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Development of polyclonal antibodies against angiotensin type 2 receptors

(immunoprecipitation/3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate/crosslinking)

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ABSTRACT **Murine neuroblastoma N1E-115 cells are a useful system in which to study neuronal angiotensin II (AngII) receptors. N1E-115 cells possess both type 1 (AT_1) and type 2 (AT_2) AngII receptor subtypes, as does mammalian brain. AT_2 receptors in brain or N1E-115 cells can be solubilized in 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate. In the present study, heparin-Sepharose chromatography was used to partially purify solubilized N1E-115 membranes to produce an enriched population of AT_2 receptors. Subsequently, an eluted peak, containing the majority of AT_2 binding activity, was used as an immunogen in the development of protein-directed polyclonal antibodies. The antibodies specifically detected immunoreactive proteins of approximately 110 and 66 kDa in both solubilized N1E-115 cells, as well as the original protein material that eluted from the heparin-Sepharose column, whereas no such immunoreactivity was detected in a kidney epithelial cell line that lacks any specific ^{125}I-labeled AngII (^{125}I-AngII) binding activity. Moreover, the antibodies immunoreacted with affinity-purified AT_2 receptors. These antibodies were also able to immunoprecipitate AT_2 receptors from solubilized N1E-115 cells, as revealed by the pharmacologic profile of ^{125}I-AngII binding to the precipitated protein. Similarly, the antibodies were able to immunoprecipitate a 66-kDa protein that had been covalently crosslinked with ^{125}I-AngII by use of the homobifunctional crosslinker dithiobis(succinimidyl propionate). Collectively, these results demonstrate the development of a specific AT_2 receptor antibody that may be used to further characterize this receptor subtype at both the cellular and molecular levels.**

The renin–angiotensin system is one of the most important regulators of body fluid and cardiovascular homeostasis (1). Angiotensin II (AngII) is known to exert its diverse effects by acting at membrane-bound receptors present on several peripheral target tissues, such as kidney, adrenal cortex, heart, and vascular smooth muscle (1). In addition to its many peripheral effects, all of the components of the renin–angiotensin system have also been localized in the central nervous system (2). Moreover, it is now recognized that many of the physiological, endocrinological, and behavioral actions of AngII are mediated by specific receptors present in the brain (3).

The diversity of actions mediated by AngII was among the first pieces of evidence leading to the suggestion of multiple receptor subtypes for AngII. The unequivocal demonstration of such multiple receptor subtypes, however, came with the development of subtype-specific antagonists (4). The existence of at least two receptor subtypes for AngII has initiated the search for a greater understanding of these receptors at the cellular and molecular levels. Such analyses have revealed that both receptor subtypes, referred to as AT_1 and AT_2, are expressed in brain and in neuron-like cell lines (2, 5, 6). The cloning of the AT_1 receptor subtype from several peripheral tissues has revealed that this receptor is a member of the guanine nucleotide binding protein-linked receptor family (7, 8) and that it is responsible for many of the cellular actions of AngII (9).

While the structural and functional properties of the AT_1 receptor subtype are now well described, similar information about the AT_2 receptor subtype has emerged more slowly. For instance, although the pharmacology of the AT_2 binding sites has permitted its localization in a variety of tissues, its functional properties have proven to be much more elusive and controversial. Recently, it has been reported that the AT_2 receptor subtype may mediate inhibition of cGMP production in neuron-enriched cultures from neonatal rats (10) and PC12 cells (11). On the other hand, AngII has been reported to increase cGMP levels in neuroblastoma cells, an effect that appears to be mediated by multiple receptor subtypes and nitric oxide production (12). Multiple effects of AngII on ionic conductances may also be mediated by AT_2 receptors (13).

An even greater paucity of information exists concerning the structural and molecular properties of AT_2 receptors. Previously, we have demonstrated the successful solubilization of functional AT_2 receptors from N1E-115 cells by using the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (14, 15). These solubilized AT_2 receptors retained their high affinity for ^{125}I-labeled AngII (^{125}I-AngII) and specificity for AT_2-selective ligands. In an effort to partially purify AT_2 receptors, solubilized N1E-115 cells were subjected to heparin-Sepharose chromatography (16). Binding studies performed on heparin-Sepharose chromatography fractions revealed three peaks of specific ^{125}I-AngII binding activity. One of these peaks, which represented the majority of specific ^{125}I-AngII binding activity, was used as an immunogen in development of a polyclonal antiserum. The antibodies were characterized and shown to selectively detect 110- and 66-kDa proteins from solubilized N1E-115 cells, as well as partially purified material from heparin-Sepharose chromatography, whereas no such immunoreactivity was detected in a kidney epithelial cell line that does not express AngII receptors of either subtype. These apparent molecular masses are consistent with independent estimates of AT_2 receptor size in N1E-115 cells (14). These antibodies also specifically reacted with affinity-purified AT_2 receptors. In addition, antibodies that

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Abbreviations: AngII, angiotensin II; AT_1 and AT_2, AngII receptor subtypes 1 and 2; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DSP, dithiobis(succinimidyl propionate; SARILE, [Sar^1,Ile^8]AngII.

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were coupled to protein A-Sepharose beads were able to immunoprecipitate specific ^{125}I-AngII binding activity from solubilized N1E-115 cells, as well as a 66-kDa protein that had been covalently crosslinked with ^{125}I-AngII by use of the homobifunctional crosslinker dithiobis(succinimidyl propionate) (DSP). These results demonstrate the development of AT_2 receptor subtype-specific antibodies, which should facilitate continued analysis of AT_2-mediated functions at the cellular, physiological, and behavioral levels.

MATERIALS AND METHODS

Materials. Monoiodinated ^{125}I-AngII was obtained from DuPont/NEN. DSP, Reacti-Gel (GF2000), and BCA protein reagents were obtained from Pierce. Heparin-Sepharose resin was purchased from Pharmacia-LKB. Losartan was a gift from Ronald Smith (DuPont) and CGP42112A was a gift from Marc DeGasparo (CIBA–Geigy). Unlabeled AngII and related peptides, Hepes, glycerol, aprotinin, 1,10-*o*-phenanthroline, Coomassie brilliant blue, polyethylenimine, CHAPS, and protein A-Sepharose beads were from Sigma. Prestained molecular size standards for SDS/PAGE were from GIBCO/BRL. Biotinylated goat anti-rabbit IgG and horseradish peroxidase streptavidin were obtained from Vector Laboratories. Enhanced chemiluminescence (ECL) Western blotting reagents were obtained from Amersham. All other chemicals and reagents were purchased from Fisher Scientific and were of the highest obtainable grade.

Cell Culture Techniques. N1E-115 cells were plated on T150 plastic dishes as described (17). Briefly, N1E-115 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (high glucose) supplemented with 10% fetal bovine serum and gentamicin (50 μg/ml) in a humidified atmosphere of 5% CO_2/95% O_2 at 37°C. Three days after subculturing, N1E-115 cells were induced to differentiate by replacement of the normal growth medium with DMEM supplemented with 0.5% serum and 1.5% dimethyl sulfoxide (18). Cells were subsequently maintained for an additional 3–4 days before harvesting.

Membrane Preparation and Solubilization. N1E-115 membranes were prepared and solubilized essentially as described (18, 19). Briefly, culture dishes were rinsed three times with ice-cold 20 mM Tris·HCl, pH 7.4/150 mM NaCl. The cells were then incubated in this buffer without the NaCl for 10 min at 0°C. At the end of this incubation, cells were harvested, homogenized with a Dounce homogenizer, and centrifuged at 48,000 × *g* for 30 min. Pelleted cells were resuspended in 50 mM Tris·HCl, pH 7.4/150 mM NaCl/5 mM $MgCl_2$ and centrifuged at 48,000 × *g* for 20 min. Pelleted cell membranes were resuspended in the same buffer, and protein concentration was determined by the method of Bradford, using bovine serum albumin (BSA) as the standard (20). Cell membranes were washed once by centrifugation at 48,000 × *g* for 20 min. The resulting pellets were resuspended to a final protein concentration of 6–8 mg/ml in solubilization buffer [25 mM sodium phosphate, pH 7.4/5 mM EGTA/25% (vol/vol) glycerol/500 mM KCl/aprotinin (0.3 TIU/ml)/1,10-*o*-phenanthroline (100 μg/ml)/0.2% heat-inactivated BSA] plus 1% CHAPS. In selected experiments, rat liver membranes were solubilized as described except that 0.2% CHAPS was used and glycerol was omitted from the buffer. The CHAPS homogenates were incubated for 1 hr at 4°C on an orbital shaker and subsequently centrifuged at 10500 × *g* for 90 min at 4°C. The resulting supernatant was then removed and used immediately or stored at −70°C.

Heparin-Sepharose and Affinity Chromatography. Heparin-Sepharose chromatography was performed as described (16). Briefly, heparin-Sepharose resin was extensively washed on a sintered glass funnel and then poured into a column (1 × 20 cm) and equilibrated at room temperature with buffer A (20 mM Bistris, pH 7.4/1 mM EDTA/0.05% CHAPS/10% glycerol). The column was placed in line with an HPLC gradient system (Waters) and the flow rate was set at 0.2 ml/min. Fractions were collected with a Pharmacia-LKB fraction collector set at drop count. Forty drops were collected, which was equivalent to ≈0.9 ml per fraction. Two milliliters of solubilized N1E-115 cell membranes (3.5–4.5 mg per ml of protein) was added to the column and proteins were eluted off the heparin-Sepharose column by buffer A alone or with buffer A plus 1.5 M NaCl.

In selected experiments, affinity purification of AT_2 receptors was performed as described (14). Briefly, an affinity column was constructed by covalently linking unlabeled AngII to Reacti-Gel (GF2000) according to the manufacturer's instruction. All proteins were monitored at 280 nm and quantified in each fraction by using the BCA protein assay reagent (Pierce). Measurement of AT_2 receptors in each fraction was determined by ^{125}I-AngII binding in the absence (total) or presence (nonspecific) of the AT_2-selective antagonist CGP42112A.

Production of Antibodies. Prior to injection of immunogen, two New Zealand rabbits were bled to isolate preimmune serum. Peak I material eluted from heparin-Sepharose chromatography was used as the immunogen. New Zealand rabbits received subcutaneous injections of 1 mg of immunogen emulsified in Freund's complete adjuvant. Booster injections were administered every 6 weeks and the rabbits were bled 2 weeks after each injection. Since the original antigen was not purified AngII receptors, antibodies selective for the AT_2 receptor were isolated by absorption of nonreceptor antibodies on agarose columns containing peak I proteins from which AngII receptors had been removed by prior affinity chromatography.

Immunoblotting of AT_2 Receptors. Protein samples were boiled for 3 min in electrophoresis sample buffer. Samples were subjected to SDS/10% PAGE and transferred to nitrocellulose membranes, and nonspecific sites were blocked with 3% gelatin in TBS (10 mM Tris·HCl, pH 8.0/150 mM NaCl) for 60 min with gentle shaking at 22°C. The blocking solution was discarded and replaced with antisera in TBS (1:500) for 90 min with gentle shaking. Blots were then washed with TBS plus 0.05% Tween 20 (TBST) and then incubated with biotinylated goat anti-rabbit IgG in TBS (1:30,000) for 60 min. After washes with TBST, blots were incubated with horseradish peroxidase streptavidin D in TBS (1:30,000) for 30 min. After washes with TBST, blots were developed by Amersham ECL Western blotting reagents as described by the manufacturer.

AngII Receptor Immunoprecipitation. AT_2 receptors were immunoprecipitated with antibody-coated protein A-Sepharose beads. Preimmune or immune serum (45 μl) was incubated for 4 hr at 4°C with 50 μl of protein A-Sepharose beads [50% (vol/vol)]. The antibody–protein A complexes were then washed three times with assay buffer and 1 ml of solubilized N1E-115 cells was added. Samples were immunoprecipitated overnight at 4°C with constant shaking. After microcentrifugation, the immunoprecipitates were washed three times in assay buffer and resuspended in assay buffer. Resuspended immunoprecipitates (100 μl) were then added to 150 μl of assay buffer [50 mM Tris·HCl, pH 7.4/150 mM NaCl/5 mM $MgCl_2$/0.2% heat-inactivated BSA/aprotinin (0.3 TIU/ml)/1,10-*o*-phenanthroline (100 μg/ml)] containing ^{125}I-AngII and unlabeled peptides as needed. Reaction mixtures were incubated for 60 min and terminated by dilution of the reaction mixture and rinsing of the assay tubes three times with wash buffer (150 mM NaCl/5 mM Tris·HCl, pH 7.4), followed by vacuum filtration using a Skatron cell harvester. Subsequently, the glass-fiber filters were counted for 60 sec in an LKB γ scintillation counter at 80.5% efficiency.

Covalent Crosslinking Studies. ^{125}I-AngII binding assays were performed in solubilized N1E-115 cell membranes essentially as described above. At the end of the incubation period, the homobifunctional crosslinker DSP was added (final concentration, 9 mM) for 30 min on ice. This reaction was terminated by the addition of 1 M Tris (pH 8.0) (final concentration, 250 mM) and allowed to sit at room temperature for 10 min. After microcentrifugation, supernatants were removed and immunoprecipitated as described above. Immunoprecipitates were resuspended and boiled in 25 μl of double-distilled H_2O (ddH_2O) and 5 μl of electrophoresis reducing sample buffer. After microcentrifugation, the supernatants were subjected to SDS/7.5% PAGE. Gels were fixed in silver stain solution (ethanol/glacial acetic acid/ddH_2O, 3:1:5) for 1 hr, washed with ddH_2O, and fixed in 2% glycerol, dried, and exposed for autoradiography.

RESULTS

Immunoblotting Studies. In previous studies, heparin-Sepharose chromatography was used to isolate AT_2 receptors from solubilized N1E-115 membranes (16). This chromatographic procedure results in the elution of three distinct protein peaks, the first and last of which contain virtually all of the membrane-associated ^{125}I-AngII binding activity. The early-eluting peak contains the majority of this binding activity and the smallest amount of total protein. Hence, in light of this substantial enrichment of AT_2-selective binding activity this protein peak, referred to as peak I, was used to generate specific antibodies for AT_2 receptors. Peak I was emulsified in Freund's complete adjuvant, and injected into New Zealand rabbits. The rabbits were bled and the partially purified serum was initially tested for its ability to immunoreact with AT_2 receptors.

In an effort to characterize and demonstrate specificity of these antibodies, their ability to detect AT_2 receptors by immunoblotting was examined. Solubilized N1E-115 cells were subjected to SDS/10% PAGE analysis. As is shown in Fig. 1*A*, the immune antisera identified immunoreactive proteins of 110 and 66 kDa in solubilized N1E-115 membranes, while preimmune sera did not. These molecular masses are in good agreement with previous estimates of the apparent size of AT_2 receptors in N1E-115 cells (14). Moreover, the antisera also reacted with proteins of the same size in peak I isolated by heparin-Sepharose chromatography (see Fig. 1*B*). In marked contrast, the antisera did not immunoreact with any proteins in membranes prepared from V2 cells, a kidney epithelial cell line that does not possess specific ^{125}I-AngII binding activity (Fig. 1*C*).

The use of partially purified membranes from N1E-115 cells raises the possibility that the immune-specific bands do not represent authentic AT_2 immunoreactivity but instead reflect the detection of proteins of similar molecular sizes. To address these concerns, affinity-purified AT_2 receptors were tested for immunoreactivity in immunoblotting studies. AngII was covalently linked to Sepharose beads to produce an AngII affinity column as described (14). Crude, solubilized N1E-115 cells were applied to an AngII affinity column and AT_2 receptors were eluted with excess agonist or warm buffer solution. Previously we have demonstrated that these procedures result in the elution of both a 110- and a 66-kDa protein that can be covalently crosslinked with ^{125}I-AngII (14), and this result was verified in the present studies (data not shown). Affinity-purified receptor protein was subjected to SDS/10% PAGE and transferred to nitrocellulose; nonspecific sites were blocked and blotted with antisera. Once again, immune but not preimmune antisera detected proteins of 110 and 66 kDa present after affinity purification (Fig. 2), confirming the specificity of these antisera for the AT_2 receptor subtype.

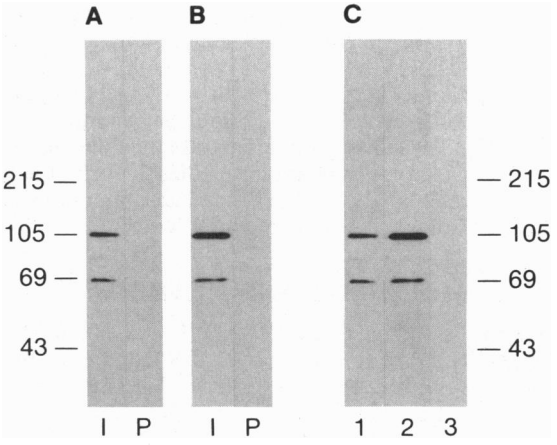

FIG. 1. Immunodetection of AT_2 receptors in crude, solubilized N1E-115 cell membranes and peak I material eluted from heparin-Sepharose chromatography. (*A*) Solubilized N1E-115 cells (5 μg) were subjected to SDS/10% PAGE, transferred to nitrocellulose membranes, blocked with 3% gelatin, and blotted with AT_2-directed antibodies at a dilution of 1:500 in TBS. Immune serum (lane I) detected proteins of approximately 110 and 66 kDa, which were not detected by preimmune serum (lane P). (*B*) Peak I material (5 μg) eluted from heparin-Sepharose chromatography was immunoblotted with AT_2-directed antibodies at a dilution of 1:500 in TBS. As seen with solubilized N1E-115 cells, immune serum (lane I) was able to detect proteins at apparent molecular masses of 110 and 66 kDa, whereas preimmune serum (lane P) did not. (*C*) AT_2-directed antibodies were able to recognize both 110- and 66-kDa proteins from solubilized N1E-115 cells (lane 1; 5 μg) and peak I material (lane 2; 5 μg) but not membranes prepared from V2 cells (lane 3; 5 μg). V2 cells lack any specific ^{125}I-AngII binding activity and also do not demonstrate any immunoreactivity with immune serum (1:500 dilution). Numbers on left and right are kDa.

Immunoprecipitation Studies. The AT_2-directed antibodies were also tested for their ability to immunoprecipitate AT_2 receptors from solubilized N1E-115 cell membranes. Antibodies were coupled to protein A-Sepharose beads and incubated with solubilized N1E-115 cells. After microcentrifugation, the presence of functional AT_2 receptors in the immunoprecipitate and the supernatant was determined by ^{125}I-AngII binding assays. Nonspecific binding was defined in the presence of 1 μM [Sar^1,Ile^8]AngII (SARILE), a high-affinity, nonselective AngII receptor antagonist or by using the more selective AT_2 antagonist CGP42112A. As shown in

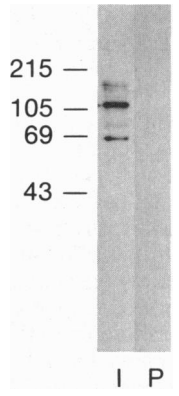

FIG. 2. AT_2-directed antibodies immunoreacted with affinity-purified AT_2 receptors from N1E-115 cells. AT_2 receptors were affinity purified as described and subjected to SDS/10% PAGE analysis followed by immunoblotting. Immune serum (lane I) (1:500) detected affinity-purified AT_2 receptor proteins at apparent molecular masses of 110 and 66 kDa. Preimmune serum (lane P) did not detect these proteins. Numbers on left are kDa.

Fig. 3A, antibodies coupled to protein A-Sepharose were able to immunoprecipitate ≈7 times more AT_2 binding activity than that removed by the preimmune serum. As expected, the appearance of binding activity in the precipitated pellet was associated with a corresponding loss of specific ^{125}I-AngII binding activity in the supernatants of solubilized N1E-115 cells that had been treated with immune serum (Fig. 3B). Interestingly, the immune-specific loss of binding activity from the supernatant consistently exceeded that detected in the precipitated pellet, suggesting that the antisera may interfere with the binding of ^{125}I-AngII. Finally, the antibody did not immunoprecipitate any significant ^{125}I-AngII binding activity from solubilized rat liver membranes that contain exclusively AT_1 receptors (data not shown).

To further document the efficacy of these antisera in immunoprecipitating AT_2 receptors from solubilized N1E-115 membranes, the homobifunctional crosslinker DSP was used to covalently couple ^{125}I-AngII to its binding sites. In previous studies with this crosslinking agent, we have demonstrated that the vast majority of specific binding of ^{125}I-AngII was to a 66-kDa protein in crude solubilized and heparin-Sepharose chromatographed N1E-115 membranes (16, 21). Moreover, covalent crosslinking of ^{125}I-AngII to affinity-purified AT_2 receptors similarly revealed a single 66-kDa protein (14). In the present experiments, solubilized AT_2 receptors were crosslinked with ^{125}I-AngII by DSP, followed by immunoprecipitation of these labeled receptors. The crosslinking studies were performed in the presence and absence of 10 μM SARILE in order to define nonspecific binding. After crosslinking with DSP, radiolabeled proteins were immunoprecipitated with antibody-coated protein A-Sepharose beads, followed by electrophoretic analysis of the precipitated proteins and subsequent autoradiography. As shown in Fig. 4, a 66-kDa protein crosslinked with ^{125}I-AngII was immunoprecipitated by the AT_2-directed antibody. Moreover, the immunoprecipitated binding protein was specific for AngII insofar as it was completely eliminated by excess SARILE when it was added before DSP. Collectively, the results of the immunoprecipitation studies demonstrate that the antibody recognizes a 66-kDa protein that specifically binds ^{125}I-AngII and that it will precipitate proteins capable of binding ^{125}I-AngII in the absence of covalent crosslinking.

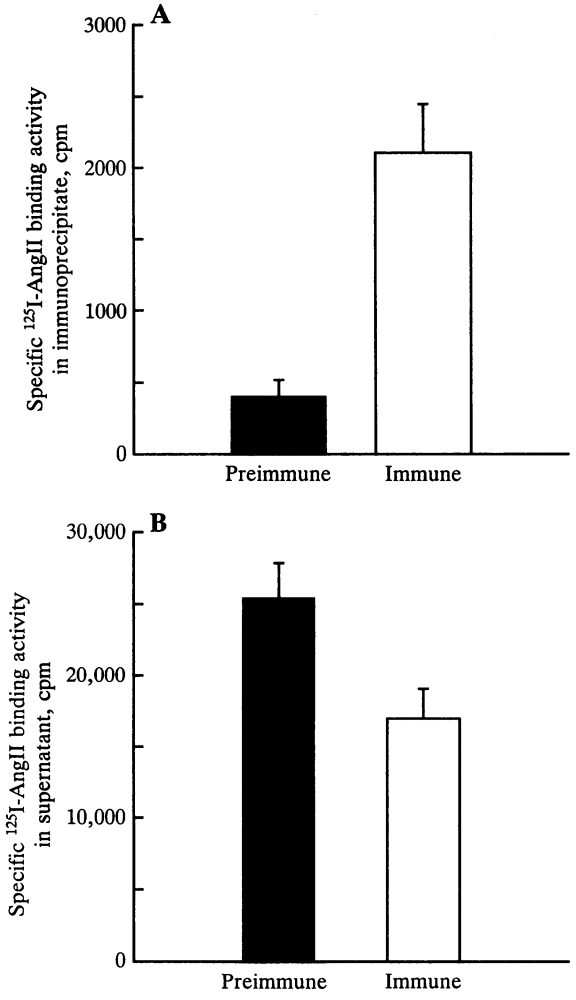

FIG. 3. Immunoprecipitation of AT_2 receptors from solubilized N1E-115 cells. Immune and preimmune sera were coupled to protein A-Sepharose beads and tested for their ability to immunoprecipitate AT_2 receptors as determined in ^{125}I-AngII binding assays as described. (A) Specific ^{125}I-AngII binding activity in the immunoprecipitate was determined by removal of supernatants and thorough washing of the protein A–antibody–AT_2 receptor complex. Washed immunoprecipitates were resuspended and used in ^{125}I-AngII binding assays. (B) Removal of specific ^{125}I-AngII binding activity from solubilized N1E-115 cells. After incubation with antibody-coated protein A beads and microcentrifugation, supernatants were removed and used in ^{125}I-AngII binding assays as described. Data represent means ± SE of at least six separate experiments.

DISCUSSION

The physiological, endocrinological, and behavioral actions of AngII are mediated by cell-surface receptors for this peptide. Recently, it has become clear that there are at least two main subtypes of these receptors, referred to as AT_1 and AT_2. The AT_1 receptor has been cloned from several peripheral sources (7, 8) and is a member of the superfamily of guanine nucleotide binding protein-linked receptors. As a consequence of its successful cloning, a great deal of structural and functional information about the AT_1 receptor has emerged, including the recent observation that there are two subtypes of AT_1 receptors, referred to as AT_{1a} and AT_{1b} (21–24), based on sequence differences in their 5′ untranslated regions. On the other hand, much less information is available about the AT_2 receptor family. Recently, several laboratories have chosen to study the properties of AngII receptors in cloned neuronal cell lines, including murine neuroblastoma N1E-115 cells (14, 17, 18, 25). The use of these alternative neuronal cell lines has provided a greater understanding of the actions of AngII in the central nervous system; the cell lines have also proven to be a rich source of AT_2 receptors.

Studies utilizing neuroblastoma and related cells have resulted in progress in the biochemical analysis of the AT_2 receptor subtype. For instance, in previous studies AngII receptors from rat brain (15) and from N1E-115 cells (14) have been successfully solubilized by using the zwitterionic de-

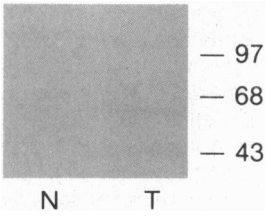

FIG. 4. Covalent crosslinking of ^{125}I-AngII followed by immunoprecipitation of AT_2 receptors from solubilized N1E-115 cells. Covalent crosslinking of ^{125}I-AngII by use of the homobifunctional crosslinker DSP was performed as described. Covalently crosslinked AT_2 receptors were immunoprecipitated, subjected to SDS/7.5% PAGE, and exposed for autoradiography. Total binding (lane T) was defined in the absence of 10 μM SARILE, while nonspecific binding (lane N) included 10 μM SARILE. Numbers on right are kDa.

tergent CHAPS. Subsequent analyses with subtype-specific antagonists revealed that these receptors were pharmacologically identical to the AT_2 receptor subtype present on the intact membrane. Moreover, chemical crosslinking studies using solubilized N1E-115 membranes followed by SDS/PAGE analysis revealed two distinct ^{125}I-AngII binding species, with apparent molecular masses of 110 and 66 kDa. In an effort to further purify AT_2 receptors, crude solubilized N1E-115 membranes were subjected to AngII affinity purification (14). These studies revealed that the AT_2 binding site has an apparent molecular mass of 66 kDa as estimated by covalent crosslinking and SDS/PAGE analysis.

In a related group of studies, solubilized N1E-115 cell membranes were partially purified by heparin-Sepharose chromatography (16). Three distinct protein peaks eluted from the heparin-Sepharose column, two of which bound ^{125}I-AngII with high affinity and saturability. One of these binding peaks eluted rapidly and represented ≈80% of the total specific binding activity. As expected from earlier solubilization studies, pharmacologic analysis revealed that the binding activity was exclusively AT_2 receptors insofar as they exhibited high affinity for CGP42112A and little or no affinity for the AT_1-selective antagonist losartan. Since heparin-Sepharose chromatography was an effective way to isolate and enrich AT_2 receptors it was subsequently used in the development of protein-directed polyclonal antisera.

Initial characterization of these antisera was performed in immunoblotting studies. These antisera detected immunoreactive proteins of approximately 110 and 66 kDa from both solubilized N1E-115 cells and heparin-Sepharose generated material, but not with membranes prepared from V2 cells, which lack any specific binding activity for ^{125}I-AngII. As indicated above, previous studies have demonstrated that N1E-115 AT_2 receptors exhibit molecular masses of 110 and 66 kDa (14). More significantly, however, was the ability of the antibody to detect affinity-purified AT_2 receptor from N1E-115 membranes. Affinity-purified AT_2 receptors eluted with excess agonist lead to the stabilization of the 110-kDa complex, as demonstrated in immunoblotting and silver stain analysis (27). This 110-kDa protein may represent a multimeric AT_2 receptor composed of a 66-kDa binding site and another subunit that is stabilized by agonist exposure. These results eliminate the possibility that the partially purified protein immunogen generated antibodies that were reacting with proteins of a molecular mass similar to that of the AT_2 receptor, but not with the AT_2 receptor itself. Immunoprecipitation studies using antibodies coupled to protein A-Sepharose revealed that they were able to immunoprecipitate AT_2 receptors from solubilized N1E-115 cell membranes, as detected by ^{125}I-AngII binding assays. Immunoprecipitates possessed a significant amount of specific ^{125}I-AngII binding activity when compared to preimmune serum and there was a corresponding loss of binding activity in immune supernatants when compared to the preimmune condition. In addition, these antibodies immunoprecipitated a 66-kDa protein that had been covalently crosslinked with ^{125}I-AngII.

Taken together, these results demonstrate the successful development of specific antibodies raised against AT_2 receptors. These antibodies should provide an invaluable tool in further characterizing AT_2 receptors. For instance, a cellular response mediated solely by the AT_2 receptor subtype remains controversial, and there is increasing evidence for heterogeneity within the AT_2 receptor family (16, 26). Related to this confusion is the relative paucity of information concerning the three-dimensional structure of this intrinsic membrane protein and the degree of cell-specific posttranslational processing. These and related structural issues can now be more directly addressed with the development of these antibodies. Lastly, the availability of AT_2 receptor antibodies should allow for the eventual cloning of this receptor by antibody screening of an expression library. The cDNA can then be used to determine the relation of this receptor to previously cloned AT_1 receptors as well as the transcriptional regulation of AT_2 receptor expression in the brain.

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