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Biochemical Analysis of Solubilized Angiotensin II Receptors from Murine Neuroblastoma N1E-115 Cells by Covalent Cross-linking and Affinity Purification

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SUMMARY

Angiotensin II (Ang-II) receptors were solubilized from differentiated N1E-115 neuroblastoma cell membranes with the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), whereas other detergents, such as digitonin, sodium cholate, and Triton X-100, were much less effective. Binding of ^{125}I-Ang-II or the antagonist ^{125}I-Sar^{1},Ile^{8}-Ang-II to 1% CHAPS-solubilized membranes was saturable and of high affinity. Moreover, these solubilized receptors retained the pharmacological specificity characteristic of particulate receptors. Covalent cross-linking of ^{125}I-Ang-II to either particulate or solubilized membrane fractions, with the homobifunctional cross-linker disuccinimidyl suberate, followed by size exclusion chromatography or sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, resulted in the identification of the same two distinct ^{125}I-Ang-II binding entities, with approximate molecular masses of 111 kDa and 68 kDa. The estimated molecular weights of the Ang-II binding sites in differentiated N1E-115 cells are in good agreement with the molecular weights obtained previously from solubilized rat brain membranes, suggesting that the N1E-115 Ang-II receptors are similar to those present in the brain. Finally, solubilized N1E-115 membranes could be purified by Ang-II affinity chromatography, resulting in only a single protein (66 kDa), which retained its ability to specifically bind ^{125}I-Ang-II.

Ang-II, the biologically active peptide of the renin-angiotensin system, has a broad range of physiological effects, including the maintenance of blood pressure and fluid homeostasis. High affinity receptors that appear to mediate these effects have been identified in a number of peripheral tissues such as the adrenal cortex (1–5), liver (6–10), heart (11, 12), mesenteric artery (13), aorta (14, 15), uterus (16, 17), bladder, and pituitary (18). Moreover, investigations using photoaffinity labels (19–24), covalent cross-linkers (9, 12, 25), affinity chromatography (26–28), and immunoaffinity purification (27, 29) have begun to provide information about the structure of Ang-II receptors within these tissues.

In addition to the well characterized actions of Ang-II in the periphery, it is now clear that the central nervous system is an important target organ for this peptide (30–32). In this regard, radioligand binding studies and autoradiographic techniques have clearly established the presence of Ang-II receptors in the brain (33–39). Moreover, recent experiments using newly developed nonpeptidic antagonists have indicated that there are multiple Ang-II receptor subtypes (40–43) and that the receptor subtypes present on most peripheral cells may differ from those located in the brain (44, 45). Therefore, continued progress in understanding the molecular mechanism of Ang-II action in the brain requires that Ang-II receptors be purified and characterized from neuronal cells. However, whereas extensive efforts have been made to biochemically characterize peripheral Ang-II receptors, only a few investigators have reported attempts to solubilize and purify the binding protein from neuronal membranes (46–49).

In this study, we have used the N1E-115 neuroblastoma cell line to study the biochemical characteristics of neuronal Ang-II binding sites. In general, these cells have proven to be very useful models of neurons, possessing many of the biochemical, physiological, and morphological properties of differentiated neurons in culture. The use of these differentiated cells eliminates some of the limitations commonly associated with the exclusive use of brain membranes, such as low receptor density, heterogeneous cell population, and tissue availability. Previously we have reported that N1E-115 cells possess Ang-II

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ABBREVIATIONS: Ang-II, angiotensin II; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; 1,10-PA, 1,10-*o*-phenanthroline; DSS, disuccinimidyl suberate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; BCA, bicinchoninic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

receptors that are up-regulated by *in vitro* differentiation (50, 51). In the present study, we report that high affinity Ang-II receptors can be solubilized from N1E-115 cell membranes by the synthetic detergent CHAPS and that the solubilized material contains two distinct Ang-II binding sites with dissimilar molecular weights. Moreover, these molecular weights are comparable to those of Ang-II binding sites that we recently isolated from rat brain membranes (48), suggesting that N1E-115 cells are a useful model in which to pursue further characterization of neuronal Ang-II receptors. Affinity purification of these binding sites further suggested that the 66-kDa protein represents the smallest protein that can specifically bind ^{125}I-Ang-II. These results have been published previously in preliminary form (49).

Experimental Procedures

Materials

Monoiodinated ^{125}I-Ang-II or ^{125}I-Sar^1,Ile^8-Ang II (2200 Ci/mmol) were purchased from DuPont-New England Nuclear (Boston, MA). Unlabeled Ang-II, HEPES, Na_2EDTA, glycerol, aprotinin, 1,10-PA, Coomassie Brilliant Blue, and polyethylenimine were obtained from Sigma (St. Louis, MO). Reacti-Gel (6×) and DSS were from Pierce, and the TSK 3000 SW (300 mm) size exclusion column was from Beckman. All other chemicals and reagents were purchased from Fisher Scientific (Pittsburgh, PA) and were of the highest obtainable grade.

Cell Culture Techniques

N1E-115 cells were grown for 3 days in T75 or T150 plastic plates in Dulbecco's modified Eagle's medium with high glucose, supplemented with 10% fetal calf serum, 2.5 μg/ml fungizone, 50 units/ml penicillin, and 50 μg/ml streptomycin, at 37° in a humidified 5% CO_2/95% O_2 atmosphere. Cell passages 15–30 were used throughout this study. In order to increase the density of Ang-II receptors, N1E-115 cells were differentiated by replacement of normal growth medium with medium containing 0.5% fetal calf serum and 1.5% dimethyl sulfoxide, for 3–4 days before harvesting (51). Differentiated N1E-115 cells were used for all experiments.

Preparation of Cell Membranes, Solubilization, and Affinity Purification

Cultured N1E-115 cells were placed on ice, the medium was removed, and the cells were rinsed three times in ice-cold 20 mM Tris·HCl, pH 7.4, 150 mM NaCl, and incubated for 10–15 min at 4° in 20 mM Tris·HCl, pH 7.4, without NaCl. The lysed cells were then removed with a rubber policeman and homogenized with a Dounce homogenizer. The homogenate was centrifuged at 1000 × *g* for 5 min, and the supernatant was collected and centrifuged at 48,000 × *g* for 30 min. The membrane pellet was resuspended in either 50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$ (buffer A), for subsequent solubilization and radioligand binding analysis, or 50 mM HEPES, 5 mM EDTA, 5 mM Na_2EGTA, 120 mM NaCl, pH 7.4 (buffer B), for cross-linking studies. The membranes were recentrifuged for 30 min at 48,000 × *g*, and the pellet was subsequently frozen at −70° until use. These washes were necessary to eliminate any trace of soluble Ang-II binding activity associated with the cytosol of N1E-115 cells (52). For solubilization, the membrane pellets in buffer A were resuspended in a solubilization buffer containing 25 mM Na_2HPO_4, 5 mM EGTA, 5 mM Na_2EDTA, 25% glycerol, 0.5 M KCl, 0.3 trypsin inhibitor units/ml aprotinin, 100 μg/ml 1,10-PA (buffer C), and 1% CHAPS, pH 7.4, at a protein concentration of 6–8 mg/ml, as determined by the method of Bradford (53). The homogenate was solubilized for 1 hr at 4°, on an orbital shaker, and subsequently centrifuged at 105,000 × *g* for 90 min at 4°. The resulting supernatant was filtered through a 0.2-μm filter and stored at −70° until use.

The affinity column was prepared according to the manufacturer's instruction (Pierce) for the Reacti-Gel (6×). Approximately 8–10 ml of gel slurry were washed extensively in a Buchner funnel on Whatman No. 1 filter paper. The gel (approximately 1.5 ml of settled gel) was removed from the filter paper and added to 3 ml of coupling buffer (125 mM Na_2CO_3, pH 9.5) containing 16 mg of unlabeled Ang-II. The mixture was incubated for 48 hr at 22°, the gel was poured into a column (10 cm × 0.6 cm), and the eluant was collected and quantitatively assayed for unbound Ang-II. The column was washed with 2 liters of double-distilled water and subsequently stored in water containing 0.02% sodium azide, at 4°. Before affinity chromatography, the column was warmed to room temperature and washed with 10 ml of double-distilled water. The column was then equilibrated, using a flow rate of 12 drops/min (approximately 0.3 ml/min), with 4 ml of mobile phase consisting of buffer C and 0.1% CHAPS. After the column was equilibrated, 3 ml of solubilized material (6–9 mg of protein) were loaded and passed through the column at a flow rate of 12 drops/min. The column eluant was collected in a plastic container that was placed on ice. The collected solubilized proteins were reloaded onto the column and, after the proteins had passed through the column for the third time, the column was washed with 6 ml of buffer C. The eluant was collected in 0.5-ml fractions using a Pharmacia-LKB ReadyFrac fraction collector (set for drop counts), and each fraction was monitored for proteins on a UV spectrophotometer set at 280-nm wavelength (Spectronic 1001). Residual proteins in the column not bound to the gel eluted completely in the first three or four fractions (1.5–2 ml of buffer).

Two different protein elution protocols were used to remove the purified proteins from the affinity column. The first protocol involved the use of excess amounts of unlabeled Ang-II to displace the proteins bound to the column. The second protocol involved the use of buffer C, warmed up to 37°, to dissociate the proteins from Ang-II that was covalently linked to the column. Although the first protocol was effective in removing the proteins from the affinity column, the second protocol yielded similar protein extraction efficiency as the first and yet was less time consuming and more economical. About 5 ml of buffer C (at 37°) were added to the column and incubated for 15 min. Subsequently, 12 ml of buffer C were added to the column and the mixture was passed through at 12 drops/min. The collected eluant was transferred into a Centriprep 30 concentrator (Amicon) and concentrated to approximately 1.5 ml, which was frozen in small aliquots at −70° until use. The affinity-purified Ang-II-binding proteins exhibited ^{125}I-Ang-II binding activity after 1 month of storage at −70°.

In order to determine the rate of removal of Ang-II binding sites during affinity purification, 3 ml of solubilized tissue were first checked for protein content (see below) and 200 μl were extracted from the 3-ml pool and evenly distributed over four tubes, two of which contained unlabeled Ang-II to define nonspecific binding. This procedure was then repeated each time the solubilized cell membranes were passed through the affinity column. All tubes received 30 μl of buffer C containing ^{125}I-Ang-II (500,000 cpm). After the binding reached equilibrium (i.e., 60 min at 25°), the assays were terminated, on glass fiber filters, by vacuum filtration. The protein content of the affinity-purified material was determined with the BCA protein assay reagent (Pierce), by following the manufacturer's instructions for enhanced protein detection. The BCA protein assay was not affected by 1% CHAPS.

Receptor-Ligand Binding Assays

Radioligand binding assays were performed essentially as described previously (50, 51). One hundred microliters of particulate or solubilized material were added to 150 μl of buffer B with 0.2% heat-inactivated BSA, aprotinin, and 1,10-PA and containing ^{125}I-Ang-II or ^{125}I-Sar^1,Ile^8-Ang-II (specific activity, 2200 Ci/mmol; NEN/DuPont). The reaction was terminated after 60-min incubation at 25° by rapid dilution and rinsing of the assay tubes three times in 5 mM Tris·HCl, pH 7.4, 150 mM NaCl, followed by filtration through 0.1% polyethylenimine-presoaked glass fiber filters, using a Skatron cell harvester. Dried filters

were counted for 1 min in a LKB γ scintillation counter at 80.5% efficiency. In saturation isotherms, ^{125}I-Ang-II concentrations ranged from 0.1 to 2.2 nM, and nonspecific binding was defined in the presence of 1 μM unlabeled Ang-II. Competition curves, using 0.3 nM ^{125}I-Sar^1,Ile^8-Ang-II and 8–14 concentrations of unlabeled competitor spanning at least 5 orders of magnitude, were performed using the binding assay conditions described above. The Ang-II-related peptides examined were Ang-I, Ang-II, Ang-III, Sar^1-Ang-II, and Sar^1,Ile^8-Ang-II. In all experiments, binding data were analyzed by LIGAND.

^{125}I-Ang-II or ^{125}I-Sar^1,Ile^8-Ang-II (DuPont-New England Nuclear) was routinely purified before use, in order to remove the BSA present in the buffer containing the radiolabel. The lyophilized radioligand was resuspended in 50% acetonitrile and water and incubated for 2 hr at room temperature. The mixture was then passed through a solid-phase preparatory column (Baker 10-SPE C_{18}), which was preequilibrated with a mobile phase containing 0.1 M phosphate buffer, adjusted to pH 3 with triethylamine, and 50% acetonitrile (mobile phase B). The entire column was centrifuged at 1000 × g for 3 min at 4°. The column was washed one time with 2 ml of mobile phase B, and the eluant was collected and dried down under vacuum in a Speed Vac (Savant). The radiolabel was either used immediately or stored lyophilized at −20° for no more than 2 days before use. This procedure effectively removed all BSA, as determined by gel filtration on a TSK 3000 SW size exclusion column.

Covalent Cross-linking of ^{125}I-Ang-II with DSS

Approximately 0.8–2 nM purified ^{125}I-Ang-II (1–2 × 10^6 cpm) was added to either washed N1E-115 cell membranes (0.8–1.4 mg of protein), CHAPS-solubilized fractions (150–700 μg of protein), or affinity-purified Ang-II-binding proteins (0.5–5 μg), in the presence of protease inhibitors. After binding had reached equilibrium, ^{125}I-Ang-II was cross-linked to its binding sites with the homobifunctional cross-linker DSS (9 mM final concentration), which was predissolved in dimethyl sulfoxide at 100 times the final assay concentration. The samples were incubated for 30 min at room temperature, and the cross-linking reaction was terminated by addition of 1 M Tris buffer (pH 8) (final assay concentration, 250 mM) to each tube. In a series of preliminary studies, we determined that, under optimal conditions, 9 mM DSS covalently cross-linked approximately 5% of the bound ^{125}I-Ang-II to particulate or solubilized membrane fractions. Varying the concentration of DSS, the time of incubation, or the presence of unbound ligand during the cross-linking reaction did not result in the detection of additional proteins (data not shown).

Size Exclusion Chromatography of Ang-II Receptors

Solubilized as well as affinity-purified N1E-115 cell membranes proteins were fractionated by gel filtration on a TSK 3000 SW (300-mm) (Beckman) column, which had been equilibrated in a mobile phase containing 25 mM HEPES, 0.5 M NaCl, and 0.1% CHAPS (mobile phase A). A guard column (DuPont Zorbax Reliance 5-μm cartridge column), which was placed in line with the TSK 3000 SW column, provided physical filtration and chemical adsorption protection. The columns were attached to a Waters high performance liquid chromatography gradient system consisting of two Waters 501 pumps, an automatic gradient controller, one liquid chromatography spectrophotometer (Lambda Max 481), and a Waters 740 data module. In all chromatographic experiments, the flow rate was set at 0.5 ml/min, and the column was run at room temperature. The fractions were collected in a Pharmacia-LKB fraction collector, and the fraction size was set at 0.25 ml/min. The column eluant was monitored with the spectrophotometer set at 280 nm. All samples were filtered through a 0.2-μm filter before column injection. All proteins eluted between 10 and 22 min, and the free label eluted between 27 and 36 min.

^{125}I-Ang-II prebound to particulate membrane fraction. Aliquots of N1E-115 cell membrane suspension (0.8–1.4 mg of protein/ml) in buffer B were prelabeled with ^{125}I-Ang-II (1–1.4 nM), 0.3 trypsin inhibitory units/ml aprotinin, and 100 μg/ml 1,10-PA, in the presence or absence of unlabeled Ang-II. After binding reached equilibrium, ^{125}I-Ang-II was covalently cross-linked with DSS and the membranes were solubilized with 1% CHAPS. Aliquots of the 105,000 × g supernatant were then injected onto the TSK 3000 SW size exclusion column.

^{125}I-Ang-II bound to solubilized membrane proteins. Aliquots of solubilized membrane proteins containing 150–700 μg of protein were incubated with ^{125}I-Ang-II (1–1.4 nM), cross-linked, and chromatographed as described above for particulate membrane preparations. In the experiments in which individual fractions were screened for ^{125}I-Ang-II binding, aliquots of the solubilized cell membranes (100–150 μg) were repeatedly injected onto the size exclusion column equilibrated with mobile phase A. Each fraction was then split into two equal volumes, to assess total and nonspecific binding.

^{125}I-Ang-II-bound to affinity-purified membrane proteins. One hundred microliters of a buffer containing 25 mM HEPES, 10 mM Na_2HPO_4, 5 mM Na_2EDTA, 500 mM KCl, 10% glycerol, 0.1% CHAPS, and 1.5–2 nM ^{125}I-Ang-II were added to affinity-purified proteins (5–10 μg of protein). After the binding, the radioligand was cross-linked and the incubation was chromatographed with the size exclusion column.

SDS-PAGE

Gel electrophoresis for all preparations was performed essentially as described by Laemmli (54). Samples were incubated in a sample buffer containing 0.5 M Tris·HCl, pH 6.8, glycerol, 10% SDS, 0.05% (w/v) bromophenol blue, and 2-mercaptoethanol, unless otherwise stated. The samples were boiled for 3–4 min and then put on a Bio-Rad discontinuous slab gel consisting of a 4% polyacrylamide stacking gel and 10% polyacrylamide separation gel. The gels either were stained with 0.125% Coomassie Brilliant Blue in methanol/acetic acid/water (50:10:40, by volume) for 60 min and then destained with methanol/acetic acid/water (50:10:40, by volume) or were silver stained as previously described (55). Autoradiography of the dried gels was performed by exposing them to Kodak XAR-5 film at −70°, typically for 6–14 days.

^{125}I-Ang-II bound to particulate membrane fraction. Membrane aliquots suspended in buffer B (0.3–1.0 ml) were incubated with ^{125}I-Ang-II (1–1.4 nM) in the presence and absence of unlabeled agonist. After equilibrium was reached, unbound ligand was removed by centrifugation at 48,000 × g for 20 min at 4°. The pellets were washed one more time by resuspension and centrifugation in buffer B and were subsequently cross-linked with DSS before SDS-PAGE.

^{125}I-Ang-Ii bound to solubilized membrane proteins. Aliquots of solubilized membranes, containing 150–700 μg of protein, were incubated with ^{125}I-Ang-II (1–1.4 nM) in the presence or absence of unlabeled agonist. The incubations were subsequently cross-linked with DSS, and the free label was removed from the bound fraction by the dextran-coated charcoal method (9). Briefly, dextran-coated charcoal slurry was prepared by addition of 1 g of prewashed charcoal and 0.1 g of dextran T70 to 20 ml of 100 mM HEPES buffer, pH 7.5, and the mixture was stirred overnight at 4°. One milliliter of the slurry was then added to each assay tube. The tubes were incubated for 10 min on ice and then spun down at 1000 × g for 20 min. The clear supernatant, containing the covalently cross-linked ^{125}I-Ang-II, was concentrated with a Centricon 30 concentrator (Amicon) and subsequently used for SDS-PAGE.

^{125}I-Ang-II bound to affinity-purified membrane proteins. Aliquots of affinity-purified proteins (5–10 μg of protein) were incubated in a buffer containing 1.5–2 nM ^{125}I-Ang-II, 25 mM HEPES, 10 mM phosphate, 5 mM Na_2EDTA, 500 mM KCl, 10% glycerol, and 0.1% CHAPS, for 60 min at room temperature. After the binding reaction, the radioligand was covalently cross-linked with DSS, and the entire incubation was injected onto the TSK 3000 SW size exclusion column equilibrated with mobile phase A. The radiolabeled peaks were collected, pooled, concentrated, and subsequently used for SDS-PAGE.

Results

Assessment of Optimum Solubilization Conditions

In a preliminary series of studies, solubilization of Ang-II binding sites from N1E-115 membranes was attempted with a number of detergents, including CHAPS, digitonin, Nonidet P-40, sodium cholate, and Triton X-100. The efficiency of solubilization of specific ^{125}I-Ang-II or antagonist ^{125}ISar^1,Ile^8-Ang-II binding was highest with CHAPS. Further studies indicated that optimal solubilization was achieved by incubation of 6–8 mg/ml membrane proteins for 1 hr at 4° with 1% CHAPS (Fig. 1). Under these conditions, 40–55% of specific ^{125}I-Ang-II binding and approximately 50% of total protein were solubilized. Similar yields of Ang-II binding proteins were obtained when binding sites were labeled with ^{125}I-Sar^1,Ile^8-Ang-II.

Radioligand Binding Studies in Solubilized N1E-115 Membrane Preparations

Saturation isotherms and subsequent Scatchard analysis indicated that the binding of ^{125}I-Sar^1,Ile^8-Ang-II to solubilized N1E-115 membranes was saturable and of high affinity, exhibiting a K_D of 1.03 nM and a B_{max} of 122 fmol/mg of protein (Fig. 2). CHAPS also solubilized binding sites for the agonist ^{125}I-Ang-II, with similar efficiency (data not shown). Moreover, the rank order potency of ^{125}I-Sar^1,Ile^8-Ang-II binding exhibited in intact membranes (50, 51), Sar^1-Ang-II > Sar^1,Ile^8-Ang-II > Ang-II > Ang-III ≫ Ang I, was retained in the solubilized membranes (Fig. 3). The K_i values obtained from these competition curves are summarized in Table 1. As expected, the unrelated peptide bradykinin did not compete for ^{125}I-Sar^1,Ile^8-Ang-II-labeled sites in solubilized membranes.

Size Exclusion Chromatography of the ^{125}I-Ang-II Binding Sites Derived from N1E-115 Cell Membranes

Particulate membrane fraction. N1E-115 cell membranes (0.8–1.4 mg protein/ml) were prelabeled with ^{125}I-Ang-II (1–1.4 nM), subsequently cross-linked with DSS, and then solubilized with 1% CHAPS. Aliquots of the 105,000 × *g* supernatant were injected onto the TSK 3000 SW size exclusion column, and two radioactive peaks were eluted from the column (Fig. 4). Both radiolabeled peaks specifically bound ^{125}I-Ang-II, as determined by subtraction of nonspecific chromatographic counts from total counts. The estimated molecular weights for the two peaks were 125,000 ± 12,000 (peak a) and 73,000 ± 4,500 (peak b) (mean ± standard deviation; five experiments). The nonspecific binding represented no more than 10% of total binding.

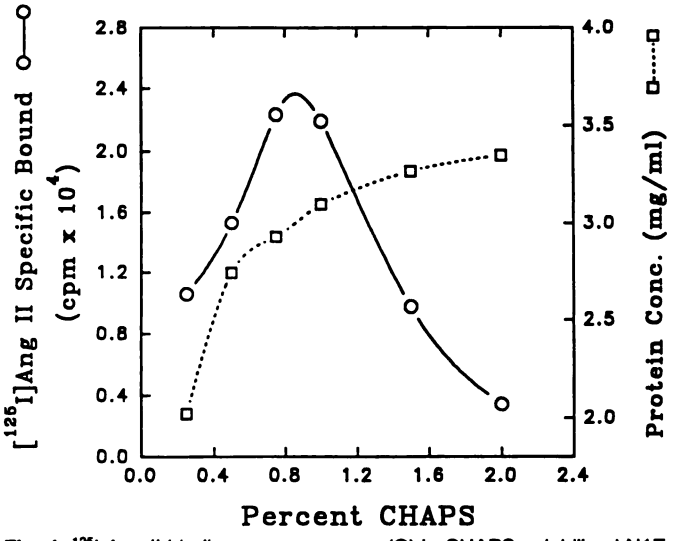

Fig. 1. ^{125}I-Ang-II binding recovery curve (○) in CHAPS-solubilized N1E-115 cell membranes. Cell membrane homogenates were incubated with CHAPS at various concentrations (0.25–2%) for 60 min at 4°, and the resulting supernatant was then used for binding assays with ^{125}I-Ang-II. Protein concentration in each solubilization tube (□) was monitored by the method of Bradford (53). The data presented are the means of triplicate determinations and are representative of three separate experiments.

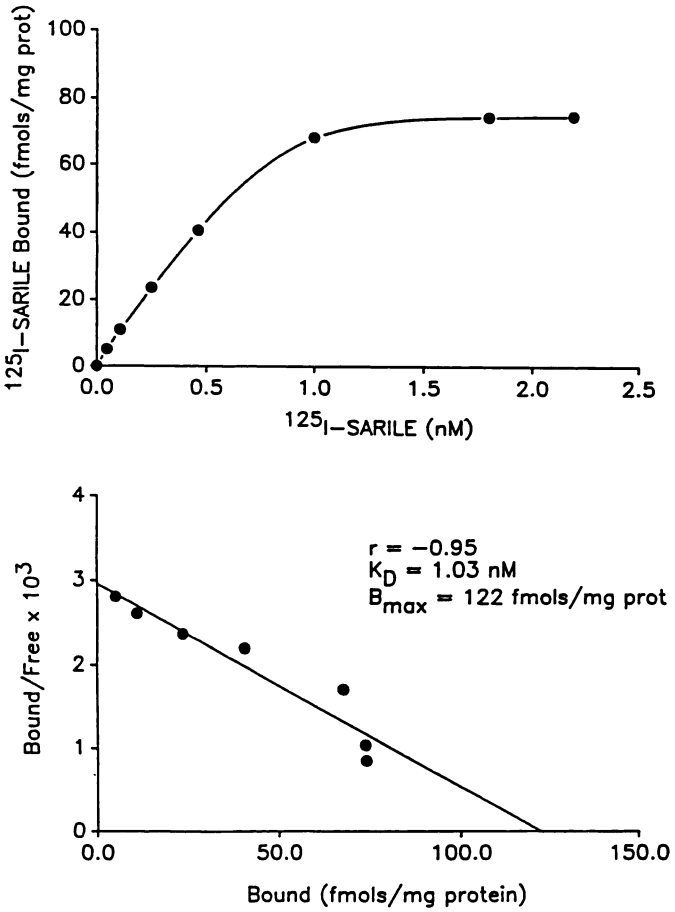

Fig. 2. Saturation isotherm and Scatchard analysis of ^{125}I-Sar^1,Ile^8-Ang-II (^{125}*I-SARILE*) binding to solubilized N1E-115 cell membranes. *Upper*, approximately 300 μg of 1% CHAPS-extracted N1E-115 cell membranes were incubated for 60 min with 0.1–2.2 nM ^{125}I-Sar^1,Ile^8-Ang-II, and nonspecific binding was defined in the presence of 1 μM unlabeled Ang-II. *Lower*, corresponding Scatchard analysis. Each *point* represents the mean of triplicate determinations, and the data are representative of three separate experiments.

Solubilized membrane proteins. When solubilized cell membrane proteins (150–700 μg of proteins) were prelabeled with ^{125}I-Ang-II (1–1.4 nM), cross-linked with DSS, and subsequently chromatographed on the TSK 3000 SW size exclusion column, two radioactive peaks eluted from the column (Fig. 5A), one minor peak (peak a) and one major peak (peak b), with estimated molecular weights of 112,000 ± 10,000 and 67,000 ± 4,000, respectively (mean ± standard deviation; nine experiments). Both radioactive peaks specifically bound ^{125}I-Ang-II, and nonspecific binding represented <10% of total binding. The results from the solubilized membrane proteins correlated well with gel filtration data obtained from the particulate membrane fraction, suggesting that the proteins labeled

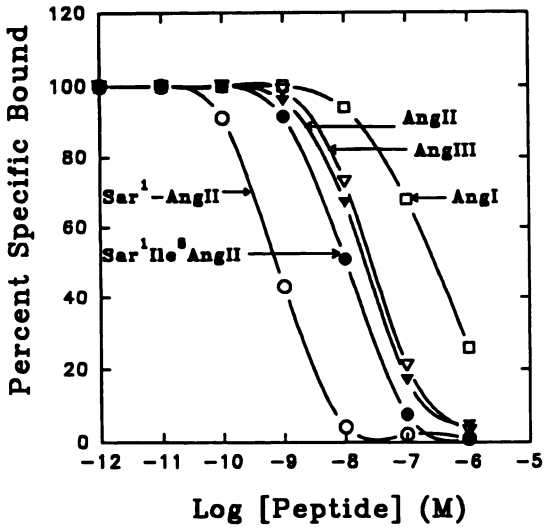

Fig. 3. Competition curves depicting the ability of Ang-II-related peptide analogs to inhibit the binding of ^{125}I-Sar^1,Ile^8-Ang-II in solubilized N1E-115 cell membranes. The binding assays were carried out in the presence of ^{125}I-Sar^1,Ile^8-Ang-II (0.3 nM) and various concentrations of unlabeled competitors for 60 min, as described in Experimental Procedures. Each *point* represents the mean of triplicate determinations, and the data are representative of three to five separate experiments.

TABLE 1

Inhibition of ^{125}I-Sar^1,Ile^8-Ang-II binding to solubilized N1E-115 membranes by Ang-II-related peptides

^{125}I-Sar^1,Ile^8-Ang-II (0.3 nM) was incubated with solubilized N1E-115 membranes and various concentrations of unlabeled competitors for 60 min, as described in Experimental Procedures. Each experiment was analyzed separately by LIGAND, in order to obtain the values for K_i. Subsequently, the values from several different membrane preparations (n) were averaged. The values reported are means ± standard errors.

Competitor	n	K_i
		nM
Sar^1-Ang-II	3	1.8 ± 0.5
Sar^1,Ile^8-Ang-II	4	12.0 ± 2.6
Ang-II	5	18.5 ± 2.1
Ang-III	3	28.6 ± 1.3
Ang-I	3	676 ± 152
Bradykinin	2	ND[a]

[a] ND, not detected.

in the solubilized preparation are likely to be the same as those labeled on intact cell membranes.

Additional experiments were done to determine whether these radiolabeled chromatography peaks would bind ^{125}I-Ang-II in the absence of covalent cross-linking. Aliquots of unlabeled solubilized cell membranes (100–150 μg of protein) were repeatedly injected onto the TSK 3000 SW column. Fractions were collected and assayed for ^{125}I-Ang-II binding. The binding profile (Fig. 5B) showed two specific binding peaks, which eluted between 14 and 18 min. These binding peaks were the same as those observed when intact (Fig. 4) or solubilized membranes (Fig. 5A) were covalently cross-linked with ^{125}I-Ang-II.

SDS-PAGE of the ^{125}I-Ang-II Binding Sites Derived from Particulate and Solubilized N1E-115 Cell Membrane Fractions

In these studies, ^{125}I-Ang-II was covalently cross-linked to its binding sites before electrophoresis. The results obtained

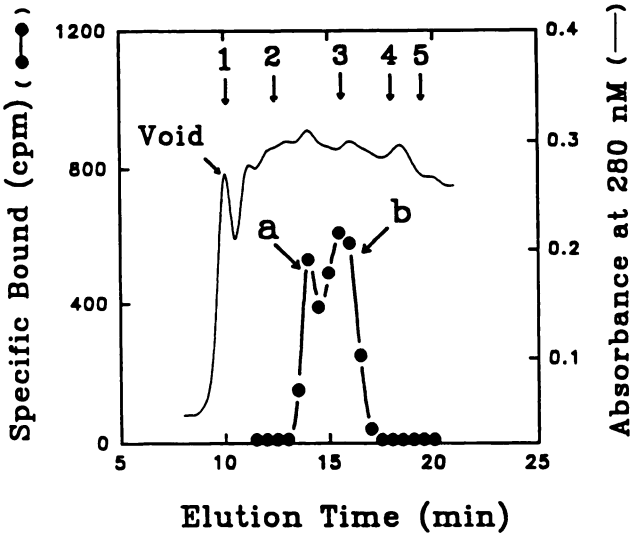

Fig. 4. Chromatographic analysis of ^{125}I-Ang-II binding proteins derived from particulate cell membrane fractions. Aliquots of washed N1E-115 cell membrane homogenate (0.8–1.4 mg of protein) were incubated with ^{125}I-Ang-II (1–1.4 nM) in the presence and absence of 1 μM unlabeled Ang-II. After the binding reached equilibrium, the radioligand was covalently cross-linked with DSS, and the membranes were solubilized with the detergent CHAPS. Aliquots of the solubilized material were put through a TSK 3000 SW size exclusion column, which was adjusted to a flow rate of 0.5 ml/min, and 0.25-ml fractions were collected. Two radioactive peaks, representing specific ^{125}I-Ang-II-binding proteins, eluted from the column. The approximate molecular weights for the two peaks were 125,000 ± 12,000 (*peak a*) and 73,000 ± 4,500 (*peak b*) (mean ± standard deviation; five experiments). Molecular weight standards depicted in the graph are as follows: *1*, fibrinogen (344,472); *2*, aldolase (160,000); *3*, BSA (67,000); *4*, β-lactoglobulin (36,000); and *5*, myoglobin (17,000).

from solubilized, as well as particulate, fractions were similar, in that two proteins of dissimilar molecular weights were consistently labeled by ^{125}I-Ang-II. More specifically, in the particulate fractions, covalent cross-linking of ^{125}I-Ang-II resulted in identification of two membrane components, with estimated molecular weights of 105,000 ± 12,000 and 72,000 ± 3,500 (mean ± standard deviation; eight experiments) (Fig. 6, *lane A*). Similarly, covalent cross-linking of ^{125}I-Ang-II in CHAPS-solubilized membranes resulted in consistent labeling of two proteins, one minor protein of 102,000 ± 10,000 Da and one major protein of 69,000 ± 5,000 Da (mean ± standard deviation; 10 experiments) (Fig. 7, *lane A*). In both intact and solubilized preparations, the binding of ^{125}I-Ang-II to high and lower molecular weight sites was almost completely displaceable by 1 μM unlabeled Ang-II (Figs. 6 and 7, *lanes B*). As was the case in the experiments using size exclusion chromatography, the high molecular weight protein in solubilized preparations was not as extensively labeled as the high molecular weight protein from intact membrane experiments. This suggests that the binding of ^{125}I-Ang-II to the high molecular weight protein is less efficient in solubilized preparations.

Affinity Purification of the Ang-II Binding Site from Solubilized Murine Neuroblastoma N1E-115 Cells

The procedures used to construct the affinity column resulted in the efficient coupling of Ang-II to the 1,1-carbonyl diimidazole-activated cross-linked 6% agarose gel, through a stable *N*-alkylcarbamate linkage. The quantitative spectrophotometric analysis of the coupling buffer eluant at 276 nm revealed that,

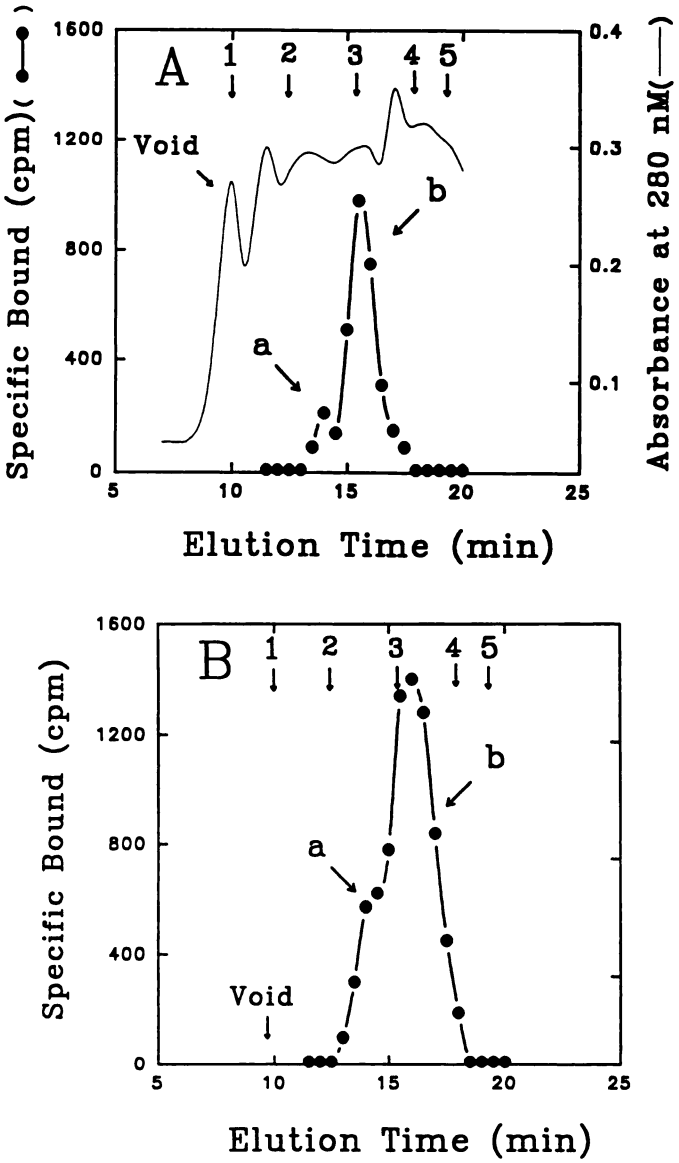

Fig. 5. Chromatographic analysis of solubilized N1E-115 cell membrane proteins labeled with ^{125}I-Ang-II either before (A) or after (B) chromatographic separation. A, Aliquots of solubilized cell membrane proteins (0.6–0.8 mg of protein) were incubated with ^{125}I-Ang-II (1–2 nM) until binding reached equilibrium. The radiolabel was covalently cross-linked with DSS and put through a high performance size exclusion column. Nonspecific binding was determined with 1 μM unlabeled Ang-II. Flow rate was 0.5 ml/min, and fraction size was 0.25 ml. Molecular weight standards used to calibrate the column were the same as described for Fig. 4. ^{125}I-Ang-II was specifically bound to two proteins, one major protein with an estimated molecular weight of 67,000 ± 4,000 and one minor protein of approximately 112,000 ± 10,000 (mean ± standard deviation; nine experiments). B, Aliquots of solubilized membrane proteins (100–150 μg of protein) were repeatedly injected onto the size exclusion column, and the pooled fractions were checked for ^{125}I-Ang-II specific binding. The binding profile in B shows the presence of one major and one minor ^{125}I-Ang-II specific binding peak, which eluted in the same fractions as observed when solubilized membranes were prelabeled with ^{125}I-Ang-II.

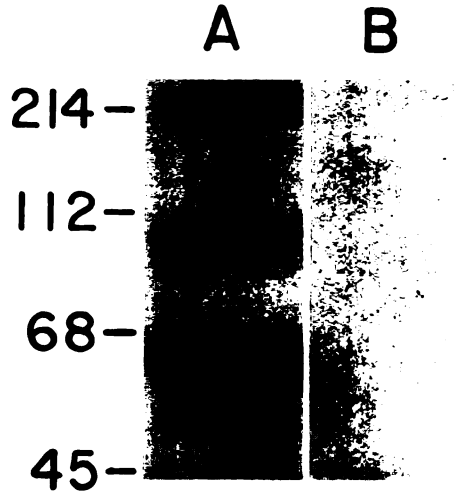

Fig. 6. Gel electrophoresis of cross-linked ^{125}I-Ang-II protein complex derived from particulate cell membrane fractions. *Lanes A* and *B*, autoradiograms of total and nonspecific ^{125}I-Ang-II binding, respectively. The autoradiograms were developed after exposure to film at −70° for 9 days. Particulate cell membrane fractions were covalently cross-linked with ^{125}I-Ang-II, the unbound radioligand was removed by repeated centrifugation, and the washed pellet was resuspended in SDS buffer containing 5% 2-mercaptoethanol. The samples were subjected to slab gel electrophoresis as described in Experimental Procedures. The molecular weight markers are myosin (214,000), phosphorylase B (112,000), BSA (68,000), and ovalbumin (45,000) and are indicated to the *left* of the autoradiogram.

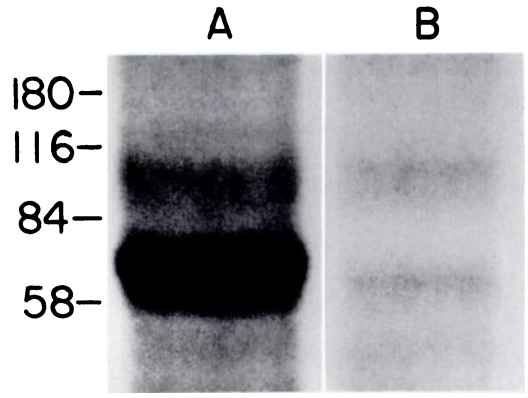

Fig. 7. SDS-PAGE analysis of cross-linked ^{125}I-Ang-II protein complex from solubilized cell membranes. ^{125}I-Ang-II (1–1.4 nM) was incubated with CHAPS-solubilized membrane proteins (150–700 μg) until binding reached equilibrium. This was followed by covalent cross-linking and removal of the unbound radiolabel by the dextran-coated charcoal method, as described in Experimental Procedures. The mixture was concentrated and subjected to gel electrophoresis in a SDS buffer containing 5% 2-mercaptoethanol. *Lanes A* and *B*, autoradiograms of total and nonspecific ^{125}I-Ang-II binding, respectively. The autoradiograms were developed after exposure to film at −70° for 7 days. The standards used were α_2-macroglobulin (180,000), β-galactosidase (116,000), fructose-6-phosphate kinase (84,000), and pyruvate kinase (58,000).

from the original 5 mg/ml Ang-II, on the average 0.24 mg/ml (three experiments) remained in the buffer, indicating approximately 99% coupling efficiency. We also examined (three experiments) the rate of removal of the Ang-II binding sites from solubilized tissue by determining the amount of ^{125}I-Ang-II bound as a function of the number of times that the soluble material was applied to the column. Approximately 40–50% of ^{125}I-Ang-II binding sites were removed after the extract had completely passed through the column for the first time (column flow rate was 12 drops/min or approximately 0.3 ml/min). The second pass through the column removed 80–90% of ^{125}I-Ang-II-binding activity. No further reduction in binding was observed after the third passage of the solubilized proteins through the column. Accordingly, routine harvesting of Ang-II

binding sites was accomplished by passing the solubilized membranes through the affinity column three times. Using this approach, 8–9 mg of solubilized cell membrane proteins yielded approximately 0.5–5 μg of partially purified Ang-II binding sites, as measured with the BCA protein assay reagent (Pierce) and the protocol for enhanced detection. In general, the yields of affinity-purified material obtained from solubilized N1E-115 membranes, as well as the gel filtration and SDS-PAGE analytical results described below, have been consistent for more than 15 preparations, so long as a new affinity column was constructed after every three purifications.

Gel Filtration of affinity-purified tissue. ^{125}I-Ang-II (1.5–2 nM) was incubated with affinity-purified tissue (5–10 μg of protein), and binding was performed as described in Experimental Procedures. Subsequently, ^{125}I-Ang-II was covalently cross-linked with DSS and the entire incubation was injected onto the TSK 3000 SW size exclusion column. The column was run with the same flow rate and mobile phase as used with intact and solubilized membrane fractions. One radioactive peak eluted from the column, with an estimated molecular weight of 63,000 ± 9,000 (mean ± standard deviation; five experiments) (Fig. 8). The eluted peak specifically bound ^{125}I-Ang-II, and nonspecific binding represented <5% of total binding.

Gel electrophoresis of affinity-purified Ang-II binding proteins. In the absence of ^{125}I-Ang-II labeling, SDS-PAGE in the absence of 2-mercaptoethanol revealed one predominant band upon silver staining, with a corresponding molecular weight of 66,000 ± 3,000 (mean ± standard deviation; three experiments) (Fig. 9, *lane A*).

In order to determine whether the affinity-purified proteins would specifically bind ^{125}I-Ang-II, we incubated aliquots of the affinity-purified proteins with ^{125}I-Ang-II, followed by covalent cross-linking with DSS and removal of the unbound radiolabel by size exclusion chromatography on a TSK 3000 SW column.

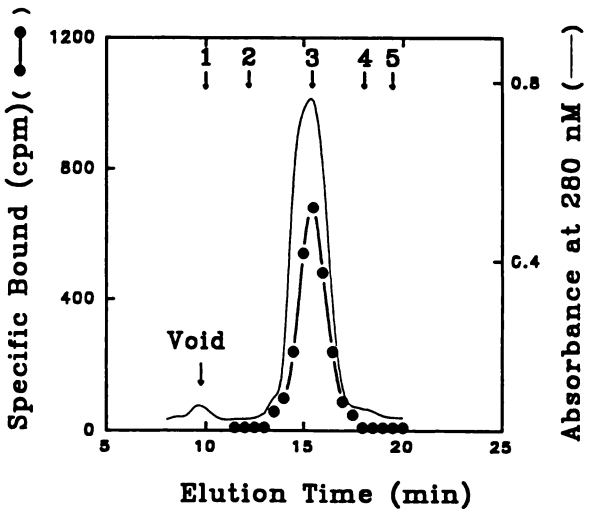

Fig. 8. Chromatographic analysis of affinity-purified Ang-II binding sites labeled with ^{125}I-Ang-II before chromatographic separation. Aliquots of affinity-purified tissue (5–10 μg of protein) were incubated with ^{125}I-Ang-II (1.5–2 nM), and the radioligand was then covalently cross-linked with DSS and subsequently chromatographed on a size exclusion column. The flow rate was 0.5 ml/min, and 0.25-ml fractions were collected. The molecular weight standards were the same as stated in Fig. 4. One radioactive peak eluted from the column, with a molecular weight of approximately 63,000 ± 9,000 (mean ± standard deviation; five experiments).

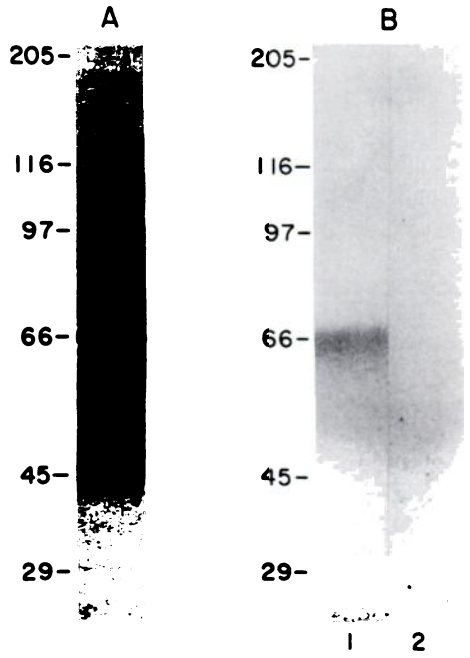

Fig. 9. *Lane A*, gel electrophoresis of affinity-purified Ang-II-binding proteins incubated in SDS in the absence of the reducing agent 2-mercaptoethanol. Silver-staining indicated one predominant protein band, with a molecular weight of 66,000 ± 3,000 (mean ± standard deviation; three experiments). *Lane B*, autoradiogram of affinity-purified Ang-II-binding proteins cross-linked with ^{125}I-Ang-II. The autoradiogram was developed after exposure to film at −70° for 8 days. The molecular weight markers were myosin (205,000), β-galactosidase (116,000), phosphorylase B (97,400), bovine plasma albumin (66,000), egg albumin (45,000), and carbonic anhydrase (29,000).

The radioactive fractions were pooled, concentrated, and subjected to gel electrophoresis in a SDS buffer containing 5% 2-mercaptoethanol. Autoradiography of the electrophoresed samples (Fig. 9, *lane B1*) revealed one radiolabeled band, with an apparent molecular weight of 66,000 ± 4,000 (mean ± standard deviation; three experiments). The radiolabeled band was completely displaceable with 1 μM unlabeled Ang-II (Fig. 9, *lane B2*). Thus, the molecular weight of the cross-linked radiolabeled band in the affinity-purified preparation was in general agreement with that of the protein bands obtained from intact as well as solubilized membrane fractions, with the important exception that the higher molecular weight site was not detected after affinity purification.

Discussion

The renin-angiotensin system is one of the principal systems for the regulation of cardiovascular and body fluid homeostasis, and Ang-II is known to produce a number of responses, including a direct contractile effect on vascular smooth muscle, stimulation of adrenal zona glomerulosa cells to release aldosterone, and renal conservation of water and sodium (56). In addition to these actions on peripheral tissues, it is well established that Ang-II has important effects on the brain, including the release of vasopressin and adrenocorticotropic hormone from the pituitary (57, 58), a centrally mediated pressor response (32, 59), and the elicitation of thirst and sodium appetite (60, 61). Despite the obvious importance of these actions of Ang-II in cardiovascular homeostasis, the receptors for Ang-II, particularly those present in the nervous system, remain the least well understood component of the renin-angiotensin system.

Much of what is known about the structural features of Ang-II receptors is based upon studies of peripheral binding sites. Although Ang-II receptors are present in the brain (33, 39), our understanding of their biochemical and molecular nature is far less complete. Progress in this area has been hampered, in part, because the highest densities of Ang-II receptors are found in restricted regions of the brain where they are part of a nexus of heterogeneous cell types and thus are not easily amenable to most biochemical assays. As an alternative approach, we and others have examined the properties of Ang-II receptors in clonal neuronal cell lines (46, 50, 51, 62). In general, these cells have proven to be very useful models of neurons, exhibiting many of their biochemical, physiological, and morphological properties.

One cell line that we have studied extensively is the murine neuroblastoma N1E-115 line. We have demonstrated that these cells possess membranous Ang-II receptors similar in specificity, affinity, and molecular weight to those in rat brain and that their density is substantially increased by neuronal differentiation (50, 51). Moreover, Ang-II receptors in differentiated N1E-115 cells are coupled to inositol polyphosphate production and calcium mobilization through a pertussis toxin insensitive GTP-binding protein (63). Thus, differentiated N1E-115 cells are a useful system in which to investigate the structural and functional properties of Ang-II receptors in the brain.

The importance of studying Ang-II receptors of neuronal origin has recently become clear, because evidence has accumulated that there are distinct subtypes of Ang-II receptors and that those present on some peripheral cells are different from those found in the brain (40, 44, 45). Because it is possible that some of the apparent differences between putative Ang-II receptor subtypes might result from cell-specific interactions among the receptor molecule and other integral membrane proteins, continued progress requires that Ang-II receptors be removed from the complex membrane environment in which they reside and studied in a relatively simpler state. To that end, we developed procedures for the solubilization of Ang-II receptors from differentiated N1E-115 membranes using the synthetic detergent CHAPS, which avoided some of the problems commonly associated with receptor solubilization, such as rapid dissociation kinetics and detergent-induced changes in binding activity or specificity. Our results demonstrate that the efficiency of solubilization with CHAPS is approximately 40%. Moreover, binding of ^{125}I-Sar^{1},Ile^{8}-Ang-II or ^{125}I-Ang-II to solubilized membranes was saturable and of high affinity, and the specificity of the solubilized receptors for Ang-II-related peptides was not appreciably altered.

We further characterized the N1E-115 Ang-II receptors using size exclusion and affinity chromatography, as well as covalent cross-linking techniques. After covalent cross-linking of ^{125}I-Ang-II to its binding sites with the homobifunctional cross-linker DSS, size exclusion chromatography of particulate membrane preparations or solubilized membranes consistently revealed two peaks of binding activity. One of the these binding peaks represented the major radioactive peak and had an estimated molecular mass of 63–73 kDa. The second, less prominent, peak had an estimated molecular mass of 102–125 kDa. Binding activity was also present in the void volume, suggesting that Ang-II receptors were part of even larger protein complexes that could not be resolved by the TSK 3000 SW gel filtration column.

The identification of these two distinct binding peaks did not require cross-linking, inasmuch as the same chromatographic profiles were obtained when fractions of solubilized membranes were collected and specific binding activity was assayed by incubation with ^{125}I-Ang-II, in the presence or absence of unlabeled Ang-II, followed by termination on glass fiber filters. Moreover, SDS-PAGE analysis of intact or solubilized membranes in which ^{125}I-Ang-II had been cross-linked with DSS also revealed two radioactive species, with molecular weights similar to those obtained by size exclusion chromatography. Finally, we have recently reported that covalent cross-linking of ^{125}I-Ang-II in CHAPS-solubilized rat brain membranes similarly resulted in the consistent labeling of two proteins with molecular weights virtually identical to those obtained in N1E-115 cells, supporting the contention that these neuroblastoma cells possess Ang-II receptors similar to those in the brain (48).

The demonstration of high and low molecular weight binding sites for ^{125}I-Ang-II has been reported by previous investigators using peripheral tissues as their source of Ang-II receptors. For instance, Capponi and Catt (19) used a photosensitive Ang-II analog, ^{125}I-2-nitro-5-azido-benzoyl-Ang-II, to irreversibly couple Ang-II to its receptor on adrenal cortical membranes. This stable hormone-receptor complex was then solubilized with the detergent Triton X-100 and analyzed by gel filtration and sucrose density gradient centrifugation or SDS-PAGE. In each case, two major radioactive species, with estimated molecular masses of 126 and 65 kDa, were observed. These results were in general agreement with those obtained using another photoaffinity label in bovine adrenal (22), as well as results obtained with DSS to covalently cross-link ^{125}I-Ang-II to its receptor in rabbit liver particles that had been solubilized with digitonin (28). On the basis of these findings, it was hypothesized that Ang-II receptors in these peripheral target tissues existed as a dimer of two identical 65-kDa binding subunits. However, other experiments have not consistently supported this conclusion. Indeed, systematic investigations of the molecular mass of Ang-II receptors from several cells and tissues have often yielded a single binding protein, with varying estimates of 60 kDa for liver, 72 kDa for both bladder and mesenteric artery, 78 kDa for NG108-15 cells, 79 kDa for adrenal glomerulosa cells, and 92 kDa for anterior pituitary (25, 46, 64). Collectively, these observations have added fuel to the continued speculation that distinct Ang-II receptors may exist within various target tissues.

In the present work, we have used similar techniques to examine the properties of neuronal Ang-II receptors. In these experiments, size exclusion chromatography of intact or solubilized membranes and SDS-PAGE of membranes covalently cross-linked with ^{125}I-Ang-II all supported the suggestion of high and low molecular weight binding sites for Ang-II. The explanation for the multiplicity of these binding sites, however, remains unclear. One possibility is that the higher molecular weight species represents a dimer of two smaller binding proteins, as has been suggested in studies of peripheral Ang-II receptors (19). Alternatively, the apparent presence of two dissimilar binding sites may be indicative of multiple Ang-II-receptor subtypes expressed in N1E-115 cells (65). Interestingly, the larger molecular weight species was more pronounced when intact membranes were exposed to the agonist ^{125}I-Ang-II before solubilization and chromatographic analysis. Re-

cently, we reported similar findings in rat brain, in which the higher molecular weight protein was more readily apparent in membranes and was only observed with ^{125}I-Ang-II, and not with ^{125}I-Sar^{1},Ile^{8}-Ang-II, in solubilized preparations (48). These results suggest that the higher molecular weight site may degrade more easily in solubilized preparations or that it is less stable in CHAPS, resulting in a significant reduction in its affinity for ^{125}I-Ang-II.

Regardless of the precise nature of the higher molecular weight binding site present within intact and solubilized membranes, it is clear that its integrity was not maintained through the affinity column. More specifically, size exclusion chromatography of the affinity-purified material revealed not two but one distinct peak of binding activity, which eluted from the column with an estimated molecular mass of 63 kDa. Similarly, when affinity-purified membranes were subjected to SDS-PAGE analysis, a single 66-kDa band was detected by silver staining, and this protein retained its ability to specifically bind ^{125}I-Ang-II in the presence of the covalent cross-linker. Collectively, these data suggest that the 66-kDa protein is the major membrane-associated binding site for Ang-II in N1E-115 cells. However, at present it is difficult to precisely quantify the fold purification of Ang-II receptors achieved by affinity chromatography, because equilibrium binding of ^{125}I-Ang-II or ^{125}I-Sar^{1},Ile^{8}-Ang-II to the affinity-purified material is not yet possible.

In summary, we have successfully solubilized Ang-II receptors from murine neuroblastoma N1E-115 cells in CHAPS. Further biochemical analysis indicated that there appeared to be at least two distinct ^{125}I-Ang-II binding sites present in both intact and solubilized membranes, with molecular masses of approximately 111 kDa and 68 kDa, as estimated by a variety of techniques including size exclusion chromatography and covalent cross-linking. These data are comparable to the molecular masses of the Ang-II binding sites obtained from rat brain membranes (48), suggesting that N1E-115 cells possess Ang-II receptors similar to those present in the brain. On the other hand, when membranous Ang-II receptors were isolated by affinity purification only one major protein, of 66 kDa, was detected that retained its capacity to specifically bind ^{125}I-Ang-II. The ability to generate microgram quantities of partially purified Ang-II binding proteins will undoubtedly facilitate a more detailed biochemical and molecular analysis of neuronal Ang-II receptors.

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References

1. Akiyama, F., N. Imai, S. Hirose, and K. Murakami. Solubilization and characterization of active angiotensin II receptors from the bovine adrenal cortex. *Biochem. Res.* **5**:9–18 (1984).
2. Birabeau, M. A., A. M. Capponi, and M. B. Vallotton. Solubilized adrenal angiotensin II receptors: studies on the site of action of sodium and calcium ions, and on the role of disulfide bridges. *Mol. Cell. Endocrinol.* **37**:181–189 (1984).
3. Capponi, A. M., M. A. Birabeau, and M. B. Vallotton. Solubilization of adrenal angiotensin II receptors with a zwitterionic detergent. *J. Recept. Res.* **3**:289–299 (1983).
4. Chang, R. S., and V. J. Lotti. Solubilization of angiotensin II receptors in bovine adrenal. *Life Sci.* **29**:613–618 (1981).
5. Glossmann, H., A. J. Baukal, and K. J. Catt. Properties of angiotensin II receptors in the bovine and rat adrenal cortex. *J. Biol. Chem.* **249**:825–834 (1974).
6. Campanile, C. P., J. K. Crane, M. J. Peach, and J. C. Garrison. The hepatic angiotensin II receptor. I. Characterization of the membrane-binding site and correlation with physiological response in hepatocytes. *J. Biol. Chem.* **257**:4951–4958 (1982).
7. Hagiwara, H., N. Sugiura, K. Wakita, and S. Hirose. Purification and characterization of angiotensin-binding protein from porcine liver cytosolic fraction. *Eur. J. Biochem.* **185**:405–410 (1989).
8. Rosenberg, E., M. A. Ravi Kiron, and R. L. Soffer. A soluble angiotensin II-binding protein from rabbit liver. *Biochem. Biophys. Res. Commun.* **151**:466–472 (1988).
9. Sen, I., K. F. Jim, and R. L. Soffer. Solubilization and characterization of an angiotensin II binding protein from liver. *Eur. J. Biochem.* **136**:41–49 (1983).
10. Sen, I. Evidence of essential disulfide bonds in angiotensin II binding sites of rabbit hepatic membranes: inactivation by dithiothreitol. *Biochim. Biophys. Acta* **813**:103–110 (1985).
11. Anand Srivastava, M. B. Angiotensin II receptors negatively coupled to adenylate cyclase in rat myocardial sarcolemma: involvement of inhibitory guanine nucleotide regulatory protein. *Biochem. Pharmacol.* **38**:489–496 (1989).
12. Rogers, T. B. High affinity angiotensin II receptors in myocardial sarcolemmal membranes: characterization of receptors and covalent linkage of [^{125}I]-angiotensin II to a membrane component of 116,000 daltons. *J. Biol. Chem.* **259**:8106–8114 (1984).
13. Gunther, S., M. A. Gimbrone, Jr., and R. W. Alexander. Identification and characterization of the high affinity vascular angiotensin II receptor in rat mesenteric artery. *Circ. Res.* **47**:278–286 (1980).
14. Rosenberg, E., C. Russo, M. A. Kiron, and R. L. Soffer. Biochemical and immunological similarity of soluble angiotensin II-binding proteins in different organs. *Biochem. Biophys. Res. Commun.* **156**:1230–1236 (1988).
15. Devynck, M. A., M. G. Pernollet, P. Meyer, S. Fermandjian, Fromageot, P., and F. M. Bumpus. Solubilization of angiotensin II receptors in rabbit aortae membranes. *Nature (Lond.)* **249**:67–69 (1974).
16. Capponi, A. M., and K. J. Catt. Angiotensin II receptors in adrenal cortex and uterus: binding and activation properties of angiotensin analogues. *J. Biol. Chem.* **254**:5120–5127 (1979).
17. Capponi, A. M., and K. J. Catt. Solubilization and characterization of adrenal and uterine angiotensin II receptors after photoaffinity labeling. *J. Biol. Chem.* **255**:12081–12086 (1980).
18. Carson, M. C., C. M. Harper, A. J. Baukal, G. Aguilera, and K. J. Catt. Physicochemical characterization of photoaffinity-labeled angiotensin II receptors. *Mol. Endocrinol.* **1**:147–153 (1987).
19. Capponi, A. M., and K. J. Catt. Solubilization and characterization of adrenal and uterine angiotensin II receptors after photoaffinity labeling. *J. Biol. Chem.* **255**:12081–12086 (1980).
20. Carson, M. C., C. M. Harper, A. J. Baukal, G. Aguilera, and K. J. Catt. Physicochemical characterization of photoaffinity-labeled angiotensin II receptors. *Mol. Endocrinol.* **1**:147–153 (1987).
21. Cantau, B., G. Guillemette, D. Chicot, and G. Devilliers. Vasopressin, angiotensin and adrenergic receptors of rat liver Golgi fractions: molecular weight of the angiotensin-receptor irreversible complex after *in vitro* and *in vivo* labelling. *Mol. Cell. Endocrinol.* **51**:59–69 (1987).
22. Guillemette, G., and E. Escher. Analysis of the adrenal angiotensin II receptor with the photoaffinity labeling method. *Biochemistry* **22**:5591–5596 (1983).
23. Guillemette, G., G. Guillon, J. Marie, *et al.* Angiotensin-induced changes in the apparent size of rat liver angiotensin receptors. *J. Recept. Res.* **4**:267–281 (1984).
24. Guillemette, G., G. Guillon, J. Marie, M. N. Balestre, E. Escher, and S. Jard. High yield photoaffinity labeling of angiotensin II receptors. *Mol. Pharmacol.* **30**:544–551 (1986).
25. Paglin, S., and J. D. Jamieson. Covalent crosslinking of angiotensin II to its binding sites in rat adrenal membranes. *Proc. Natl. Acad. Sci. USA* **79**:3739–3743 (1982).
26. Bonnafous, J. C., R. Seyer, M. Tence, J. Marie, and M. Kabbaj. Design of angiotensin II derivatives suitable for indirect techniques: potential applications to receptor studies. *J. Recept. Res.* **8**:295–309 (1988).
27. Elton, T. S., L. D. Dion, K. L. Bost, S. Oparil, and J. E. Blalock. Purification of an angiotensin II binding protein by using a peptide encoded by angiotensin II complementary RNA. *Proc. Natl. Acad. Sci. USA* **85**:2518–2522 (1988).
28. Sen, I., H. G. Bull, and R. L. Soffer. Isolation of an angiotensin II-binding protein from liver. *Proc. Natl. Acad. Sci. USA* **81**:1679–1683 (1984).
29. Couraud, P. O. Anti-angiotensin II anti-idiotypic antibodies bind to angiotensin II receptor. *J. Immunol.* **138**:1164–1168 (1987).
30. Ganong, W. F. The brain renin-angiotensin system. *Annu. Rev. Physiol.* **46**:17–31 (1984).
31. Ganten, D., M. I. Fuxe, M. I. Phillips, J. F. E. Mann, and U. Ganten. The brain isorenin-angiotensin system: biochemistry, localization, and possible role in drinking and blood pressure regulation. *Front. Neuroendocrinol.* **5**:61–99 (1978).
32. Severs, W. B., and A. E. Daniels-Severs. Effects of angiotensin on the central nervous system. *Pharmacol. Rev.* **25**:415–449 (1973).
33. Bennett, J. P., and S. H. Snyder. Angiotensin II binding to mammalian brain membranes. *J. Biol. Chem.* **251**:7423–7430 (1976).
34. Harding, J. W., L. P. Stone, and J. W. Wright. The distribution of angiotensin II binding sites in rodent brain. *Brain Res.* **205**:265–274 (1981).

35. Healy, D. P., and M. P. Printz. Autoradiographic localization of angiotensin II binding sites in the brain, pituitary, kidney and adrenal gland of the rat. *J. Hypertens.* **2 (Suppl. 3)**:57–61 (1984).
36. Mendelsohn, F. A. O., R. Quirion, J. M. Saavedra, G. Aguilera, and K. J. Catt. Autoradiographic localization of angiotensin II receptors in rat brain. *Proc. Natl. Acad. Sci. USA* **81**:1575–1579 (1984).
37. Rogulja, I., J. W. Harding, and S. Ritter. Reduction of [^{125}I]-angiotensin II binding sites in rat brain following monosodium glutamate treatment. *Brain Res.* **419**:333–335 (1987).
38. Simonnet, G., P. Legendre, A. Carayon, M. Allard, F. Cesselin, and J. D. Vincent. Angiotensin II in the central nervous system in the rat. *J. Physiol. (Paris.)* **79**:453–460 (1984).
39. van Houten, M., E. L. Schiffrin, J. F. E. Mann, B. I. Posner, and R. Boucher. Radioautoradiographic localization of specific binding sites for blood-borne angiotensin II in the rat brain. *Brain Res.* **186**:480–485 (1980).
40. Chiu, A. T., J. V. Duncia, D. E. McCall, *et al.* Nonpeptide angiotensin II receptor antagonists. III. Structure-function studies. *J. Pharmacol. Exp. Ther.* **250**:867–874 (1989).
41. Chiu, A. T., W. F. Herblin, D. E. McCall, *et al.* Identification of angiotensin II receptor subtypes. *Biochem. Biophys. Res. Commun.* **165**:196–203 (1989).
42. Chiu, A. T., D. E. McCall, T. T. Nguyen, *et al.* Discrimination of angiotensin II receptor subtypes by dithiothreitol. *Eur. J. Pharmacol.* **170**:117–118 (1989).
43. Whitebread, S., M. Mele, B. Kamber, and M. de Gasparo. Preliminary biochemical characterization of two angiotensin II receptor subtypes. *Biochem. Biophys. Res. Commun.* **163**:284–291 (1989).
44. Chang, R. S., V. J. Lotti, T. B. Chen, and K. A. Faust. Two angiotensin II binding sites in rat brain revealed using [^{125}I]Sar1,Ile8-angiotensin II and selective nonpeptide antagonists. *Biochem. Biophys. Res. Commun.* **171**:813–817 (1990).
45. Koepke, J. P., P. R. Bovy, E. G. McMahon, *et al.* Central and peripheral actions of a nonpeptidic angiotensin II receptor antagonist. *Hypertension (Dallas)* **15**:841–847 (1990).
46. Carrithers, M. D., K. A. Koide, V. K. Raman, S. Masuda, and J. A. Weyhenmeyer. Covalent crosslinking analysis of angiotensin receptors on differentiated NG108-15 cells. *Biochem. Biophys. Res. Commun.* **170**:1096–1101 (1990).
47. Laribi, C., M. Allard, J. D. Vincent, and G. Simonnet. Solubilization and characterization of covalently labeled angiotensin II receptors in cultured mouse spinal cord cells. *Neuropeptides* **9**:345–356 (1987).
48. Siemens, I. R., G. N. Swanson, S. J. Fluharty, and J. W. Harding. Solubilization and partial characterization of angiotensin II receptors from rat brain. *J. Neurochem.* **57**:690–700 (1991).
49. Siemens, I. R., H. J. Adler, L. P. Reagan, R. Mir, and S. J. Fluharty. Solubilization and characterization of angiotensin II receptors from murine neuroblastoma N1E-115 cells. *Neuroscience* **16**:684 (1990).
50. Fluharty, S. J., and L. P. Reagan. Characterization of binding sites for the angiotensin II antagonist [^{125}I]-[Sarc1,Ile8]-angiotensin II on murine neuroblastoma N1E-115 cells. *J. Neurochem.* **52**:1393–1400 (1989).
51. Reagan, L. P., X. H. Ye, R. Mir, L. R. DePalo, and S. J. Fluharty. Up-regulation of angiotensin II receptors by *in vitro* differentiation of murine N1E-115 neuroblastoma cells. *Mol. Pharmacol.* **38**:878–886 (1990).
52. Moody, K. M., K. Addya, S. Y. Chow, R. M. A. Kiron, R. L. Soffer, and S. J. Fluharty. Cytosolic binding proteins from angiotensin II: relation to membranous receptors in murine neuroblastoma N1E-115 cells. *Neuroscience* **16**:685 (1990).
53. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
54. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.)* **227**:680–683 (1970).
55. Heukeshoven, J., and R. Dernick. Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver stains. *Electrophoresis* **6**:103–112 (1985).
56. Peach, M. J. Renin-angiotensin system: biochemistry and mechanism of action. *Physiol. Rev.* **57**:313–370 (1977).
57. Keil, L. C., J. Summy Long, and W. B. Severs. Release of vasopressin by angiotensin II. *Endocrinology* **96**:1063–1065 (1975).
58. Ramsay, D. J., L. C. Keil, M. C. Sharpe, and J. Shinsako. Angiotensin II infusion increases vasopressin, ACTH, and 11-hydroxycorticosteroid secretion. *Am. J. Physiol.* **234**:R66–R71 (1978).
59. Phillips, M. I. Angiotensin in the brain. *Neuroendocrinology* **25**:354–377 (1978).
60. Epstein, A. N., J. T. Fitzsimons, and B. J. Rolls. Drinking induced by injection of angiotensin into the brain of the rat. *J. Physiol. (Lond.)* **210**:457–474 (1970).
61. Fluharty, S. J., and A. N. Epstein. Sodium appetite elicited by intracerebroventricular infusion of angiotensin II in the rat. II. Synergistic interaction with systemic mineralcorticoids. *Behav. Neurosci.* **97**:746–758 (1983).
62. Weyhenmeyer, J. A., and C. J. Hwang. Characterization of angiotensin II binding sites on neuroblastoma × glioma hybrid cells. *Brain Res. Bull.* **14**:409–414 (1985).
63. Monck, J. R., R. E. Williamson, I. Rogulja, S. J. Fluharty, and J. R. Williamson. Angiotensin II effects on the cytosolic free Ca^{+2} concentration in N1E-115 neuroblastoma cells: kinetic properties of the Ca^{+2} transient measured in single fura-2-loaded cells. *J. Neurochem.* **54**:278–287 (1990).
64. Catt, K. J., M. C. Carson, W. P. Hausdorff, *et al.* Angiotensin II receptors and mechanisms of action in adrenal glomerulosa cells. *J. Steroid Biochem.* **27**:915–927 (1987).
65. Ades, A. M., F. Slogoff, and S. J. Fluharty. Differential regulation of Type 1 and Type 2 angiotensin II receptors in murine neuroblastoma N1E-115 cells. *Neuroscience* **17**:809 (1991).

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