buffered saline, and then rapidly lysed in 1 ml of 10% trichloroacetic acid. Insoluble materials were pelleted at 16,000g for 30 min. The pellets were solubilized in 500 μl of 1% sodium dodecylsulfate in 0.1 M NaOH for protein quantification. The supernatant from each lysate was extracted five times with 2 volumes of water-saturated ether. Following the final extraction, the aqueous layers were neutralized by addition of sodium bicarbonate and EDTA to final concentrations of 6 and 15 mM, respectively. The aqueous supernatants were added to 1 ml of AG 1-X8 anion exchange resin columns (Bio-Rad, Hercules, CA) and inositol phosphates were separated by stepwise elution with buffers of increasing ammonium formate concentration in 0.1 M formic acid, as previously described (Berridge et al., 1982; Bokkala and Joseph, 1997). The amount of [3H]IP3 eluted from each column was quantified by liquid scintillation counting in Tru-Count scintillation cocktail (IN/US Systems, Inc., Tampa, FL).

**Data Analysis.** All radioligand binding and receptor signaling data were analyzed using GraphPad Prism software (GraphPad Software, San Diego, CA). The results are presented as means ± standard error. Statistical analysis was performed with the aid of SuperANOVA software (Abacus Concepts, Berkeley, CA). ANOVA was performed on receptor signaling and competition binding data, and the Student-Newman-Keuls test was used as a post hoc test with the significance level set at P < 0.01.

**Results**

A series of chimeric AngII receptors was synthesized as depicted in Fig. 1. The cDNAs encoding these chimeras as well as the wild-type AT2 and AT1 receptors were separately transiently transfected into COS cells and the level of expression for each receptor was measured by saturation binding assay using [125I]-AngII. Representative saturation iso-

- **AT2**
- **AT1**
- **AT[AT2, TM6-CT]**
- **AT[AT2, ECL3-CT]**
- **AT[AT2, TM7-CT]**
- **AT[AT2, ECL3]**
- **AT2 Receptor Domains for CGP 42112A Binding and Activation**

![Fig. 1. Schematic of wild-type AT2, AT1, and chimeric AT2/AT1 receptors. The chimeric receptors were constructed by a modified version of the splicing by overlap extension technique as described under Experimental Procedures.](image)

 thermographs for each of the wild-type and chimeric AngII receptors are shown in Fig. 2, and the calculated mean K_D and B_max values are listed in Table 1. The wild-type AT2 and AT1 receptors both exhibited similarly high affinities for AngII (K_D = 4.7 ± 1.8 and 4.6 ± 1.3 nM, respectively) and similar levels of expression (B_max = 4.1 ± 0.6 and 6.3 ± 1.5 pmol/mg of protein, respectively).

However, the affinity for AngII and level of expression showed greater variation among the chimeric receptors. AT1[AT2 TM6-CT], a chimera consisting of AT1 receptor sequence through the third intracellular loop and then AT2 receptor from the sixth transmembrane-spanning domain to the end of the cytoplasmic tail, displayed a reduction in affinity for AngII, approximately 3-fold relative to the wild-type AT2 receptor, as well as an approximately 10-fold reduction in expression level relative to the wild-type AT2 receptor. While AT1[AT2 ECL3-CT], which replaces the third extracellular loop through the end of the cytoplasmic tail of the AT1 receptor with the corresponding AT2 receptor domains, also showed measurable specific binding of [125I]-AngII (Fig. 2), the binding was nonsaturable and thereby prohibited precise determination of its expression level and affinity for AngII (Table 1). AT1[AT2 TM7-CT], which is comprised of AT1 receptor sequence from the amino terminus up until the extracellular face of the seventh transmembrane-spanning domain, and then AT2 sequence through the end of the cytoplasmic tail, showed the greatest measurable shift in affinity for AngII, an approximately 6-fold reduction compared with the wild-type AT2 receptor. However, its expression level was over 2-fold greater than the wild-type AT2 receptor. AT1[AT2 ECL3] is constructed of the AT2 third extracellular loop placed on the AT1 receptor, and while it did show measurable specific binding of [125I]-AngII (Fig. 2), the binding was also nonsaturable (Table 1). The subsequent competition binding results and functional responses that are later seen with these chimeras, nevertheless, are clear evidence of their expression and insertion into the cell membrane. Of all the chimeras constructed, AT1[AT2 ECL3], a mirror chimera of AT1[AT2 ECL3], most closely approxi-
mated the affinities and expression levels of the wild-type receptors.

Competition radioligand binding between the AT2-selective agonist CGP 42112A and [125I]-AngII was used to measure the relative affinity of each of the receptors for CGP 42112A (Fig. 3). The concentration of CGP 42112A at which 50% of the maximum level of specifically bound [125I]-AngII was displaced (the IC50) was determined for each of the receptors. Not surprisingly, the wild-type AT2 receptor displayed a very high affinity for CGP 42112A, with an IC50 of 4.0 ± 2.2 nM, while the wild-type AT1 receptor displayed the equally anticipated low affinity for CGP 42112A, with an IC50 of 11.4 ± 1.9 μM, almost 3000-fold less than the AT2 receptor. The first chimeric AngII receptor of this study, AT1[AT2 TM6-CT], showed a remarkably high affinity for CGP 42112A (IC50 = 52.9 ± 5.1 nM) even though only a relatively small portion of that chimera is comprised of AT2 receptor sequence. Clearly, some domain(s) in the transplanted portion of the AT2 receptor had increased CGP 42112A affinity more than 200-fold over that of the wild-type AT1 receptor. In fact, the CGP 42112A affinity of AT1[AT2 TM6-CT] was only about 10-fold less than the wild-type AT2 receptor itself. Upon moving to AT1[AT2 ECL3-CT] and its

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<tr>
<th>Receptor</th>
<th>Kd</th>
<th>Bmax</th>
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<tr>
<td>AT2</td>
<td>4.7 ± 1.8</td>
<td>4.1 ± 0.6</td>
</tr>
<tr>
<td>AT1[AT2 TM6-CT]</td>
<td>16.6 ± 3.2</td>
<td>0.40 ± 0.02</td>
</tr>
<tr>
<td>AT1[AT2 TM7-CT]</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>AT1[AT2 ECL3]</td>
<td>28.0 ± 1.7</td>
<td>10.9 ± 3.1</td>
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<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>AT1[AT2 ECL3]</td>
<td>2.1 ± 0.4</td>
<td>1.3 ± 1.1</td>
</tr>
<tr>
<td>AT1</td>
<td>4.8 ± 1.3</td>
<td>8.3 ± 1.5</td>
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N.D., not determined. While these chimeras possessed demonstrable specific binding of [125I]-AngII, the binding was not saturable over the achievable range of radioligand concentrations (see Fig. 2). A precise quantitation of receptor expression and ligand affinity could not be made.
concomitant replacement of the AT₂ sixth transmembrane-spanning domain with that of the AT₁ receptor, the same high affinity for CGP 42112A was retained, with an IC₅₀ of 56.5 ± 14.2 nM. Thus, it appeared the sixth transmembrane-spanning domain of the AT₂ receptor does not play a critical role in high-affinity interactions with CGP 42112A.

In comparison, when the AT₂ third extracellular loop was also removed and replaced with that of the AT₁ receptor, the result was a drastic reduction in affinity for CGP 42112A: AT₁[AT₂ TM7-CT] possesses an IC₅₀ of 13.8 ± 3.4 μM, similar to that of the wild-type AT₁ receptor. Therefore, the third extracellular loop of the AT₂ receptor is an important determinant for CGP 42112A binding. This idea is further supported by the competition binding results obtained with AT₁[AT₂ ECL3], a chimeric AngII receptor where the third extracellular loop of the AT₂ receptor is the only AT₂ domain present: AT₁[AT₂ ECL3] also possessed moderately high affinity for CGP 42112A (IC₅₀ = 131 ± 28.5 nM). While the affinity of AT₁[AT₂ ECL3] for AT₂-selective PD 123319 was significantly lower than that seen with either AT₁[AT₂ ECL3-CT] or AT₁[AT₂ TM6-CT] (by ANOVA and Student-Newman-Keuls post hoc test, P < 0.01), it was still 100-fold greater than that observed with AT₁[AT₂ TM7-CT] or the wild-type AT₁ receptor. This confirms that the AT₂ third extracellular loop is, by itself, sufficient for dramatic improvement in the binding of CGP 42112A. It also suggests that the seventh transmembrane-spanning domain of the AT₂ receptor may have an enhancing effect on high-affinity interactions of the AT₂ third extracellular loop with CGP 42112A. Note, however, that by itself the AT₂ seventh transmembrane-spanning domain had no appreciable enhancement on CGP 42112A affinity, as seen with AT₁[AT₂ TM7-CT].

The third extracellular loop of the AT₂ receptor is, however, even by itself clearly an important determinant for CGP 42112A binding affinity. Surprisingly, a mirror chimera of AT₁[AT₂ ECL3], AT₂[AT₁ ECL3], retains affinity for CGP 42112A (IC₅₀ = 2.6 ± 0.7 nM) equivalent to that of wild-type AT₂ despite the absence of the AT₂ third extracellular loop, indicating the probable existence of more than one binding determinant in forming the CGP 42112A binding site within the AT₂ receptor.

To demonstrate that these affinity shifts observed between the chimeras were specific for CGP 42112A and not indicative of more global changes in overall angiotensinergic binding activity, similar competition binding was performed using the nonselective AngII analog SARILE (Fig. 4). Unlike the results observed in the CGP 42112A competition assays, the SARILE competition assays showed that most of the receptors exhibited no significant differences in affinity for SARILE: the wild-type AT₂ and AT₁ receptors possessed IC₅₀ values of 2.9 ± 0.7 and 2.1 ± 0.6 nM, respectively; AT₁[AT₂ TM6-CT] had an IC₅₀ of 1.8 ± 0.7 nM; AT₁[AT₂ ECL3-CT] had an IC₅₀ of 2.5 ± 0.3 nM; AT₁[AT₂ ECL3] had an IC₅₀ of 2.3 ± 0.6 nM; and AT₂[AT₁ ECL3] had an IC₅₀ of 1.8 ± 0.5 nM. Only AT₁[AT₂ TM7-CT], with an IC₅₀ of 9.9 ± 2.2 nM, possessed an affinity for SARILE that was significantly different (P < 0.01) compared with the other receptors; even so, it was far less than the affinity changes associated with CGP 42112A. The ability of other subtype-selective antagonists, the AT₂-selective PD 123319 and AT₁-selective losartan, to displace [¹²⁵I]-AngII was impaired similarly in most of the chimeric receptors (Table 2). Only the wild-type AT₂ receptor and AT₁[AT₂ ECL3] (which is itself comprised mostly of AT₂ sequence) displayed high sensitivity to 1 μM PD 123319, although AT₁[AT₂ ECL3] did possess a much lower, but still significant (P < 0.01) sensitivity to 1 μM PD 123319. Conversely, the wild-type AT₁ receptor and AT₁[AT₂ ECL3] were the only receptors that showed any sensitivity to 1 μM losartan. CGP 42112A was the only subtype-selective ligand to preferentially bind with high affinity to all the receptors possessing the AT₂ third extracellular loop. These results suggest that the binding determinants for the two AT₂ receptor-selective ligands, CGP 42112A and PD 123319, are somewhat distinct from each other.

In addition to having high affinity and selectivity for the AT₂ receptor, CGP 42112A is functionally an agonist at the AT₂ receptor (Kang et al., 1994; Nahmias et al., 1995; Stoll et al., 1999; 2001). This functional effect likely contributes to the high affinity observed for this peptide in the AT₂-selective competition assay.
response to agonists could be quantitated by measuring IP3 phospholipase C was preserved. Thus, their activation in main of the AT2 receptor showed a robust activation upon extracellular loop and seventh transmembrane-spanning do-

(Fig. 5). Furthermore, the chimeras that possessed the third intracellular loop of the AT1 receptor, coupling to spanning domain, rather than the AT2 third extracellular exception of the AT2 receptor) activated and induced signif-

icant IP3 release, as seen in a previous report on receptor chimeras (Wang et al., 1995). All the receptors (with the expected pathways (Buisson et al., 1992; Brechler et al., 1994a; Kang et al., 1995). Unfortunately, activation of the reported signaling receptors in fetal tissues (Grady et al., 1991) and the recent discovery that AT2 receptors can mediate apoptosis (Yamada al., 1995). Therefore, activation of the reported signaling pathways (Buisson et al., 1992; Brechler et al., 1994a; Kang et al., 1994; Huang et al., 1996) was not detected in our previously published study involving the AT2 receptor (Yee et al., 1997a). However, since many of the chimeras possessed the third intracellular loop of the AT1 receptor, coupling to phospholipase C was preserved. Thus, their activation in response to agonists could be quantitated by measuring IP3 release, as seen in a previous report on receptor chimeras (Wang et al., 1995). All the receptors (with the expected exception of the AT2 receptor) activated and induced significant IP3 release in response to the endogenous agonist AngII (Fig. 5). Furthermore, the chimeras that possessed the third extracellular loop and seventh transmembrane-spanning domain of the AT2 receptor showed a robust activation upon stimulation with CGP 42112A. Interestingly, very high concentrations of CGP 42112A (up to $10^{-4}$ M), which were sufficient to saturate the AT1 receptor (Fig. 3), failed to activate it (Fig. 5). The presence of the AT2 seventh transmembrane-spanning domain, rather than the AT2 third extracellular loop, was the necessary element for CGP 42112A-induced activation of the chimeric receptors: both AT1[AT2 TM6-CT] and AT1[AT2 ECL3-CT] were activated by CGP 42112A in a dose-dependent manner (Fig. 6); yet, AT1[AT2 ECL3] was not activated by a saturating concentration ($10^{-4}$ M) of CGP 42112A, despite the demonstrated ability of AT1[AT2 ECL3] to activate in response to AngII (Fig. 5). The importance of the seventh transmembrane-spanning domain of AT2 for functional activation by CGP 42112A is further underscored in that AT1[AT2 TM7-CT], which binds CGP 42112A with the same low affinity as the AT1 receptor (Fig. 3), does activate in response to CGP 42112A (Figs. 5 and 6). Again, the AT1 receptor itself did not activate upon treatment with equally high concentrations of CGP 42112A. Thus, the seventh transmembrane-spanning domain of the AT2 receptor plays an especially important role in the ability CGP 42112A to activate its target receptor. The additional presence of the AT2 third extracellular loop in the chimeras tended to shift the EC50 values for activation to lower concentrations of CGP 42112A (Fig. 6): EC50 = 251 ± 65.1 nM for AT1[AT2 TM7-CT] versus EC50 = 96.8 ± 6.0 nM and 142 ± 22.1 nM for AT1[AT2 TM6-CT] and AT1[AT2 ECL3-CT], respectively. However, the differences in these EC50 values do not reach statistical signif-

icance by ANOVA and Student-Newman-Keuls post hoc test ($P < 0.01$). Neither PD 123319 nor losartan were able to block agonist-induced activation of these chimeras (Table 3), consistent with the results of radioligand binding assay indicating an inability of either antagonist to bind to these chimeras (Table 2).

These functional assay results, which reveal the impor-

tance of the seventh transmembrane-spanning domain in mediating receptor activation by CGP 42112A, are somewhat complementary to the results of the binding assays, where the AT2 third extracellular loop was the major determinant for CGP 42112A binding, and the seventh transmembrane-spanning domain played a supporting role. Taken together, it can be concluded that the third extracellular loop and seventh transmembrane-spanning domain together form an important functional unit for specific, high-affinity binding of CGP 42112A and its subsequent ability to activate the AT2 receptor.

Discussion

While the role of the AT1 receptor has been firmly estab-

lished for regulating blood pressure and body fluid composi-

tion, the biological role of the AT2 receptor has only recently begun to be understood. The extensive distribution of AT2 receptors in fetal tissues (Grady et al., 1991) and the recent discovery that AT2 receptors can mediate apoptosis (Yamada et al., 1996) suggest a role in controlling tissue development. This role may continue into adult animals, as the AT2 recep-

TABLE 2

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<th>% of Specific [125I]-AngII Binding Remaining in Presence of</th>
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<tbody>
<tr>
<td></td>
<td>1 μM PD 123319</td>
</tr>
<tr>
<td>AT2</td>
<td>3.4 ± 0.7**</td>
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<td>AT1[AT2 TM6-CT]</td>
<td>100 ± 1.7</td>
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<td>AT1[AT2 ECL3]</td>
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<tr>
<td>AT1[AT2 TM6-CT]</td>
<td>0.90 ± 0.14**</td>
</tr>
<tr>
<td>AT1</td>
<td>92.3 ± 3.9</td>
</tr>
</tbody>
</table>

** Significant displacement of [125I]-AngII by the specified antagonist ($P < 0.01$).
Effects of subtype-selective AngII receptor antagonists on agonist-induced receptor activation

**TABLE 3**
Effects of subtype-selective AngII receptor antagonists on agonist-induced receptor activation

Transfected cells were treated with a submaximal concentration of either AngII (5 nM for AT1, 15 nM for AT1[AT2 TM6-CT], AT1[AT2 ECL3-CT], and AT1[AT2 TM7-CT]) or CGP 42112A (100 nM for AT1[AT2 TM6-CT], 150 nM for AT1[AT2 ECL3-CT], 250 nM for AT1[AT2 TM7-CT]) in the absence or presence of losartan (1 μM) or PD 123319 (1 μM) and release of IP3 was quantitated. Release of IP3 in drug-treated groups for each receptor is expressed as percentage of its control (unstimulated) level. The values reported represent the mean ± standard error of three to six independent experiments.

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>Wild-Type AT1</th>
<th>AT1[AT2 TM6-CT]</th>
<th>AT1[AT2 ECL3-CT]</th>
<th>AT1[AT2 TM7-CT]</th>
</tr>
</thead>
<tbody>
<tr>
<td>control (unstimulated)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>AngII</td>
<td>235 ± 34.6**</td>
<td>267 ± 25.4**</td>
<td>253 ± 14.4**</td>
<td>202 ± 9.7**</td>
</tr>
<tr>
<td>AngII + losartan</td>
<td>136 ± 23.7†</td>
<td>260 ± 33.2**</td>
<td>252 ± 18.3**</td>
<td>207 ± 13.3***</td>
</tr>
<tr>
<td>AngII + PD 123319</td>
<td>218 ± 27.0**</td>
<td>262 ± 21.0**</td>
<td>264 ± 4.4**</td>
<td>214 ± 11.8**</td>
</tr>
<tr>
<td>CGP 42112A</td>
<td>N.D.</td>
<td>227 ± 13.2**</td>
<td>211 ± 7.8**</td>
<td>177 ± 3.1**</td>
</tr>
<tr>
<td>CGP 42112A + losartan</td>
<td>N.D.</td>
<td>234 ± 11.1**</td>
<td>280 ± 17.2**</td>
<td>178 ± 7.4**</td>
</tr>
<tr>
<td>CGP 42112A + PD 123319</td>
<td>N.D.</td>
<td>226 ± 13.3**</td>
<td>210 ± 3.9**</td>
<td>168 ± 5.1**</td>
</tr>
<tr>
<td>Losartan</td>
<td>106 ± 6.7</td>
<td>105 ± 3.8</td>
<td>98.7 ± 8.0</td>
<td>100 ± 4.2</td>
</tr>
<tr>
<td>PD 123319</td>
<td>95.7 ± 2.3</td>
<td>106 ± 4.2</td>
<td>97.8 ± 8.0</td>
<td>96.7 ± 3.5</td>
</tr>
</tbody>
</table>

N.D., not determined.
***, significantly different from control (unstimulated) IP3 release ($P < 0.01$).
††, significantly different from agonist-induced IP3 release ($P < 0.05$).

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The AT2 receptor has been demonstrated to suppress coronary cell proliferation (Stoll et al., 1995) and neointima formation in carotid artery following balloon injury (Nakajima et al., 1995). Manipulation of these processes via the AT2 receptor may hold some clinical benefit in controlling wound healing. Given the demonstrated physiological antagonism between the two subtypes, the development of AT2 receptor agonists (along with AT1 receptor antagonists) could hold some promise in treating cardiovascular disease. Understanding the structure/function relationships of AT2 receptors would therefore be useful in designing strategies for selectively modulating AT2 receptor-mediated processes.

Understanding of structure/function relationships of the AT2 receptor has lagged behind that of the AT1 receptor, due as much to the difficulty of uncovering a consensus cellular signaling pathway for the AT2 receptor (Kambayashi et al., 1993; Brechler et al., 1994a,b) as to uncertainty regarding its larger biological role. However, there has been some recent progress in identifying AT2 receptor domains and residues involved in the binding of the endogenous ligand AngII (Heerding et al., 1997; Yee et al., 1997b, 1998; Turner et al., 1999). Moreover, the few studies where an AT2 receptor function was reliably detected have identified the third intracellular loop as crucial for coupling to effectors (Kang et al., 1995; Hayashida et al., 1996). The use of receptor chimeras has proven valuable in previous studies on AngII receptor structure/function relationships, providing insight into ligand binding and effector coupling domains of the AT1 subtype (Schambye et al., 1994; Wang et al., 1995), as well as the AngII binding domains of the AT2 subtype (Yee et al., 1998). However, none of these studies have identified determinants responsible for the specific interactions with AT2-selective ligands, or the subsequent agonist-induced transition of the inactive AT2 receptor to the activated state. These issues are important to the realization of AT2 receptors as pharmacological targets. In the present study, we used new chimeric AngII receptors to investigate the basis for the selective interaction of the AT2 receptor with the potent AT2-selective agonist CGP 42112A.

We constructed a series of chimeric AngII receptors by replacing distal portions of the AT1 receptor (Fig. 1) with those of the AT2 subtype. Transfection of these chimeric receptor cDNAs into COS cells allowed for verifying the AngII binding activity and for quantitation of expression levels (Table 1) for all but a pair of chimeras, both of which nevertheless exhibited measurable [125I]-AngII binding (Fig. 2). An approximately 3000-fold difference in affinity exists between the AT2 and AT1 receptors for the AT2-selective agonist CGP...
42112A (Fig. 3). For this ligand, the binding affinities of the chimeras varied greatly despite comparatively small changes in the portions comprised of AT2 receptor domains (Fig. 3). These results clearly revealed the AT2 third extracellular loop (ECL3) to be an important determinant for CGP 42112A binding: the three chimeras possessing the AT2 ECL3 all exhibited an approximately 100- to 200-fold increase in affinity for CGP 42112A relative to the AT1 receptor, while a chimera lacking the AT2 ECL3, i.e., AT1[AT2 TM7-CT], demonstrated the same reduced affinity for CGP 42112A as the AT1 receptor. Further binding analysis with the non-selective AngII analog SARILE (Fig. 4), as well as with the structurally dissimilar subtype-selective antagonists PD 123319 and losartan (Table 2), showed that these large changes in CGP 42112A affinity associated with the AT2 ECL3 were specific for CGP 42112A and not reflective of differences in overall ligand binding activity. This difference in affinity for CGP 42112A and PD 123319 bind to the AT2 receptor may be a factor behind the pharmacodynamic difference between them.

Furthermore, while these binding results make clear the importance of the AT2 ECL3 in determining affinity for CGP 42112A, they also suggest the existence of other CGP 42112A binding determinants: 1) none of the chimeras with the AT2 ECL3 achieved an affinity for CGP 42112A quite as high as the AT2 receptor itself; their affinities were still approximately 15- to 30-fold less; and 2) AT1[AT1 ECL3], which is comprised of AT1 receptor except for ECL3, retains high affinity for CGP 42112A. These results are best explained by the presence of more than one binding determinant in forming a CGP 42112A binding “pocket” within the AT2 receptor: removing one of them (i.e., ECL3) can minimally disrupt binding to the AT2 receptor, while introducing the same determinant into a receptor with negligible CGP 42112A binding (i.e., the AT1 receptor) dramatically increases its affinity for that ligand. This is not a surprising result because peptidic ligands like CGP 42112A, owing to their relatively large size, often bind to multiple contact points on their receptors (Strader et al., 1994; Hunyady et al., 1996). Studies are ongoing to identify other CGP 42112A binding determinants; the results of this study in fact do point toward the AT2 seventh transmembrane-spanning domain (TM7) as playing a supporting role in the binding of CGP 42112A: AT1[AT2 TM6-CT] and AT1[AT2 ECL3-CT], which possess both the AT2 ECL3 and TM7, have significantly higher affinity for CGP 42112A than AT1[AT1 ECL3], which possesses the AT2 ECL3 without the AT2 TM7. Given their ability to increase CGP 42112A affinity to greater than 200-fold over the AT1 receptor (thereby leaving only a 15- to 30-fold affinity deficit between themselves and the AT2 receptor), the AT2 ECL3 and TM7 play a comparatively important role in the binding of this agonist. However, the participation of other, as yet unidentified binding determinants should not be considered trivial, as evidenced by the results with AT1[AT1 ECL3].

Measurements of the activation of these chimeras during treatment with CGP 42112A confirms the involvement of the AT2 TM7 in interactions with this ligand (Fig. 5). Because most of the chimeric AngII receptors contained the third intracellular loop of the AT1 receptor, it was possible to measure release of IP$_3$ as a readout of receptor activation (J. Hines, S. J. Fluharty, and D. K. Yee, unpublished data; Wang et al., 1995), thereby avoiding the problems inherent with uncovering an AT2 receptor signal. All the receptors tested, except the wild-type AT1, stimulated IP$_3$ release upon treatment with the endogenous agonist AngII. Furthermore, those chimeras possessing the AT2 TM7 were able to activate and stimulate IP$_3$ release in response to CGP 42112A as well. In fact, one of the chimeras, AT1[AT2 TM7-CT], exhibited CGP 42112A-induced IP$_3$ release at agonist concentrations that had produced negligible $^{[125]}$I-AngII displacement in competition binding assays (Fig. 3). Of all the chimeras, AT1[AT2 TM7-CT] has the lowest measurable affinity for CGP 42112A (and most other ligands tested): perhaps, in this case, signaling activation assay (which depends on ligand-receptor association rate, and is enzymatic) is more sensitive to detecting CGP 42112A interaction than equilibrium competition binding assay (which depends on ligand-receptor association and dissociation rates, and is stoichiometric). This particular result underscores the importance of doing both binding and functional assays when evaluating the impact of mutations on a receptor.

Since high micromolar concentrations of CGP 42112A that completely displace $^{[125]}$I-AngII from the AT1 receptor still fail to activate that subtype, activation in response to CGP 42112A is not an AT1 receptor attribute, but rather should be considered an AT2-specific phenomenon. The inability of the AT2-specific antagonist PD 123319 to block IP$_3$ release (Table 3) likely arises from its inability to bind to the chimeras (Table 2). Possession of the AT2 TM7 is a definite prerequisite for activation in response to CGP 42112A: AT1[AT2 ECL3] binds CGP 42112A with an approximately 100-fold greater affinity than the AT1 receptor (Fig. 3), and yet neither of them activates even at saturating concentrations of CGP 42112A (Fig. 5) since both lack the AT2 TM7. Interestingly, while the presence of the AT2 TM7 had a relatively minor impact on CGP 42112A binding compared with the AT2 ECL3, the complementary situation exists from the standpoint of CGP 42112A-induced activation: the AT2 ECL3 has a minor (although statistically nonsignificant) impact on the EC$_{50}$ values (Fig. 6) of receptor activation by CGP 42112A (AT1[AT2 TM7-CT] versus AT1[AT2 ECL3-CT] and AT2[AT2 TM6-CT]), but the AT2 TM7 was of greater importance. Thus, it could be considered that the ECL3 and TM7 constitute a single combined determinant bestowing high-affinity CGP 42112A binding and receptor activation.

The ability to bind CGP 42112A with high affinity is conserved across many species isoforms of the AT2 receptor, including murine, human, and bovine (Kambayashi et al., 1993; Ouali et al., 1993; Lazard et al., 1994; Yee et al., 1997a). Not surprisingly, the amino acid sequence of the ECL3 and TM7 is also virtually identical across the cloned AT2 isoforms (Kambayashi et al., 1993; Nakajima et al., 1993; Lazard et al., 1994). Thus, the information presented herein may apply to the structure/function relationships of most, if not all, AT2 receptor species isoforms. Given the large number of candidate amino acids in these domains alone, and the absence of information regarding important pharmacophores of the ligand CGP 42112A, the ongoing investigation of their molecular interaction becomes increasingly complex.

In summary, we report on the first identification of a subtype-selective ligand binding determinant for the AT2 receptor. The AT2 ECL3 and TM7 are necessary for the
unique ability of this subtype to bind and activate in response to its selective agonist CGP 42112A. This is also the first clear demonstration that the binding determinant(s) for CGP 42112A are at least partially distinct from those of the AT₂-selective antagonist PD 123319. Detailed structural information on the AT₂ receptor should provide a valuable resource for developing better pharmacologic tools to control the antiproliferative actions of the AT₂ receptor as its physiological role becomes clearer.

References


