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Solubilization of an Angiotensin II Binding Site from Rat Liver

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Abstract □ The high affinity binding sites for angiotensin II were solubilized from rat liver membranes by treatment with CHAPS. The binding protein was also partially purified by angiotensin III inhibitor-coupled Affi-gel affinity chromatography. Binding to the intact membranes as well as to the solubilized preparation was specific and saturable. According to the Scatchard plot, the membrane preparations exhibited a single class of high affinity binding sites with a *Kd* of 0.71 nM. The solubilized preparation also showed the presence of a single class of binding sites with less affinity (*Kd* of 14 nM). Meanwhile the competition studies using angiotensin II analogues represented two separate binding sites for angiotensin II and single binding site for antagonist. These latter findings were correlated to the results provided by Garrison's research group. More works are needed to clarify this discrepancy.

Keywords □ Renin-Angiotensin system, angiotensin II receptor (binding site), solubilization, affinity chromatography

The renin-angiotensin system (RAS) is known to be an important hormonal pathway to play a key role in the regulation of cardiovascular homeostasis and Na^+ balance in normal and hypertensive subjects (Searley and Laragh, 1990). The system includes the following components and sources. Renin is synthesized by kidney and secreted into the circulation, where it cleaves the decapeptide angiotensin I from angiotensinogen. Angiotensin I is then converted to the octapeptide angiotensin II by angiotensin-converting enzyme (ACE), and enzyme found in high concentration in the pulmonary vascular bed. The active hormone of the RAS is angiotensin II, the actions of which are thought to be mediated by specific receptors located on various target organs such as adrenal cortex (Glossmann *et al.*, 1974), aorta (Devymck *et al.*, 1974), vascular (Meyer *et al.*, 1972), and uterine smooth muscle (Williams *et al.*, 1974), brain (Bennett and Snyder, 1976) and liver (Lafontaine *et al.*, 1979). In at least some of these organs, the relative potencies of peptide analogs in competing for binding parallel their biological activities, suggesting that these binding sites may be physiologically relevant angiotensin II receptors.

While extensive efforts have been made to characterize the membrane-bound angiotensin II binding sites in various organs of different species, only a few investigators have reported attempts to solubilize and purify the binding protein. Two methods empolyed in these attempts have been salt extraction and treatment with detergents. Salt extraction with 0.45 M potassium chloride was used to solubilize them from the adrenal glomerulosa layer (Brecher *et al.*, 1974). This potassium chloride extract bound [^{3}H]-angiotensin II, but the solubility of the binding sites in the extract was not defined.

Sodium deoxycholate treatment of rabbit aortic membranes reportedly released angiotensin II binding sites, but these sites showed altered peptide specificity and had a lower binding capacity than the native binding sites (Devynck *et al.*, 1974).

Since the early unsuccessful detergent solubiliza-

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tion of angiotension II receptors without the presence of a cross-linker, there have been recent reports of successful detergent solubilization procedures for adrenal receptors. There is one report of successful detergent solubilization of angiotensin II receptors (Change and Lotti, 1981) in which investigators used 1% digitonin to solubilize bovine adrenal cortical angiotensin II receptors, showing no change in peptide specificity in the solubilized preparation. However, there was no follow-up report for further purification of the receptor and physiochemical characterization was not demonstrated.

In this paper, we report on: (1) the successful solublilization of rat liver angiotensin II binding sites by treatment with CHAPS, a zwitterionic detergent; (2) a modified assay method which permits study of the binding properties of the solubilized receptor sites; (3) expermental data which suggests similarities between the solubilized and the membrane-bound angiotensin II binding sites; (4) partial purification of CHAPS-solubilized receptor by utilization of an affinity chromatography.

EXPERIMENTAL METHODS

Materials

Unlabeled angiotensin II was purchased from Bachem (Torrance, CA) and [Sar^1, Ile8]angiotensin II, des-Asp^1-angiotensin II heptapeptide (C7) were from Vega Biochemicals (Tucson, AZ). Zwitterionic detergent, 3-{(3-Cholamidopropyl)dimethyl-ammonio}-1-propanesulfonate (CHAPS), was obtained from Pierce Chemical Co. (Rockford, IL), and Hepes was from Boehringer Mannheim Biochemicals. The following chemicals were bought from Sigma Chemical Co.: bovine serum albumin, Tris, EDTA, chloramine T (N-chloro-p-toluenesulfonamide sodium), sodium metabisulfite, dithiothreitol, protease inhibitor (aprotinin). "Nuflow" glass fiber membrane filters (25 mm diameter) was purchased from Courtaulds Speciality Plastics.

Preparation and storage of monoiodo-angiotensin II

Radioiodination procedure: Angiotensin II was iodinated using the chloramine T procedure (Glossmann *et al.*, 1985): Transfer 7.5 μl of 1 mM angiotensin II into a 5×75 mm glass tube, followed by 7.5 μl of 0.5 M phosphate buffer (pH 7.4) and 3 μl of 1.5 mCi carrier free ^{125}I in 0.1 M NaOH. Add 7.5 μl of a freshly prepared solution of chloramine T (1 mg/ml), and after gently shaking for 30 second incubate for 1 min at room temparature and then stop the reaction by adding 15 μl of sodium metabisulfite (2 mg/ml) in 0.05 M phosphate buffer.

Purification and storage of tracer: To prepare labeled angiotensin II of higher specific activity and maximum bindability, HPLC using DEAE Sephadex-A25 column was employed to resolve the unlabeled peptide and diiodo-angiotensin II from monoiodo [^{125}I]angiotensin II. After injecting the contents of the radioiodination tube into HPLC, initially collected 305 drops/tube and after 10 min started a linear gradient (solution A=0.1 M ammonium acetate buffer, pH 7.8; solution B=0.1 M ammonium acetate+ 30% acetonitrile), and then switched to 30 drops/tube when solution B was reached to 30%.

Fractions containing high radioactivities were pooled and freezed immediately under acetone-dry ice mixture and kept at −70℃. Each frozen aliquot (50 μl) was used only once after thawing.

Preparation of rat liver particulate fractions

Rat liver membranes were prepared from 200 to 300 g male Wistar rats according to the protocol (through step 10) of Pohl (Pohl SL, 1976) which is based on the orignal procedure of Neville (Neville DM Jr., 1968). Membranes were stored in a deep freezer (−70℃) for up to 6 months without loss of binding activity.

Radioligand binding assay in particulate fraction

Assay mixtures composed of 25 mM Hepes (pH 7.5), 3 mM $MgCl_2$, 1 mM EDTA, 1% bovine serum albumin (BSA) and 1 nM of ^{125}I-labeled angiotensin II. The binding reaction was initiated by the addition of 10 μl of liver membrane extract (0.5-1 mg protein) to 90 μl of the assay mixture and incubated for 20 min at 32℃. The reaction was stopped by the addition of 2 ml of ice-cold 25 mM Tris-HCl buffer containing 2 mM $MgCl_2$. ^{125}I-angiotensin II bound protein was then separated from free ^{125}I-angiotensin II by vacuum filtration on glass fiber filters which have been presoaked in a 1% of BSA solution. Specific binding of ^{125}I-angiotensin II to its receptor was determined by subtraction of nonspecific binding, which was calculated in the presence of excess amount of unlabeled angiontensin II (10 μM), from total binding.

Solubilization of angiotensin II binding protein

The membrane extract was spun down for 20 min at 40,000 rpm to concentrate and the precipitate was resuspended in 1 ml of 25 mM Hepes buffer. The tube contained 485 μl of concentrated membrane extract, 300 μl of 100% glycerol, 150 μl of 66% sucrose, 25 μl of 1 M Hepes and 40 μl of 0.25 M of CHAPS in 1000 μl of total volume. The tube sitting on ice was occasionally vortexed for 30 min and centrifuged at 40,000 rpm for 60 min. Remove and save a supernatant, and the supernatant was diluted 5 times with 25 mM Hepes and 100 mM NaCl.

Binding assay in solubilized fractions

The assay mixture included following components: 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 0.1% BSA, 10 μg/ml aprotinin, 2.5 mM CHAPS, and ^{125}I-angiotensin II. To 80 μl of the assay mixture, added 20 μl of CHAPS extrat and the tubes were incubated for 20 min at room temperature. Stop the reaction by adding 320 μl of 0.125% gamma-globulin and 800 μl of 7.5% polyethylene glycol (PEG). Filter through oxoid filter, which was presoaked with 10% PEG, and wash the tubes three times with 10% PEG.

Partial purification of solubilized angiotensin II binding protein

Affi-Gel 10 affinity column was prepared as followings: Either angiotensin II or angiotensin III inhibitor (des-Asp^1-Ile^8-A II), both dissolved in DMSO, was incubated with Affi-Gel at room temperature for 1.5 hrs (2 mg ligands added to 1 ml of gel). After the coupling reaction was done, 1 M ethanolamine prepared in 25 mM Hepes was added and then incubated for 2 hrs in order to block any remaining active groups. Finally to wash away excess ligand, shaked the gel with 9 M urea for 3 hrs. Before loading CHAPS extract to this column, it was equilibrated with starting buffer containing 25 mM Hepes, 1 mM EDTA, 5 mM $MgCl_2$, 10% glycerol and 5 mM CHAPS.

Once the affinity column was equilibrated, the protein solution to be purified flowed into it and did same procedure several times at 4℃, and then collected "flow through" for later use. Next, the column was washed with buffer containing 100 mM NaCl until the effluents were free of materials absorbing UV light at 280 nm. Elution was carried out

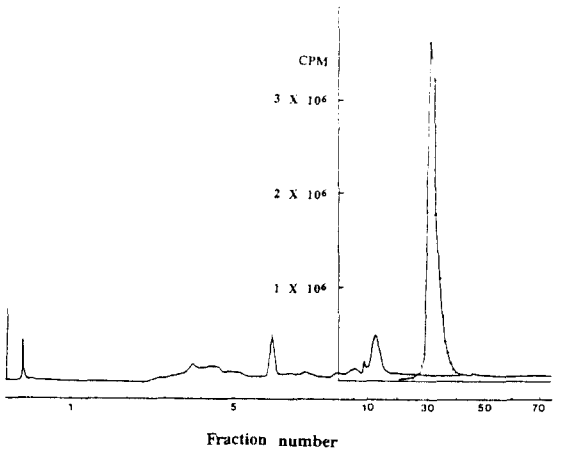

Fig. 1. Purification profile of monoiodo-angiotensin II by using DEAE Sephadex-A25 HPLC column. Highly specific radiolabeled ligand was started to come out from fraction 30, and fractions containing high radioactivity (over 1.7 million cpm value) were pooled together.

at room temperature with the elution buffer containing either 20 μM angiotensin III inhibitor or 3 M NaCl. To remove angiotensin III inhibitor or to reduce salt concentration, the eluate was subjected to dialysis thoroughly. The dialysate was then concentrated in an Amicon ultraconcentration apparatus equipped with a PM10 filter.

Protein concentration determination

The Lowry method (Lowry *et al.*, 1951) was employed using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Preparation and purification of the monoiodo[^{125}I]angiotensin II: The preparation of monoiodinated angiotensin II was achieved by the chloramine T method, and however diiodinated angiotensin II which has low biological activity is also formed during the iodination procedure, and has to be carefully separated from the monoiodinated species. We employed the DEAE Sephadex-A25 column to perform this separation: fractions were collected into 80 test tubes and we counted 5 μl of each fraction (actually from fraction number 20 to 50), and fractions containing over 1.7 million cpm were pooled together (Fig. 1). Active fractions were then divided

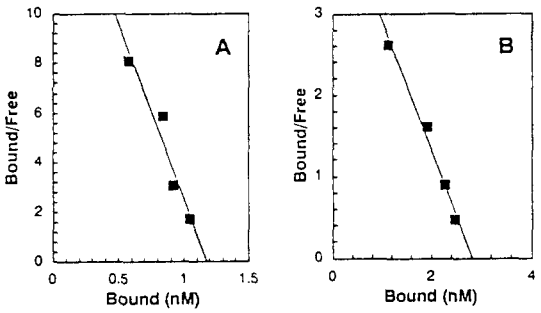

Fig. 2. Scatchard analysis for angiotensin II receptor of rat liver membrane. Plot A is for ^{125}I-angiotensin II and plot B for ^{125}I-SIAng II.

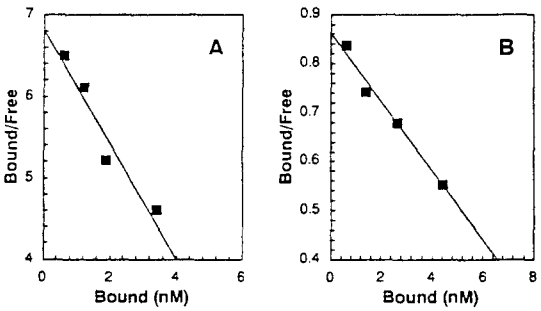

Fig. 3. Scatchard analysis for CHAPS-solubilized angiotensin II receptor. Plot A is for ^{125}I-angiotensin II and plot B for ^{125}I-SIAng II.

into 50 μl aliquot and each aliquot was freezed under dry ice-acetone mixture and then kept at −70°C.

Tritium-labeled angiotensin II has also been utilized to detect angiotensin II receptors (Devynck and Meyer, 1976). Although the tritiated hormone has higher biological activity than the monoiodinated ligand, its relatively low specific activity (30-60 μCi /μg) makes it less suitable for detecting high affinity binding sites. One potential problem with the use of iodine-labeled *vs* tritium-labeled angiotensin II is that the ^{125}I is introduced primarily at the Tyr^4 residue, and could perhaps alter the binding characteristics of the molecule. However, comparisons of the binding obtained with each type of ligand showed receptor numbers and affinities which were in good agreement (Glossman *et al.*, 1974; Gunther *et al.*, 1980).

Determination of binding parameters for angiotensin II and its analogue (Sar^1 Ile^8-Angiotensin II: SIAng II): Fig. 2 represents typical saturation isotherms for ^{125}I-angiotensin II and ^{125}I-SIAng II to rat liver membranes. The data, expressed as Scatchard plots, yielded apparent straight lines indicative of one class of binding sites. This finding was confirmed by computer analysis of the data which yielded a best fit to a single class of sites with Kd=0.71 nM and a number of sites N=1178 fmol/mg protein for angiotesin II and Kd=0.63 nM, N=2813 fmol/mg protein for SIAng II.

Scatchard plot constructed by using CHAPS-solubilized preparation is shown in Fig. 3. A single class of binding sites in solubilized receptor was also revealed, with a dissociation constant of 14 nM and a B_{max} of 48 fmol/mg protein for angiotensin II and Kd of 14 nM and a B_{max} of 1488 fmol/mg protein for SIAng II.

The affinity of the solubilized binding sites for angiotensin II is significantly less than that of membrane preparations. This reduced affinity may be due to physical manipulation of the receptor protein during solubilization or to a slight conformational change in the solubilized state. A similar observation has been noted for another solubilized preparation (Chang and Lotti, 1981).

Displacement of bound ^{125}I-angiotensin II or ^{125}I-SIAng II by analogs of angiotensin II: First, SIAng II and angiotensin II itself were tested for their ability to inhibit the binding of ^{125}I-angiotensin II to intact rat liver membrane. As shown in Fig. 4, the displacement patterns for SIAng II and angiotensin II are different indicating that there may be two separate binding sites for agonist and one single site for antagonist (SIAng II). Somehow SIAng II seemed to be equipotent in inhibiting the binding of ^{125}I-angiotensin II to receptor protein as effectively as unlabeled angiotensin II as far as high affinity sites are concerned. The fact that angiotensin II binding protein has two separate binding sites for agonist was already ascertained by Garrison's research group (Campanile *et al.*, 1982), and our present study partially confirmed the binding characteristics of angiotensin II receptor protein. To solve the discrepancy in agonist binding sites, we may need more experimental points to construct the Scatchard plot, since four experimental points employed in Fig. 2 and 3 may not enough to pass over reflection point, which is necessary to draw mathematically reasonable binding parameters of the receptor.

Second, angiotensin III inhibitor was also tested

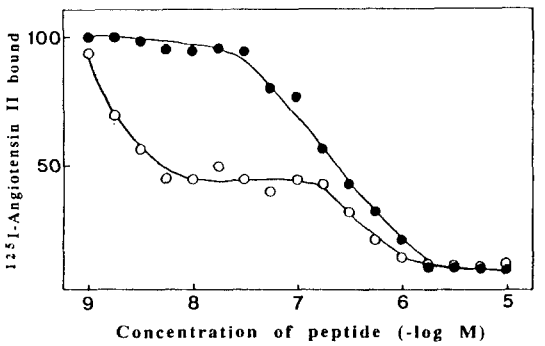

Fig. 4. The binding profiles of different unlabeled agents in competing for radioligand binding is usually expressed by calculating the IC_{50} value, i. e., the concentration of competitor that reduces specific radioligand binding detected in the absence of competitor (i. e., 100%) by half. The closed circles represent unlabeled SIAng II and open circles for unlabeled angiotensin II.

Table I. Effects of several detergents on binding of radiolabeled angiotensin II to rat liver membrane

Detergents used	% Binding
CHAPS only	100
+0.001% digitonin	91
+0.005% digitonin	89
+0.05% digitonin	48
+0.01% digitonin	32
+0.05% cholate	29
+0.1% cholate	21
+0.001% lubrol PX	43
+0.01% lubrol PX	10

for its potency to inhibit the binding of ^{125}I-SIAng II to solubilized membrane, and by using Cheng and Prusoff's equation the inhibition constant of angiotensin III inhibitor was determined to be a 272 nM, which was 20 times less potent than that of SIAng II. The fact that angiotensin III inhibitor represented relatively low affinity for angiotensin II receptor prompted us to utilize this angiotensin II analogue as an affinity column-coupling ligand, since high affinity ligand may not be suitable for affinity column-coupling agent in terms of consideration of receptor recovery.

Effects of several detergents on binding of radiolabeled angiotensin II to CHAPS-solubilized rat liver membrane: 2 m*l* of membrane batch L46 was thawed and centrifuged at 40 K for 30 min, then pellet was resuspended in 0.5 m*l* of 25 mM Hepes buffer. This concentrated membrane solution was used in CHAPS solubilization procedure. Binding assay was performed without and with several differet detergents to evaluate their effects on binding property. As seen in Table I. Lubrol PX was the worst detergent which angiotensin II receptor is unhappy with.

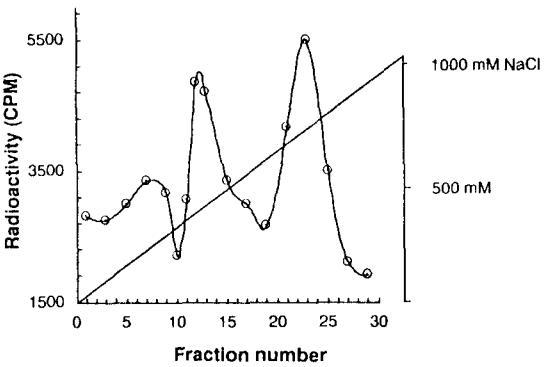

Fig. 5. Elution profile of angiotensin III inhibitor-coupled affinity column chromatography. Fractions of 0.8 m*l* volume were collected and assayed for angiotensin II recetor activity as described in the experimental methods.

Partial purification of CHAPS-solubilized angiotensin II binding protein: CHAPS-solubilized particulate fraction was loaded on angiotensin III inhibitor-coupled Affi-Gel 10 affinity column (coupling ability of angiotensin III inhibitor was slightly better than that of angiotensin II; 96% *vs* 89%). As an eluant, first we tried 20 μM of angiotensin III inhibitor or 3 M of NaCl. The percent recoveries of angiotensin II binding protein were 5 for angiotensin III inhibitor and 22 for NaCl. Secondly, a salt gradient as eluent (from 100 to 1000 mM NaCl) was employed. At 400 mM of NaCl the binding protein started to elute and two peaks containing active fractions were consecutively appeared as shown in Fig. 5. In the latter case the eluate represented approximately 10% recovery.

At this time we think that salt gradient or high concentration of salt alone may be good enough to recover angiotensin II binding sites. Other elution procedure will also be tried in the future.

In conclusion, we report the successful solubilization of angiotensin II binding protein from rat liver. This is the first step toward purification of the active

receptor protein which will enable us to study molecular properties and physiological relevance in greater detail.

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