Molecular cloning, characterization, and distribution of the gerbil angiotensin II AT₂ receptor

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Hoe, Kwang-Lae, Ines Armando, Gustavo Baiardi, Taduru Sreenath, Ashok Kulkarni, Alfredo Martinez, and Juan M. Saavedra. Molecular cloning, characterization, and distribution of the gerbil angiotensin II AT₂ receptor. Am J Physiol Regul Integr Comp Physiol 285: R1373–R1383, 2003; 10.1152/ajpregu.00008.2003.—We isolated a cDNA clone encoding the gerbil AT₂ receptor (gAT₂) gene from a gerbil adrenal gland cDNA library. The full-length cDNA contains a 1,089-bp open reading frame encoding 363 amino acid residues with 90.9, 96.1, and 95.6% identity with the human (hAT₂), rat (rAT₂), and mouse AT₂ (mAT₂) receptors, respectively. There are at least seven non-conserved amino acids in the NH₂-terminal domain and in positions Val₁₀⁹, Val₁₁⁷, and Met₂₅₀, important for angiotensin (ANG) II but not for CGP-42112 binding. Displacement studies in adrenal sections revealed that affinity of the gAT₂ receptor was 10–20 times lower for ANG II, ANG III, and PD-123319 than was affinity of the rAT₂ receptor. The affinity of each receptor remained the same for CGP-42112. When transfected into COS-7 cells, the gAT₂ receptor shows affinity for ANG II that is three times lower than that shown by the hAT₂ receptor, whereas affinities for ANG III and the AT₂ receptor ligands CGP-42112 and PD-123319 were similar. Autoradiography in sections of the gerbil head showed higher binding in muscles, retina, skin, and molars at embryonic day 19 than at 1 wk of age. In situ hybridization and emul-sion autoradiography revealed that at embryonic day 19 the gAT₂ receptor mRNA was highly localized to the base of the dental papilla of maxillary and mandibular molars. Our results suggest selective growth-related functions in late gestation and early postnatal periods for the gAT₂ receptor and provide an essential basis for future mutagenesis studies to further define structural requirements for agonist binding.

adrenal gland; brain; CGP-42112; dental papilla

ANGIOTENSIN (ANG) II is a circulating hormone and a locally formed modulatory agent (29). Two major types of mammalian ANG II receptors, AT₁ and AT₂, have been distinguished pharmacologically. Biphenyl imidazole compounds such as losartan specifically antagonize AT₁ receptors, whereas binding to the AT₂ receptors is selectively displaced by imidazole pyridine carboxylic acid compounds such as PD-123319 and by the synthetic peptide analog CGP-42112 (4).

The well-known functions of ANG II in regulating blood pressure, pituitary hormone release, and water and salt metabolism are mediated by stimulation of the G protein-coupled AT₁ receptors (4). On the other hand, the functions of the AT₂ receptor are undefined. AT₂ receptor expression is very high in peripheral fetal tissues and in many areas in the immature brain (8, 35) and markedly decreases early during postnatal life (33, 39). In adult rats, AT₂ receptors are confined to the cardiovascular and reproductive system, the adrenal gland, and a few brain areas related to regulation of sensory and motor activity (11, 35). In adult rodents, the highest AT₂ receptor expression occurs in the adrenal medulla, where this receptor type contributes 90–95% of the total ANG II receptor population (12). AT₂ receptor activation could be involved in the regulation of tissue growth, brain development, and organogenesis (33) and contributes to regulate catechol-amine release (17). Cross-talk mechanisms have been proposed between AT₁ and AT₂ receptors, with AT₂ activation leading to dilation of blood vessels, inhibition of growth, and induction of apoptosis (20). However, apoptosis appears to be a constitutive function of the AT₂ receptor, independent of ANG II stimulation or PD-123319 blockade (22), and the physiological functions of AT₂ receptors are still a matter of controversy (4).

Mouse (mAT₂), rat (rAT₂), and human AT₂ (hAT₂) receptors have been cloned and show >90% amino acid sequence identity between species (16, 25, 36). However, there is only 34% amino acid homology with AT₁ receptors (16, 25, 36). The AT₂ Receptor has a putative seven-transmembrane topology that is typical of the G protein-coupled receptors and activates the Gᵢ α-sub-unit (10). However, the AT₂ receptor does not internalize upon agonist stimulation (24) and does not always demonstrate the guanosine 5’-O-(3-thiotriphosphate)-induced shift to a low-affinity form characteristic of G
protein-linked receptors (34), and its signal transduction mechanisms are poorly understood (10). In addition, the AT2 receptor recognizes ANG II analogs and ANG II with equal affinity, although some of these analogs do not have physiological effects (2, 21). Although many of the amino acid residues involved in ligand binding and coupling to intracellular proteins and their signal transduction mechanisms in AT1 receptors have been identified (9, 15), the structure-activity requirements for ligand binding and coupling to intracellular proteins of AT2 receptors are only beginning to be studied (14, 21, 27).

There are wide variations in AT2 receptor expression among closely related rodent species (5, 11, 33). Among adult rodents, gerbils show the highest expression of AT2 receptors, not only in the adrenal gland but also in the brain (5). Our preliminary studies on adrenal gerbil AT2 (gAT2) receptors revealed large differences in agonist affinity compared with the rat. We previously found large differences in affinity for losartan between gerbil AT1 and rat AT1 receptor subtypes (23) and established that the differences were the result of the presence of nonconserved amino acids in positions crucial for the binding of the nonpeptidic antagonist (15). To attempt to further clarify the possible physiological role of AT2 receptors and the reasons for the large differences in agonist affinity between gerbil and rat receptors, we cloned the gAT2 receptor, characterized the receptor pharmacologically, and studied in some detail its expression during late-gestation and early postnatal life.

MATERIALS AND METHODS

Animals and tissue preparation. We obtained adult male Mongolian gerbils (Meriones unguiculatus), weighing 65–80 g, pregnant (embryonic day 19 (E19)) female gerbils, and gerbil mothers with 1-wk-old pups from Tumblebrook Farm (West Bookfield, MA). Adult male Wistar-Kyoto rats, 8 wk of age, were obtained from Taconic Farms (Germantown, NY). The National Institutes of Health Animal Care and Use Committee approved all animal procedures. The animals were maintained under controlled conditions (12:12-h light-dark cycle) with free access to standard food and water. Gerbils were killed by decapitation, males 1 wk after arrival and females and pups on the day of arrival. Brains, pituitary glands, adrenal glands, and kidneys were quickly removed from adult animals, and whole heads were dissected from animals at E19 and 1 wk of age. Tissues were frozen by immersion in isopentane on dry ice and kept at −80°C until use. Tissue sections, 16 μm thick, were cut at −20°C in a cryostat, mounted on gelatin-coated glass slides for binding experiments or on silanated glass slides (Digene Diagnostics, Beltsville, MD) for in situ hybridization, and stored at −80°C. Adjacent sections were used for binding experiments and in situ hybridization.

Southern and Northern blot analyses. Frozen gerbil adrenals were homogenized, and genomic DNA was isolated using DNeatol (Life Technologies, Rockville, MD). After digestion with KpnI/Sacl or Sacl/XhoI, digested DNA was separated on a 1% agarose gel (10 μg/lane) and transferred to a nylon membrane (Schleicher and Schuell, Keene, NH). An EcoRI fragment (2.3 kb) of the hAT2 receptor cDNA (a gift from Dr. Tadashi Inagami, Vanderbilt University School of Medicine, Nashville, TN) was labeled using a random labeling kit (Boehringer Mannheim, Germany) with [α-32P]dCTP (Amersham, Arlington Heights, IL). The blots were hybridized to the labeled probe overnight at 42°C in Hybrisol (Oncor, Gaithersburg, MD), washed under lower-than-usual stringency conditions [2× saline-sodium citrate (SSC) at 42°C], and subjected to autoradiography for 3 days with Hyperfilm MP (Amersham). For Northern blots, total RNA was prepared from gerbil hippocampus, pituitary gland, adrenal gland, or kidney using TRizol reagent (Life Technologies). Aliquots of 10 μg of RNA were separated on a 1% denaturing agarose gel containing 2.2 M formaldehyde and blotted onto the nylon membrane (Schleicher and Schuell). The probes, hybridization conditions, and washes were similar to those used for Southern analysis (see above).

cDNA cloning and sequencing. Total RNA was extracted from gerbil adrenal gland using TRizol reagent. Polyadenylated RNA was purified by an oligo(dT)-cellulose column (Life Technologies). First- and second-strand cDNAs were synthesized with 5 μg of polyadenylated RNA following the manufacturer’s recommendation (Stratagene, La Jolla, CA) using a cDNA synthesis kit. Fractions of cDNA (1.5–4 kb) were pooled by agarose gel electrophoresis followed by gel elution. Size-fractionated cDNA fragments were purified by phenol and chloroform extraction and ligated to an EcoRI/XhoI pre-cut λ-vector. Approximately 3.6 × 105 independent clones were screened through cDNA sequence comparison with results of the genomic Southern blot. Genomic regions confirmed by the cDNA

![Fig. 1. Southern blot analysis of gerbil genomic DNA and its deduced restriction map. A: gerbil genomic DNA was isolated from frozen spleens and hybridized with an EcoRI fragment (2.3 kb) of human ANG II type 2 (AT2) receptor cDNA. B: genomic restriction map was speculated through cDNA sequence comparison with results of the genomic Southern blot. Genomic regions confirmed by the cDNA sequence are represented by open boxes. Open reading frame (ORF) of gerbil AT2 (gAT2) receptor is shown in hatched box with 5’ and 3’- untranslated regions (UTR).](image)
Fig. 2. Amino acid sequence comparison between gAT2, human AT2 (hAT2), rat AT2 (rAT2), and mouse AT2 (mAT2) receptors. A: amino acid sequence of gAT2, hAT2, rAT2, and mAT2 receptors. The gAT2 receptor has 33 amino acids different from the hAT2 receptor, only 14 different from the rAT2 receptor, and 16 different from the mAT2 receptor. B, C, and D: 2-dimensional representations of hAT2, rAT2, and mAT2 receptors, respectively. ● Amino acid residues different in the gAT2 receptor.
were screened with a conventional in situ plaque hybridization method, with 32P-labeled hAT2 receptor open reading frame (ORF) cDNA as a probe. The membranes (NEN, Boston, MA) with the gerbil adrenal gland cDNA plaques were hybridized overnight at 42°C in HybriSolv solution containing 40% formamide and washed at 40°C with 0.1× SSC-0.1% SDS. Four positive clones were selected, two of which were of the same kind. Three different kinds of positive clones, designated α, β, and γ, were treated for in vivo conversion from the λ-vector to a phagemid vector (pBK-CMV, Stratagene) and subjected to further nucleotide analysis. The nucleotide sequences were determined using an ABI Prism 310 sequencing machine (PE Applied Biosystems, Foster City, CA).

Cell culture and transfection. The monkey kidney epithelial COS-7 cell line (CRL-1651, American Type Culture Collection, Manassas, VA) was cultured in Dulbecco’s modified Eagle’s medium containing 4.5 g/l glucose with 10% fetal bovine serum, 100 U/ml penicillin, and 100 g/ml streptomycin (Life Technologies) in a humidified atmosphere of 5% CO2-95% air at 37°C. To transfect COS-7 cells, BamHI/NotI fragments of the gAT2 receptor cDNA and the hAT2 receptor cDNA were cloned into the λ- vector (Stratagene) or after they were applied to tissue culture plates (2 × 105 cells/cm2), cells were washed with 10 ml of Opti-MEM (Life Technologies). Four micrograms of vector DNA in 800 μl of Opti-MEM and 16 μl of LipofectAMINE (Life Technologies) in 800 μl of Opti-MEM were mixed and incubated at room temperature for 30 min. The DNA-LipofectAMINE complex was added to each plate after it was mixed with 6.4 ml of Opti-MEM. On the next day, transfected cells were divided into wells in 24-well tissue culture plates (1 × 104 cells/well).

Binding assays in transfected cells. Binding analysis was performed 2 days after transfection using 125I-CGP-42112, a compound that labels only AT2, and not AT1, receptors, as described elsewhere (12). Cells were washed with binding buffer (0.1% BSA-HBSS) and incubated at room temperature for 1 h with 0.05–12 nM 125I-CGP-42112. In the competition study, variable amounts (10–12–10–4 M) of unlabeled peptide or nonpeptide ligands were included in the binding buffer containing 0.5 nM 125I-CGP-42112. After incubation, the cells were washed with ice-cold HBSS three times and dissolved in 0.2 M NaOH, and bound 125I-CGP-42112 was measured in a gamma counter. Each experiment was carried out at least twice in quadruplicate. The binding data were analyzed, and dissociation constants (Kd) and Bmax values were determined by computerized nonlinear regression analysis using GraphPad Prism 2.0 software (San Diego, CA).

Receptor autoradiography. Binding to AT2 receptors in tissue sections from adrenal glands and heads was performed using 125I-CGP-42112, as described elsewhere (12). Sections were preincubated for 15 min at 22°C in sodium phosphate buffer, pH 7.4, containing 120 mM NaCl, 5 mM EDTA, 0.005% bacitracin, and 0.2% proteinase-free BSA (Sigma) and then incubated for 2 h at 22°C in fresh buffer prepared as described above with the addition of 0.2 nM 125I-CGP-42112 (NEN; 2,200 Ci/mmol) to determine total binding. Nonspecific binding was determined in the presence of 1 × 10–6 M unlabeled ANG II (Peninsula Laboratories, Belmont, CA). The competition assays were conducted using 0.2 nM 125I-CGP-42112 displaced by 10–12–10–4 M unlabeled ANG II, ANG III (Peninsula Laboratories), losartan (DuPont-Merck, Wilmington, DE), CGP-42112 (Sandoz, Basle, Switzerland), or PD-123319 (Sigma). After incubation, sections were washed in 50 mM Tris-HCl, pH 7.4, rinsed in water, and dried under a cold airstream. Sections were exposed to Hyperfilm–3H (Amersham) for 1 day, developed in D-19 developer (Kodak, Rochester, NY) for 4 min at 0°C, and fixed in Rapid fixer (Kodak) for 4 min at 22°C. Films were analyzed by measuring optical densities using the NIH Image 1.61 program, quantified by comparison with [125I]micro-Scales (Amersham, Arlington Heights, IL), and transformed to the corresponding values of femtomoles per milligram of protein (26).

In situ hybridization histochemistry. Specific riboprobes directed against the 3′-untranslated region (3′-UTR) of the cloned gAT2 receptor were obtained after subcloning of a 600-bp Xhol/SacI fragment (corresponding to nucleotides 1845–2445) into the pHBluescript II KS(+) vector (Stratagene). For in vitro transcription of sense (control) and antisense riboprobes, a subclone containing plasmid was linearized with SacI or Xhol and transcribed with T3 or T7 RNA polymerase, respectively. In vitro transcription was performed in the presence of 200 μCi of [35S]UTP (>1,000 Ci/mmol; Amersham), 1 μg of linearized plasmid, and T7 (sense) or T3 RNA polymerase (antisense) using an RNA labeling kit according to the manufacturer’s protocol (Amersham). After transcription, the template DNA was digested with DNase I for 10 min at 37°C, and the labeled riboprobes were separated from unincorporated [35S]UTP by centrifugation through ProbeQuant G-50 Micro Columns (Pharmacia Biotech, Piscataway, NJ). In situ hybridization was performed as described elsewhere (23). Briefly, sections were fixed, dehydrated, and covered with hybridization buffer containing 4 × 104 cpm/μl probe. After hybridization for 18 h at 54°C, sections were treated with RNase A and washed with increasing stringency. After a final high-stringency wash in 0.1× SSC at 65°C for 1 h, sections were dehydrated and exposed to Hyperfilm–3H for 7 days, and the films were developed as described above. Specific mRNA expression was estimated after hybridization with antisense probes directed against the 3′-UTR of the gAT2 receptor mRNA. Nonspecific background was obtained with sense (control) probes.

For cellular localization of the hybridization signal, slides were dipped in Kodak NTB2 photo emulsion, exposed for 4 wk, developed in Kodak D-19 developer for 3 min at 15°C, fixed for 4 min, and counterstained with toluidine blue.

RESULTS

Southern and Northern blot analysis. As a preliminary experiment to assess the presence of the gAT2 (odk) for 4 min at 22°C. Films were analyzed by measuring optical densities using the NIH Image 1.61 program, quantified by comparison with [125I]Micro-Scales (Amersham, Arlington Heights, IL), and transformed to the corresponding values of femtomoles per milligram of protein (26).
receptor, we performed Southern blot analysis using an hAT2 ORF cDNA as a probe (Fig. 1A). The genomic restriction map was speculated through cDNA sequence comparison with the results of the genomic Southern blot. The genomic regions confirmed by the cDNA sequence are represented by open boxes. The gAT2 ORF is indicated by hatching with flanked 5'- and 3'-UTRs. In addition, the gAT2 gene has one internal KpnI site, as shown by restriction enzyme treatment with KpnI (Fig. 1B).

Molecular cloning of the gAT2 receptor. To identify the gAT2 cDNA, we constructed a cDNA library from mRNA obtained from the gerbil adrenal gland and performed conventional in situ plaque screening using an hAT2 receptor cDNA as a probe (same as in Fig. 1A). The nucleotide sequence of the longest clone of 2,827 bp, named /H9252 (GenBank accession no. AF 080066) contained an ORF of 1,089 bp encoding a protein with 363 amino acid residues (Fig. 2A). There are seven hydrophobic regions that are likely to represent membrane-spanning domains providing the seven-transmembrane region structural topology found among the G protein-linked superfamily of receptors, including other mammalian AT2 receptors. The α-clone, which started from inside the ORF, was a shortened form of the β-clone, and the γ-clone was almost the same as the β-clone, except it missed 14 nt of the 5′-UTR. It appears that the coding region is uninterrupted by introns, as is the case for many G protein-coupled receptors and mammalian AT2 receptors (16). As is the case for most ANG II receptor 3′-UTRs, a typical mammalian poly(A) additional signal motif, AAUAAA, is present from nt 2,662 to nt 2,667, 12 bp from the end of the 3′-UTR, and there are four AUUUA motifs in the 3′-UTR that may relate to rapid receptor mRNA turnover (31).

We prepared a cDNA insert selected in the range of ~1.5–4 kb. The frequency of gAT2 mRNA was as low as 1 of 90,000 clones, according to the results of our plaque screening. The mRNA frequency of the gAT2 receptor gene was 21 times lower than that of the gAT1B receptor gene (23), indicating that the mRNA frequency of the gAT2 receptor gene is comparatively low.

Comparison of the deduced amino acid sequence of the gAT2 receptor with that of hAT2, rAT2, and mAT2 receptors revealed 90–96% identity (Fig. 2A). The gAT2 receptor differs from the hAT2 receptor at only 33 residues (36). Nineteen mismatches are localized to the NH2-terminal extracellular domain, and three mismatches are localized to the COOH-terminal intracellular tail. The sequences corresponding to three of the intracellular loops are identical. There are four mis-

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Fig. 4. Autoradiography of AT2 receptor binding in sections of the gerbil head. A–C: sections from gerbil heads taken at embryonic day 19 (E19). D–F: sections from 1-wk-old gerbils. A and D: sections stained with hematoxylin-eosin. B and E: consecutive sections showing total 125I-CGP-42112 binding. C and F: consecutive sections showing binding as in B and E with addition of unlabeled ANG II (nonspecific binding). Arrows point to tongue filaments (tf), tongue accessories (ta), tongue (to), tongue muscle (tm), ocular muscle (om), retina (r), cartilage primordium of ethmoid bone (ce), skin (sk), palate (p), palate vessels/nerves (g), eyes (e), maxillary molar (mxm), mandibular molar (mdm), and facial muscle (fm).
matches in the extracellular loops (Val\textsuperscript{196}, Ser\textsuperscript{282}, Ile\textsuperscript{286}, and Met\textsuperscript{293}) and seven amino acid differences in the transmembrane (TM) domains (Val\textsuperscript{49}, Leu\textsuperscript{53}, Ala\textsuperscript{60}, Ile\textsuperscript{64}, and Ser\textsuperscript{67} in TM-I, Val\textsuperscript{163} in TM-IV, and Val\textsuperscript{217} in TM-V; Fig. 2B). The gAT\textsubscript{2} receptor differs from the rAT\textsubscript{2} receptor at an even lower number of residues, only 14. Seven mismatches are localized to the NH\textsubscript{2}-terminal extracellular domain and only one to the COOH-terminal intracellular tail. The sequences corresponding to four of the intracellular loops are identical. There are three mismatches in the extracellular loops, and three amino acid differences are found in the transmembrane domains (Leu\textsuperscript{53} and Ile\textsuperscript{64} in TM-I and Val\textsuperscript{217} in TM-V; Fig. 2C). With few exceptions, the mismatches between the gAT\textsubscript{2} and rAT\textsubscript{2} receptors are identical to some of those found between the gAT\textsubscript{2} and hAT\textsubscript{2} receptors (Fig. 2, B and C). The gAT\textsubscript{2} receptor differs from the mAT\textsubscript{2} receptor in 16 residues, and the mismatches between the gAT\textsubscript{2} and mAT\textsubscript{2} receptors are almost identical to the mismatches between the gAT\textsubscript{2} and rAT\textsubscript{2} receptors (Fig. 2D).

Transcription of the gAT\textsubscript{2} receptor in selected tissues. We performed a Northern blot analysis to determine which adult gerbil tissues could transcribe the gAT\textsubscript{2} mRNA (Fig. 3). Of the tissues studied, only the gerbil adrenal gland transcribed the gAT\textsubscript{2} receptor mRNA in a detectable amount. There was no detectable gAT\textsubscript{2} mRNA in the pituitary, kidney, and hippocampus (Fig. 3).

Expression of AT\textsubscript{2} receptor binding in the gerbil head at E19 and 1 wk of age. To clarify whether the developmental expression pattern of the gAT\textsubscript{2} receptor is similar to that of the rAT\textsubscript{2} receptor, we studied the binding of \textsuperscript{125}I-CGP-42112 to heads of fetal and 1-wk-old gerbils. In the gerbil head at E19, binding of \textsuperscript{125}I-CGP-42112, revealing AT\textsubscript{2} receptors, was highest in the dental papillas at the epithelial root sheath in maxillary and mandibular molars (Fig. 4, Table 1). Binding was also high, in descending order, in the greater palatine vessels and ocular, facial muscle, skin, and retina (Fig. 4, Table 1). AT\textsubscript{2} receptor binding in the heads of 1-wk-old gerbils revealed a distribution similar to that in the fetal gerbil, although binding was generally lower than in the fetus (Fig. 4, Table 1). In addition, postnatally, we found no AT\textsubscript{2} binding in the mandibular molars and a very pronounced decrease in AT\textsubscript{2} binding in the skin (Fig. 4, Table 1).

Localization of gAT\textsubscript{2} receptor mRNA in fetal and postnatal teeth by in situ hybridization and emulsion autoradiography. The gAT\textsubscript{2} receptor mRNA was detected by hybridization to sections from gerbils at E19 and 1 wk of age. Arrows, base of dental papilla.

![Fig. 5. Emulsion autoradiography showing in situ hybridization of AT\textsubscript{2} receptor mRNA in maxillary molar of the gerbil. A and B: hematoxylin-eosin stain and emulsion autoradiography in sections from gerbils at E19. C and D: hematoxylin-eosin stain and emulsion autoradiography in sections from 1-wk-old gerbils. Arrows, base of dental papilla.](http://ajpregu.physiology.org/)

Table 1. AT\textsubscript{2} receptor binding in selected areas of the gerbil head.

<table>
<thead>
<tr>
<th>Localization</th>
<th>AT\textsubscript{2} Binding, fmol/mg protein</th>
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<tbody>
<tr>
<td>Cartilage primordium of ethmoid bone</td>
<td>84 ± 7</td>
</tr>
<tr>
<td>Facial muscle</td>
<td>196 ± 21</td>
</tr>
<tr>
<td>Ocular muscle</td>
<td>287 ± 82</td>
</tr>
<tr>
<td>Tongue muscle</td>
<td>45 ± 7</td>
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<tr>
<td>Tongue filaments</td>
<td>120 ± 6</td>
</tr>
<tr>
<td>Palate</td>
<td>190 ± 58</td>
</tr>
<tr>
<td>Palate vessels/nerves</td>
<td>356 ± 72</td>
</tr>
<tr>
<td>Retina</td>
<td>146 ± 32</td>
</tr>
<tr>
<td>Skin</td>
<td>155 ± 20</td>
</tr>
<tr>
<td>Mandibular molar</td>
<td>455 ± 118</td>
</tr>
<tr>
<td>Maxillary molar</td>
<td>384 ± 100</td>
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Values are means ± SE for groups of 6 animals measured individually. ND, binding not detectable above nonspecific binding. *P < 0.05 vs. embryonic day 19 (E19).
autoradiography. In situ hybridization followed by emulsion autoradiography revealed the highest mRNA expression at E19 at the base of the dental papilla near the epithelial root sheath of maxillary and mandibular molars (Fig. 5, A and B). Expression of gAT2 receptor mRNA was lower in the maxillary molar of 1-wk-old animals (Fig. 5, C and D).

Binding properties of the cloned gAT2 receptor transfected to COS-7 cells. To investigate its ligand-binding properties, the cloned gAT2 receptor was transiently transfected into COS-7 cells. The receptor bound $^{125}$I-CGP-42112 specifically with a $K_d$ of 0.6 nM (Fig. 6A), similar to values obtained with hAT2 receptor cDNA-transfected COS-7 cells ($K_d = 0.3$ nM). No binding was detected in untransfected cells or cells transfected with the vector DNA alone. The ligand affinity profile of the gerbil receptor after transfection of COS-7 cells was similar to that observed for the hAT2 receptor, with highest affinity for CGP-42112, followed by ANG III and then PD-123319, and no significant displacement by the AT1 receptor-selective antagonist losartan. Conversely, ANG II displaced $^{125}$I-CGP-42112 from the gAT2 receptor with fourfold lower affinity than from the hAT2 receptor (Fig. 6B, Table 2).

### Table 2. Binding of gerbil and human AT2 receptors after transfection to COS-7 cells

<table>
<thead>
<tr>
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<th>IC$_{50}$, nM</th>
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<tbody>
<tr>
<td>CGP-42112</td>
<td>1.35</td>
</tr>
<tr>
<td>ANG II</td>
<td>9.8</td>
</tr>
<tr>
<td>ANG III</td>
<td>2.8</td>
</tr>
<tr>
<td>PD-123319</td>
<td>15.4</td>
</tr>
<tr>
<td>Losartan</td>
<td>ND</td>
</tr>
<tr>
<td>Human</td>
<td>0.65</td>
</tr>
<tr>
<td>ANG II</td>
<td>2.7</td>
</tr>
<tr>
<td>ANG III</td>
<td>2.5</td>
</tr>
<tr>
<td>PD-123319</td>
<td>22.4</td>
</tr>
<tr>
<td>Losartan</td>
<td>ND</td>
</tr>
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Values are means of experiments performed at least twice in quadruplicate samples. ND, binding was not displaced by $\leq 10^3$ nM losartan.

Binding properties of the gAT2 and rAT2 receptors in adrenal glands, as determined by autoradiography. To further characterize the affinity profile of the gerbil receptor, we carried out competition studies in consecutive sections of gerbil and rat adrenals. These studies indicated that displacement of $^{125}$I-CGP-42112 by unlabelled CGP-42112 was similar in adrenal medulla and cortex from gerbils and rats (Fig. 7, Table 3). However, ANG II, ANG III, and PD-123319 displaced $^{125}$I-CGP-42112 from the gerbil adrenal medulla and cortex with a 10- to 20-fold lower affinity than from rat adrenal medulla and cortex (Fig. 7, Table 3).

![Fig. 6. Binding characteristics of gAT2 (left) and hAT2 (right) receptors transfected to COS-7 cells. A: saturation isotherm of specific $^{125}$I-CGP-42112 (CGP) binding in AT2 receptors transfected to COS-7 cells. Insets: Scatchard plots. B: displacement of $^{125}$I-CGP-42112 binding to AT2 receptors transfected to COS-7 cells by increasing concentration of unlabeled CGP-42112, ANG III, ANG II, PD-123319, and losartan.](http://ajpregu.physiology.org/ by 10.220.33.2 on June 26, 2017)
DISCUSSION

The molecular characteristics of the gAT2 cDNA are similar to those of other mammalian AT2 receptors, with 90.9, 96.1, and 95.6% identity with the hAT2, rAT2, and mAT2 receptors, respectively (25, 36).

We found significant gAT2 mRNA transcription in adrenal gland and no significant transcription in pituitary, kidney, or hippocampus, tissues that express, in other rodent species, predominantly AT1, and not AT2, receptors (1, 33, 35). However, the amount of mRNA transcribed could be below the sensitivity of the method, because transcription may occur in small areas such as the olfactory bulb, the only region of the adult gerbil brain with significant gAT2 receptor expression (5).

We studied expression of gAT2 receptor binding and mRNA in fetal and early postnatal gerbil heads. We found massive expression of AT2 receptors in connec-

Fig. 7. Binding characteristics of AT2 receptors in gerbil and rat adrenal cortex (A) and medulla (B) as determined by quantitative autoradiography. Displacement of 125I-CGP-42112 binding to AT2 receptor by increasing concentrations of CGP-42112, ANG II, ANG III, and PD-123319 is shown.
To determine the importance of nonconserved amino acids in the NH2-terminal tail of the gAT2 receptor, we compared the affinity of the gAT2 receptor with that of the hAT2 receptor. The NH2-terminal tail contains 12 nonconserved amino acids in the gAT2 receptor, compared with the hAT2 receptor. These differences in affinity profile between the gAT2 and hAT2 receptors may be related to the influence of nonconserved amino acids in the gAT2 receptor. First, there are seven nonconserved amino acids in the NH2-terminal tail of the gAT2 receptor compared with the hAT2 receptor. The NH2-terminal tail interacts with the NH2-terminal end of ANG II (30). This receptor domain is important for ANG II, but not for CGP-42112, binding affinity (40). Second, nonconserved amino acids in the second and third extracellular loops of the gAT2 receptor (Val196 and Met293, respectively) are close to mutations Arg182Ala and Asp297Lys, also reported to decrease affinity for ANG II (13, 18, 19). The Asp297Lys mutation did not decrease receptor affinity for CGP-42112 (18), and we report that the affinity for this compound is preserved in the gerbil. However, the mutation Arg182Ala may be critical for ANG II and CGP-42112 binding (19). Third, a positively charged side chain of Lys215 in the rAT2 receptor, located in TM-V, binds to the COOH terminus of ANG II (28). Lys215 in the AT2 receptor occupies a position similar to that of Lys199 in the AT1 receptor, which binds to ANG II in a similar manner (28). Replacement of Lys215 by Glu or Gln greatly reduced affinity for ANG II, retaining CGP-42112 affinity (28). Ile217 in the rAT2 receptor is not conserved in the gAT2 receptor and is replaced by Val217. Lack of conservation in this position, located close to Lys215, could contribute to decreased ANG II and ANG III affinity. It is possible that the additional CH3 group provides a steric hindrance for the interaction with ANG II. Although replacement of Lys215 by Glu or Gln did not affect binding to PD-123319 (28), it may be possible that Ile217Val nonconservation very substantially decreased affinity for PD-123319.

The COOH-terminal cytoplasmic domain may also be important for the affinity profile of the AT2 receptor. COOH-terminal truncated AT2 receptors express reduced affinity for ANG II and enhanced CGP-42112 binding (27). However, we found only one nonconserved amino acid in the COOH-terminal domain of the gAT2 receptor compared with the rAT2 receptor (Met392). Comparison between the gAT2 and mAT2 receptors revealed similar amino acid nonconservancy.

The positions described here are not the only ones reported to influence the binding profile of the AT2 receptor. Others include position His273 (38), the inner half of TM III (30), the third intracellular loop domain (6), and the two disulfide bridges present not only in the AT1, but also in the AT2, receptor (7, 13). All these domains, however, are conserved in the gerbil compared with the rat.

There was less amino acid homology between gAT2 and hAT2 receptors than between gAT2 and rAT2 receptors, with 33 nonconserved amino acids in the gAT2 receptor. For the most part, all amino acids nonconserved in the gAT2 receptor compared with the rAT2 receptor were also nonconserved compared with the hAT2 receptor. There were 12 additional nonconserved amino acids in the NH2-terminal tail, 3 in TM-I, 1 in the third extracellular loop, and 2 in the COOH-terminal cytoplasmic domain. For these reasons, it was surprising to find that although the affinities for ANG II, ANG III, and PD-123319 for the gAT2 receptor were much lower than those for the rAT2 receptor, the gAT2 receptor exhibited only a threefold decrease in ANG II affinity compared with the hAT2 receptor, with no differences in affinity for ANG III or PD-123319. It is nevertheless entirely possible that binding profiles for receptors transfected into COS-7 cells are not identical to those of native receptors residing in their natural membrane environment. Determination of receptor binding by autoradiography has its own particular limitations. On the other hand, constructing and transfecting an isolated receptor to a cell not naturally

<p>| Table 3. Binding to AT2 receptors in gerbil and rat adrenal medulla and cortex as determined by quantitative autoradiography |
|-----------------|----------------|----------------|----------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>IC50, nM</th>
<th>ANG II</th>
<th>ANG III</th>
<th>PD-123319</th>
<th>Losartan</th>
</tr>
</thead>
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<tr>
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<td>43</td>
<td>45</td>
<td>43</td>
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<tr>
<td>Rat</td>
<td>1.3</td>
<td>9.7</td>
<td>12</td>
<td>7.3</td>
<td>ND</td>
</tr>
<tr>
<td>Gerbil</td>
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<td>82</td>
<td>170</td>
<td>80</td>
<td>ND</td>
</tr>
<tr>
<td><strong>IC50 values for displacement of 125I-CGP-42112 binding were determined by incubation of consecutive sections with 125I-CGP-42112 and increasing concentrations of CGP-42112, ANG II, ANG III, PD-123319, and losartan. ND, not displaced by ≤106 nM losartan.</strong></td>
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</tr>
</tbody>
</table>
expressing such a receptor, an excellent tool to study newly cloned or point-mutated receptors, do not reproduce the natural environment. Determination of binding profiles for natural receptors expressing nonconserved amino acids in important positions, as demonstrated for the gAT1A and gAT1B receptors (15), remains an important tool to determine the relative importance of selective positions and binding domains.

We conclude that although our observations support the hypothesis of different requirements for ANG II, the hypothesis of different requirements for ANG II, remains an additional important tool to determine the relative importance of selective positions and binding domains.

The question remains regarding the physiological significance of the findings reported here, in particular with respect to human pathology. It is of interest that, in the gerbil, Gly21Val is identical to the hAT2 receptor mutation found in some patients with profound X-linked mental retardation (38). Other substitutions reported here are close to additional amino acid substitutions (Arg22Gln and Ile39Val) and polymorphic variants in this disease (38). Whether the decreased affinity of the gAT2 receptor to ANG II reported here and the hypothesized deficiency in AT2 receptor function leading to developmental brain abnormalities in X-linked mental retardation are dependent on similar amino acid substitutions has not been established.

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3. Christensen LR, Janas MS, Mollgard K, and Kjaer I. An immunocytochemical study of the innervation of developing human fetal teeth using protein gene product 9.5 (PGP 95) [125I]-polymer standards with [125I]-brain paste standards for quantitation leading to developmental brain abnormalities in X-linked mental retardation are dependent on similar amino acid substitutions has not been established.


