A Novel Angiotensin Receptor Subtype in Rat Mesangium
Coupling to Adenylyl Cyclase

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The diversity of angiotensin II (Ang II) actions implies multiple receptor subtypes. To characterize these subtypes in rat mesangial cells, we used the angiotensin subtype LA (AT₁) antagonist losartan (DuP 753), the subtype 2/1B (AT₂/AT₆B) antagonist PD 123319, and the AT₂ antagonist CGP 42112A in radioreceptor and adenylyl cyclase assays. In radioligand binding competition experiments, approximately 25% of the specific binding sites labeled by [³⁵S]-Sar¹]Ang II were inhibited by low concentrations of PD 123319 (0.1 to 10 nM), whereas the AT₁ antagonist CGP 42112A was inactive at concentrations less than 0.1 μM. Conversely, losartan inhibited 75% of the binding at low concentrations (0.1 nM to 0.1 μM), but higher concentrations (up to 10 μM) were required to inhibit the second component of [³⁵S]-Sar¹]Ang II binding. The effects of the different antagonists on the inhibition by Ang II of forskolin-stimulated cyclic AMP production were also analyzed. Ang II inhibited forskolin-stimulated adenylyl cyclase in a concentration-dependent fashion (IC₅₀, 35±7 nM), and the maximal inhibition of adenylyl cyclase was 44±2%. In the radioligand binding experiments, both losartan and PD 123319 antagonized the inhibition of adenylyl cyclase elicited by 0.1 μM Ang II (IC₅₀, 0.5±0.2 and 1.2±0.4 μM, respectively), whereas CGP 42112A was less potent (IC₅₀, 5.7±1.6 μM). Comparison of binding affinities at AT₁ receptor sites with antagonist potencies in the adenylyl cyclase assay show good agreement for losartan and CGP 42112A, whereas PD 123319 is less potent than expected from membrane binding assays, possibly because of partial agonist properties. These results cannot be accounted for by a single AT₁ subtype and imply that there are two functionally coupled subtypes of AT₁ receptor in rat mesangial cell membranes. AT₁LA and AT₁LB subtypes differ in their ligand specificity profile. The AT₁LA subtype and an additional receptor, possibly the AT₁LB, are coupled to adenylyl cyclase through Gₛ.

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Recently, two subtypes of angiotensin II (Ang II) receptors have been described based on their differential affinities for the nonpeptide antagonists losartan (DuP 753) compared with PD 123319 or the peptide CGP 42112A.¹ The Ang II binding site antagonized by losartan, termed the AT₁ subtype, was the predominant receptor subtype found in the vascular smooth muscle, adrenal cortex, liver, and some brain regions. The Ang II binding site antagonized by PD 123319 and CGP 42112A, termed the AT₂ subtype, was the major subtype in uterine smooth muscle, ovary, adrenal medulla, the developing rat fetus, and other brain regions.² Most of the known actions of Ang II are mediated by the AT₁ receptor, such as phosphoinositide hydrolysis, regulation of calcium channels, inhibition of adenylyl cyclase, and stimulation of phospholipase A₂.³⁻⁵ The AT₂ receptor mediates inhibition of guanylyl cyclase and regulation of potassium channels⁶⁻⁸; however, the signaling mechanism for the AT₂ receptor remains undefined. Recently, complementary DNA clones for bovine⁹ and rat¹⁰ AT₂ receptors have been isolated and characterized. The cloned AT₂ receptors have the typical features of G protein-coupled receptors and are linked to phosphoinositide-specific phospholipase C.

Assessments of binding kinetics and signal transduction mechanisms in the kidney suggest that more than one population of high-affinity Ang II receptors exists.⁵ We have recently observed a novel pharmacologically distinct Ang II receptor subtype in glomerular mesangial cells that is sensitive to PD 123319 and coupled to G protein.¹¹ This receptor is distinct from the AT₂ subtype, which is not expressed by mesangial cells. As adenylyl cyclase is an important G protein-coupled signal transduction system in the kidney, the present studies were designed to determine whether one or both receptor subtypes are linked to it.

Methods

Mesangial Cell Culture and Membrane Preparation

Glomerular isolation and tissue culture of mesangial cells were performed as previously reported.¹¹ Mesangial cells were cultured in RPMI 1640 with 20% fetal calf serum. Cells were used for studies of Ang II
receptor interaction between the third and eighth passages and were harvested at 4°C by gentle scraping and rinsing with calcium-free Hank's solution and pelleting at 250g for 5 minutes. Mesangial cells were homogenized in 15 mL ice-cold HEPES-Tris buffer, pH 7.4, containing 100 μM 1,10-phenanthroline, 1 mM phenylmethanesulfonyl fluoride, 5 μM leupeptin, 7 μM phosphoramidon, 4 μM pepstatin, 1 mM EDTA, and 250 mM sucrose. Homogenates were centrifuged at 1,000g for 5 minutes at 4°C. The pellets were resuspended in 20 mL homogenization buffer and homogenized and centrifuged at 1,000g for 5 minutes. The combined supernatants were centrifuged at 48,000g for 20 minutes at 4°C, and resulting pellets were resuspended in 35 mL of 50 mM Tris-HCl buffer, pH 7.7. After being recentrifuged at 4,000g for 20 minutes, the membranes were used in binding and adenylyl cyclase assays.

Radio-receptor Binding Assay

Binding assays were performed with 125I-[Sar1]Ang II for determination of specific binding to Ang II receptor subtypes as previously described. Membranes (20 to 50 μg protein) were incubated with 0.1 to 0.5 nM 125I-[Sar1]Ang II and varying amounts of unlabeled hormone in a total volume of 250 μL containing 50 mM Tris-HCl (pH 7.7), 120 mM NaCl, 0.5 mM MgCl2, 0.5 mg/mL bovine serum albumin, 100 μM bacitracin, 10 μM phosphoramidon, 50 μM phenylmethanesulfonyl fluoride, and 10 μg/mL soybean trypsin inhibitor for 60 minutes at 23°C. The incubation was stopped by vacuum filtration through Whatman GF/C filters (presoaked in 0.1% bovine serum albumin) to isolate receptor-bound radioactivity. The filters were washed four times with 5 mL ice-cold Tris-HCl containing NaCl and MgCl2 and counted at 80% efficiency in a gamma counter. Nonspecific binding was defined in the presence of 2.0 μM unlabeled Ang II.

Adenylyl Cyclase Assay

Adenylyl cyclase was determined by measuring the formation of cyclic AMP by radioimmunoassay as described previously in this laboratory with some modifications. Briefly, the assay system for the determination of adenylyl cyclase activity contained 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 4 mM MgCl2, 4 mM ATP, 0.01 mM GTP, 2 mM isobutylmethylxanthine, 7.5 mM creatine phosphate, and 15 U/mL creatine phosphokinase. Reaction was initiated by adding 40 μg mesangial cell membrane protein or regeneration system at 26°C for 30 minutes and was stopped by 0.5 mL of 50 mM sodium acetate buffer, pH 6.2. The amount of cyclic AMP formed was determined by radioimmunoassay. 

Calculations

Results are expressed as mean±SEM. Data were entered as disintegrations per minute into the EQUILIBRIUM BINDING DATA ANALYSIS PROGRAM to obtain estimates of half-maximal inhibitory concentration (IC50) and Hill slope and to create input files for the LIGAND program. Subsequently, 4 to 10 experiments were analyzed simultaneously by use of the LIGAND program for nonlinear curve fitting. Dose–response curves were analyzed by nonlinear curve fitting using logistic equations (INPLOT, GRAPHPAD Software, San Diego, Calif.).

Materials

Losartan was donated by Du Pont Merck Pharmaceutical Co., Wilmington, Del. PD 123319 was donated by Parke-Davis, Ann Arbor, Mich., and CGP 42112A by CIBA-GEIGY, Basel, Switzerland. Iodine-125 was purchased from Amersham Corp., Arlington Heights, Ill. Other compounds were obtained from Research Biochemicals International, Natick, Mass.; Sigma Chemical Co., St. Louis, Mo.; Peninsula Laboratories, Belmont, Calif.; and Boehringer Mannheim Biochemicals, Indianapolis, Ind.

Results

Binding Studies of Mesangial Cell Membranes

In agreement with our previous studies, 125I-[Sar1]Ang II bound to rat mesangial cell membranes in a specific and monophasic manner as indicated by a Hill slope approximating 1. The apparent Kd value and the number of sites (Bmax) were 1.4±0.13 nM and 72±6 fmol/mg protein, respectively. In competition experiments, both of the inhibitors of Ang II binding by losartan and PD 123319 were biphasic. Approximately 25% of the specific binding sites labeled by 0.2 nM 125I-[Sar1]Ang II were inhibited by low concentrations of PD 123319 (0.1 to 10 nM), whereas the remaining 75% of the sites were not inhibited even by 1,000-fold higher concentrations.

The interaction of losartan and PD 123319 with 125I-[Sar1]Ang II binding could be modeled by nonlinear curve fitting as two distinct populations of sites. The major proportion of sites showed higher affinity for losartan (nanomolar) and lower affinity for PD 123319 (micromolar). Conversely, the minor site displayed a higher affinity (nanomolar) for PD 123319 and lower affinity (micromolar) for losartan. Losartan showed 82-fold higher affinity for the majority site; in contrast, PD 123319 showed more than 10,000-fold selectivity in the opposite direction. The peptide agonist angiotensin III and [des-Phe1]Ang II bound more avidly to this smaller population of sites (700-fold and 40-fold, respectively). Ang II itself showed similar affinities for the two components (within one-half log unit). The AT1 antagonist CGP 42112A was relatively weak (Kd, >1 μM) at both sites. The subpopulation of specific binding sites with high affinity for losartan was termed the AT1a site, and the subpopulation with high affinity for PD 123319 was termed the AT1b site. The relative affinities (pIC50) of Ang II, losartan, PD 123319, and CGP 42112A at the AT1b site are shown in Figure 1 (squares).

Inhibition of Adenylyl Cyclase

The effects of the different antagonists on the inhibition by Ang II of forskolin-stimulated cyclic AMP production were analyzed in rat mesangial cell membranes. Ang II inhibited forskolin-stimulated adenylyl cyclase in a concentration-dependent fashion (IC50, 35±7 nM), and the maximal inhibition of adenylyl cyclase was 44±2% (Figure 2). Much higher concentrations of Ang II (100-fold) are required to inhibit adenylyl cyclase than to occupy either the AT1a or AT1b receptor subtypes (Figure 1). Losartan could antagonize completely the inhibition of adenylyl cyclase elicited by 0.1 μM Ang II but with low potency relative to AT1a (IC50, 0.5±0.2 μM). PD 123319 also competed for Ang II-induced inhibition of adenylyl cyclase (IC50, 35±7 nM).
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1.2±0.4 μM). CGP 42112A was less potent (IC50, 5.7±1.6 μM) (Figure 3). Comparison of binding affinities at AT1B receptor sites with antagonist potencies in the adenylyl cyclase assay show good agreement for losartan and CGP 42112A, whereas PD 123319 is less potent than expected from membrane binding assays (Figure 1). Approximately 1,000-fold difference was noted between binding affinity and adenylyl cyclase regulation. The latter discrepancy may be explained by a partial agonist action of PD 123319 at AT1B receptors. These data further support the heterogeneity of renal angiotensin receptors.

Discussion

In the present study we demonstrate that specific 125I-[Sar1]Ang II binding to cultured rat mesangial cells corresponds to two subgroups of receptor sites, here proposed as AT1A and AT1B. AT1A showed a higher affinity for losartan (nanomolar) and lower affinity for PD 123319 (micromolar). In contrast, AT1B displayed a higher affinity (nanomolar) for PD 123319 and lower affinity for losartan (micromolar). GTPγS and pertussis toxin interfere with binding to both sites, suggesting that one or more G proteins may modulate receptor interactions.11 Masking experiments suggested that GTPγS was fourfold more potent in inhibiting the AT1A component of binding relative to the AT1B component. When a G protein is involved in a signal transduction system, GTP is required for agonist-induced second messenger production. In our experiment, Ang II-induced inhibition of Ang II on adenylyl cyclase required the presence of GTP (data not shown), consistent with observations of Crawford et al,18 who used 7315c cells derived from rat anterior pituitary tumor. In mesangial cell membranes stimulated with GTP and forskolin, Ang II inhibited adenylyl cyclase activity by a maximum of 44% at 10 μM. Similar results were reported by Bauer and colleagues19 using rat liver membranes. Ang II-elicited adenylyl cyclase inhibition appeared to be mediated through an AT1A-like receptor, because losartan but not PD 123177 blocks the effect of Ang II.18-19 However, losartan is a relatively weak antagonist (IC50, approximately 1 μM) relative to its inhibitory action on vascular smooth muscle inositol phosphate metabolism and calcium mobilization (IC50, approximately 10 nM).20 The subtype of Ang II receptor

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Plot shows comparison of IC50 values for angiotensin II (Ang II) and selective antagonists in radioligand binding assays at AT1B receptors and in adenylyl cyclase assays. For 125I-Ang II binding assays, affinities at AT1B sites were determined by nonlinear iterative curve fitting as previously described,11 and IC50 values were determined with the Cheng-Prusoff equation as reported previously.16 For adenylyl cyclase assays, IC50 values were determined by nonlinear curve fitting to a logistic equation (see "Methods").

![Figure 2](http://hyper.ahajournals.org/)

**Figure 2.** Plot shows dose-dependent inhibition of forskolin-stimulated (5 μM) adenylyl cyclase activity in mesangial cell membranes by angiotensin II. Each point represents mean±SEM of triplicate determinations. Similar results were obtained in two other independent experiments. The curve was computer generated based on the best fit to a logistic function (INPLLOT, GRAPHPAD). Half-maximal inhibition (22%) was attained at 35 nM.

![Figure 3](http://hyper.ahajournals.org/)

**Figure 3.** Plot shows effects of angiotensin II antagonists on inhibitory action of 0.1 μM angiotensin II on forskolin-stimulated adenylyl cyclase activity in rat mesangial cell membranes. Each point represents mean±SEM of triplicate determinations. Curves were generated and IC50 values determined by nonlinear curve fitting (INPLLOT, GRAPHPAD) as follows: for losartan, 0.5±0.2 μM; for PD123319, 1.2±0.4 μM; for CGP42112A, 5.7±1.6 μM.
coupled to adenylyl cyclase inhibition in various cell types remains to be fully defined. Because a 100-fold higher concentration of Ang II is required to inhibit mesangial cell adenylyl cyclase than to occupy either the AT1A or AT1B receptor subtypes (Figure 1), it would seem that more Ang II receptors must be occupied by an agonist for inhibition of adenylyl cyclase than for inositol trisphosphate–induced calcium mobilization.

A similar discrepancy was noted in the liver. 19 This differs from observations in 7315c cells, where the binding affinity to a single site and IC50 values for adenylyl cyclase inhibitions agreed within one log unit. 18

Our results suggest that the proposed AT1B subtype is coupled to effects typically associated with the AT1 receptor. Ang II inhibited the activity of forskolin-stimulated adenylyl cyclase, and losartan, PD 123319, CGP 42112A was the least potent. In recent studies in our laboratories, we have shown that PD 123319 is a potent antagonist (IC50, <1 nM) of the calcium response to Ang II in mesangial and renal epithelial cells, implying functional coupling of AT1B receptors to signaling pathways regulating intracellular calcium. 17 However, PD 123319 also elicited agonist actions when administered alone, which may account for its reduced potency in functional assays relative to its binding affinity (1,000-fold difference). We cannot rule out the possibility that AT1A receptors also contribute to the adenylyl cyclase response. However, the low potency of losartan in the present study, relative to other studies, suggests that a renal subtype with low affinity for losartan as identified in binding assays may be primarily responsible for adenylyl cyclase inhibition. This novel subtype coupled to adenylyl cyclase inhibition may be the AT1B or a previously unknown subtype.

Multiple subtypes of AT1 have recently been cloned, and Northern blot analyses have revealed that the messenger RNA for AT1B is expressed in aortic vascular smooth muscle cells, lung, and ovary; the messenger RNA for a putative AT1B is expressed in adrenal, uterus, and anterior pituitary; and these two RNAs are expressed in liver, kidney, and spleen at similar levels. 21–25 But this AT1B differs substantially from our AT1B because it did not bind PD 123319 and is pharmacologically indistinguishable from the AT1A subtype.

In the present study using rat mesangial cell membranes, we demonstrate clearly that there are two subtypes of AT1 receptor. AT1A has higher affinity for losartan, whereas AT1B has higher affinity for PD 123319. Two distinct receptor types appear to be coupled to adenylyl cyclase through Gs. One of these receptors appears to be an AT1B subtype, whereas the functional role of AT1B in the kidney remains to be defined.

References


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