CHARACTERIZATION OF THE FUNCTIONAL ANGIOTENSIN II-
RECEPTOR COMPLEX ISOFORM IN RAT LIVER PLASMA
MEMBRANE

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Summary

In rat liver plasma membrane a single angiotensin II (Ang II) binding site (K_d of 3.71 ± 0.33 nM and B_max of 1143.7 ± 83.9 fmol/mg protein) was identified using radioligand binding assay. Pharmacologically, this receptor match with the AT_1 receptor subtypes in term of affinity for the selective antagonist Losartan, and probably with the AT_1A receptor form in term of insensitivity for the antagonist PD123319. Nevertheless, using polyacrylamide gel isoelectric focusing, two 125I-Ang II binding sites migrating to pl 6.8 and 6.5 were found in these membrane preparations. Monophasic displacement of 125I-Ang II bound to isoform migrating at pl 6.8 clearly indicate that this isoform represents a functional Ang II-receptor complex. In contrast, the high concentrations of agonist and peptidic derivates of Ang necessary to displace 125I-Ang II bound to isoform migrating at pl 6.5 indicate that this atypical 125I-Ang II binding site represents a biologically nonfunctional Ang II binding molecule, presumably a nonspecific 125I-Ang II binding site.

Key Words: angiotensin II receptor, angiotensin II receptor subtypes, angiotensin II receptor isoforms, liver plasma membranes

Angiotensin II (Ang II), the principal mediator of the physiological actions of the renin-angiotensin system (RAS), exerts its function by interacting with membrane-bound receptors located on target tissues. Physiological and pharmacological evidences have suggested the existence of a heterogeneous population of Ang II receptors. Two distinct Ang II receptor subtypes, termed AT_1 and AT_2 (1), based on the differences in sensitivity of binding sites to nonpeptide and peptide ligands (2,3), have been described in various species and tissues.

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In addition, numerous evidences based upon biochemical and molecular biology techniques exist to suggest that there may be more than two Ang II receptor subtypes. Two distinct cDNAs encoding AT₁ receptor subtypes (AT₁A and AT₁B) have been cloned and sequenced (4,5), and polyacrylamide gel isoelectric focusing of solubilized receptors showed different Ang II-receptor complexes in various rat tissues (6,7) and human placental syncytiotrophoblast plasma membranes (8), pointing to heterogeneity of Ang II binding sites. In this paper, a characterization of the functional Ang II binding sites expressed in rat liver plasma membrane was carried out using pharmacological and biochemical approaches.

**Material and Methods**

**Preparation of plasma membranes:** Male adult Wistar rats (CRIFFA) were killed by cervical dislocation and livers were quickly removed. Plasma membrane preparations were obtained as previously described by Glossmann et al. (9). In brief, tissue was homogenized in ice-cold 20 mM sodium bicarbonate, containing 1 μg/ml of leupeptin, 1 μg/ml aprotinin, 0.1 μg/ml of bacitracin and 0.1 μg/ml phenylmethanesulphonyl fluoride (Sigma Chemical Co.). The homogenate was filtered through two layers of gauze and centrifuged at 900 g for 15 min at 4°C. The pellet was resuspended in the same buffer, recentrifuged, and the pooled supernatants were centrifuged at 30000 g for 30 min at 4°C. The pellet was resuspended in assay buffer (50 mM Tris-HCl, pH 7.4, containing 120 mM NaCl, 6 mM MgCl₂, 0.1% BSA and proteinase inhibitors).

**¹²⁵I-Ang II binding assay:** Binding of ¹²⁵I-Ang II (specific activity 2200 Ci/mmol; Du Pont-New England Nuclear) to membranes (50 μg protein) was conducted at room temperature for 30 min with 0.1 nM ¹²⁵I-Ang II in the presence or absence of varying concentrations of unlabeled Ang II (30 pM-100 nM, Sigma Chemical Co.) or selectives Ang II receptor subtype antagonists Losartan (30 pM-1 μM), CGP42112A and PD123319 (30 pM-5 μM). Bound and free ¹²⁵I-Ang II were separated by adding 3.0 ml ice-cold saline to the assay tube, followed by vacuum filtration through Millipore filters presoaked with assay buffer. Nonspecific was defined in the presence of 10 μM unlabeled Ang II. The equilibrium dissociation constant (Kᵦ), the inhibiting constant for Ang II receptor antagonists (Kᵢ), and the concentration of receptor sites (Bₘₐₓ) were determined from competition curves using the iterative curve-fitting program "Ligand".

**Isoelectric focusing analysis:** In IEF studies bound and free ¹²⁵I-Ang II were separated by centrifugation at 10000 g for 5 min. The pellet was washed once more with assay buffer, resuspended in 100 μl of Tris buffer, containing 0.5% Triton X-100, and incubated for 20 min at 4°C. The solubilized membrane suspensions, collected after centrifugation at 10000 g for 5 min, were fractionated by IEF as previously described (10). Briefly, polyacrylamide slab gels, containing 12% glycerol and a pH 3.5-10.0 gradient, were prepared using 1% (v/v) ampholine pH 3.5-10.0 and 1.6% (v/v) ampholine pH 5.0-8.0 (LKB). Gels were prefocused at 4°C for 40 min at 2000 V, 20 mA and 20 W, and runs carried out
for 4 h at 2500 V, 20 mA and 20 W. A mixture of natural proteins (Bio-Rad) for pH calibration was used. Gels were then cut into 3 mm width slices and counted in a γ-counter.

**Protein determination:** Protein concentrations were determined according to Lowry et al. (11), using BSA as standard.

**Results and Discussion**

**Binding assays:** Competition binding experiments of $^{125}$I-Ang II to rat liver plasma membrane in the presence of varying concentrations of unlabeled Ang II are depicted in Fig. 1. The data demonstrated the presence of a single binding site for Ang II with a $K_d$ of 3.71±0.33 nM and a $B_{max}$ of 1143.7±83.9 fmol/mg protein. A Hill coefficient of 0.98±0.02 (mean ± SD, n=4) was indicative of a single binding site. In agreement with previous findings (12,13), these results do not support the evidence of second Ang II binding site on rat liver plasma membrane, as several authors have reported (14,15), even when our binding assay conditions were similar.

![Graph showing competition binding of Ang II](image)

**FIG. 1**

Competition studies of $^{125}$I-Ang II binding to rat liver plasma membrane in the presence of varying concentrations of unlabeled Ang II. Each point represents the mean ± SD of four separate experiments. Scatchard analysis of the data is shown in the inset. Nonspecific binding was determined in the presence of 10 μM unlabeled Ang II.

Monophasic displacement of $^{125}$I-Ang II using selective Ang II receptor antagonists are showed in Fig. 2. Losartan, an AT$_1$ receptor antagonist, was capable of competing for this binding site ($K_i$ values of 173.0 ± 50.8 nM), while no changes were observed in the presence of lower concentrations of both AT$_2$ receptor ligands CGP42112A and PD123319. The order of potency for inhibiting $^{125}$I-Ang II binding in rat liver plasma membrane was Ang
The binding-inhibition studies reveal the presence of only one type of Ang II binding site in rat liver membrane preparations, corresponding to the AT$_1$ receptor subtype. In all competition binding experiments the best fit was to one-site model.

Two closely related AT$_1$ receptor forms (AT$_{1A}$ and AT$_{1B}$) encoded by different genes have been identified (4,5). At the moment, it is unclear if only one or both AT$_1$ receptor forms are present in plasma membrane from rat hepatocytes. Tissue measurements of AT$_{1A}$ and AT$_{1B}$ mRNA levels using reverse transcriptase/polymerase chain reactions showed a similar expression level for the two mRNAs in rat liver (5). In contrast, in situ hybridization studies using specific AT$_{1A}$ and AT$_{1B}$ riboprobes for the 3' noncoding region of the mRNAs provided evidence that only AT$_{1A}$ was expressed in rat hepatocytes (16).

Recently, it has been suggested that it is possible to subdivide the AT$_1$ receptors expressed in rat mesangial cells into two subpopulations of receptor termed also AT$_{1A}$ and AT$_{1B}$ (17). The AT$_{1A}$ receptor subtype has a high affinity for Losartan and is PD123319 and CGP42112A insensitive, while AT$_{1B}$ receptor subtype shows a lower affinity for Losartan than for PD123319 and lower affinity by CGP42112A. On these pharmacological basis our data suggest that the AT$_{1A}$ receptor form could be predominantly expressed in the rat liver. It is important to note that the AT$_{1A}$ and AT$_{1B}$ defined here are different from the cloned receptor subtypes.

![FIG. 2](image)

**FIG. 2**

Competition of specific $^{125}$I-Ang II binding to rat liver plasma membrane in the presence of the designated concentrations of unlabeled Ang II (●), Losartan (○), CGP42112A (▲) and PD123319 (▼). Each point represents the mean ± SD of four separate experiments.

*Isoelectric focusing assays:* Previous studies have demonstrated that there are tissue-specific Ang II binding isoform profiles (6,7). Particularly, in the rat liver,
two differently charged isoforms can be found (6) which under the conditions described in this paper correspond to peaks at pI 6.8 and 6.5 (data not shown). \(^{125}\)I-Ang II binding-inhibition analyses to these isoforms in the presence of unlabeled Ang II are showed in Fig. 3.

In membrane preparations from rat lung three specific \(^{125}\)I-Ang II binding complexes have been found (Montiel et al. 1995). The displacement of these isoforms in the presence of unlabeled Ang II showed a dose-dependent manner characteristic of receptor binding. In the present study, the displacement of \(^{125}\)I-Ang II from isoform migrating at pI 6.8 showed also a dose-dependent fashion characteristic of the Ang II receptor (IC\(_{50}\) = 2.38 nM), while higher concentrations of agonist was necessary to displace \(^{125}\)I-Ang II bound at the isoform migrating to pI 6.5 (IC\(_{50}\) = 5.6 \mu M). These results, and our previous observation indicating that the agonist induces an internalization process of the \(^{125}\)I-Ang II-bound protein migrated to pI 6.8 (19), clearly indicate that this isoform represents a functional Ang II-receptor complex.

Although it is generally believed that Ang II is the main biologically active final product of the RAS, recent observations indicate that proteolytic cleavage of Ang I and Ang II may result in the formation of even shorter fragments, such as Ang III (a 2-8 fragment of Ang II), Ang (1-7), and Ang IV (a 3-8 fragment of Ang II). These two latter peptides appear to interact with specific receptors which are not blocked by selective antagonists for either AT\(_{1}\) and AT\(_{2}\) receptors (20,21). In order to investigate the possibility that \(^{125}\)I-Ang II binding site to pI 6.5 could be related with some of these Ang receptors, further competition studies were carried out using a single concentration (10 \mu M) of these Ang derivatives. The results are showed in Fig. 4.
The order of potency for inhibiting $^{125}$I-Ang II binding to pI 6.8 was Ang III > Ang IV > Ang (1-7), and Ang III = Ang IV > Ang (1-7) for $^{125}$I-Ang II bound to pI 6.5.

The absence of displacement at physiological concentrations of agonist and high concentrations of peptidic derivatives of Ang raised the question of the Ang II-related nature of this atypical $^{125}$I-Ang II binding site migrating at pI 6.5. Presumably, this biologically nonfunctional isoform represents a nonspecific $^{125}$I-Ang II binding site.

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**References**


