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ACCELERATED COMMUNICATION

A Cloned Angiotensin Receptor Isoform from the Turkey Adrenal Gland Is Pharmacologically Distinct from Mammalian Angiotensin Receptors

T. J. MURPHY, YASUYUKI NAKAMURA, KAZUHISA TAKEUCHI, and R. WAYNE ALEXANDER

Department of Medicine, Division of Cardiology (T.J.M., Y.N., K.T., R.W.A.), and Department of Pharmacology (T.J.M.), Emory University School of Medicine, Atlanta, Georgia 30322

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SUMMARY

A 2046-base pair cDNA clone, homologous to mammalian angiotensin (AT) AT_1 receptors, was isolated from a library prepared from adrenal glands of the domestic turkey (*Meleagris gallopavo*). Sequence analysis of the cDNA insert in clone pTAT2′ reveals a 1077-base pair open reading frame predicting a 359-amino acid protein ~75% homologous to mammalian AT_1 receptors. Saturation radioligand binding studies performed in membranes of COS-7 cells transfected with pTAT2′ show high affinity specific binding of ^{125}I-angiotensin II, with a K_d of 172 pM. The rank order of affinities for a series of ligands determined by competition binding studies is angiotensin II ≥ [Sar^1,Ile^8]-angiotensin II > angiotensin III ≈ [Sar^1,Ala^8]-angiotensin II ≈ CGP42112A > angiotensin I > Dup753 > PD123177. This rank order of affinity series differs substantially from that for mammalian AT_1 receptors and AT_2 binding sites. Angiotensin II (100 nM) can stimulate inositol phosphate production similarly in COS-7 cells transfected with pTAT2′ and in COS-7 cells transfected with the AT_{1a} receptor cDNA pCa18b. This response in pTAT2′-transfected cells is not attenuated in the presence of 30 μM Dup753. In contrast, this concentration of antagonist attenuates >90% of the inositol phosphate response to angiotensin II in COS-7 cells transfected with the rat AT_{1a} receptor cDNA. These results demonstrate an avian structural homologue of mammalian AT_1 receptors possessing distinct pharmacological properties with both peptide and nonpeptide AT receptor ligands.

Two classes of mammalian receptors for angiotensins (AT receptors), termed AT_1 receptors and AT_2 binding sites, differ in their affinities and selectivity for synthetic analogues developed relatively recently (1). AT_1 receptors have high affinity (1–10 nM) for biphenylimidizoles such as Dup753 and low affinity (millimolar) for imidazopyridine carboxylic acids such as PD123177. AT_2 binding sites have low affinity (micromolar) for Dup753 and 10–100 nM affinity for PD123177. The modified peptide derivative CGP42112A further discriminates AT_1 receptors (~1000 nM affinity) from AT_2 binding sites (~1 nM affinity). AT_1 receptors are thought to mediate most of the cardiovascular actions of angiotensins (2–4), which involve coupling of the heptahelical AT_1 receptor proteins to effector pathways through heterotrimeric GTP-binding proteins. Although little consensus exists on the functional relevance of AT_2 binding sites (Ref. 5 and references cited therein), the failure to observe guanine nucleotide-induced shifts in affinity for agonist competition binding curves may be evidence that these sites are either weakly coupled to or uncoupled from heterotrimeric GTP-binding proteins (6–8).

Isoforms of AT_1 receptors have now been cloned from several mammalian species (9–14). Their predicted amino acid sequences reveal them to be highly related members of the large family of seven-transmembrane domain GTP-binding protein-coupled receptors. Two isoforms of AT_1 receptors that are 95% identical to each other, termed AT_{1a} and AT_{1b} receptors, are encoded by apparently separate genes in rat and mouse. These cloned isoforms share high amino acid homology (~90%) with isoforms cloned from bovine and human sources, which so far appear to contain only a single gene for AT_1 receptors (15).

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ABBREVIATIONS: AT receptor, angiotensin receptor; angiotensin I, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu; angiotensin II, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe; angiotensin III, Arg-Val-Tyr-Ile-His-Pro-Phe; [Sar^1,Ile^8]-AngII, Sar-Arg-Val-Tyr-Ile-His-Pro-Ile; [Sar^1,Ala^8]-AngII, Sar-Arg-Val-Tyr-Ile-His-Pro-Ala; CGP42112A, *N*-α-nicotinoyl-Tyr-(*N*-α-Cbz-Arg)-Lys-His-Pro-Ile-OH; Dup753, 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole, potassium salt; PD123177, 1-(4-amino-3-methylphenyl)methyl-5-diphenylacetyl-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridine-6-carboxylic acid·2HCl; DMEM-PS, high-glucose Dulbecco's modified Eagle's medium with glutamine plus penicillin and streptomycin supplementation; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kb, kilobase(s); bp, base pair(s); PCR, polymerase chain reaction.

When expressed in surrogate systems, the cloned mammalian isoforms demonstrate virtually identical pharmacological properties where examined (9–14, 25).

AT receptors have been described in some tissues of a few nonmammalian vertebrates, including *Xenopus laevis* and *Aves* (16, 17). These receptors appear to have pharmacological properties quite distinct from those of mammals. The AT receptors found in adrenal glands of domestic turkeys stimulate aldosterone production and induce Ca^{2+} mobilization, similar to the known responses of AT_1 receptor activation in mammalian adrenal glands (18). A notable difference, however, is that AT receptors in turkey adrenal glands appear to have lower affinity for Dup753 than do their mammalian counterparts. Taken together, these data led us to explore the possibility that the AT receptors expressed in the domestic turkey adrenal gland may share topographical similarities with the AT_1 receptors of mammals yet differ significantly in their domains involved in ligand recognition. To begin to test this hypothesis, we sought to isolate cDNAs encoding the AT receptors expressed in the domestic turkey adrenal gland. Here we demonstrate a cDNA clone that, when expressed in COS-7 cells, binds angiotensin peptides with high affinity and stimulates inositol phosphate production in response to angiotensin II. Nevertheless, it is pharmacologically and structurally distinct from cloned mammalian AT_1 receptor isoforms. These differences between the turkey AT receptor isoform and mammalian AT_1 receptors may prove useful in providing insights regarding structural determinants of AT receptors necessary for ligand recognition.

Materials and Methods

Library construction, screening, and sequencing. Enzymes for molecular cloning were purchased from GIBCO-BRL (Gaithersburg, MD), Stratagene (La Jolla, CA), or United States Biochemicals (Cleveland, OH). Chemicals for buffers and media were purchased from Sigma Chemical Co. (St. Louis, MO) and radiolabeled nucleotides from Amersham, Inc., (Arlington Heights, IL). Standard molecular cloning protocols were used throughout this study (19). Frozen domestic turkey adrenal glands were homogenized in a 4.4 M guanidinium isothiocyanate buffer, and total RNA was isolated using a CsCl step gradient. Polyadenylated RNA was extracted by oligo(dT)-cellulose chromatography, with two passes of total RNA over a 0.5-ml oligo(dT)-cellulose column. First- and second-strand cDNAs were synthesized in parallel reactions with 5 μg of polyadenylated RNA and either oligo(dT) or random hexamer primers, using the Choice cDNA synthesis system (GIBCO-BRL). After the two cDNA mixtures were combined, a ligation was performed with a 250-fold molar excess of *Bst*XI adapters made from the oligonucleotide pair 5′-PO_4-CTGGCGCG-3′ and 5′-PO_4-CGCGCCAGCACA-3′. Fractions of cDNA greater than 1 kb were selected after fractionation on a 1.0-ml Sephacryl S-500 column, pooled, and ligated to stuffer-free, *Bst*XI-cut, vector pCDM8 (20). A library of 4×10^6 independent clones in *Escherichia coli* MC1061/p3 was obtained by electro-transformation using a Bio-Rad Gene Pulser with settings of 25 μF, 400 Ω, and 2.47 kV.

Approximately 1×10^6 independent clones were fixed to nitrocellulose membranes after amplification on agar plates and were hybridized overnight at 42° in 50% formamide, 6× standard saline citrate (150 mM sodium chloride, 15 mM sodium citrate), 0.1% sodium dodecyl sulfate, 5× Denhardt's solution, 100 μg/ml sheared salmon sperm DNA, with a random-primed, [^{32}P]dCTP-labeled, PCR fragment generated from turkey adrenal cDNA (21). This PCR fragment contains nucleotides from bp 432–1148 (see Fig. 1 below) and was a generous gift from Rocco Carsia (University of Medicine and Dentistry of New Jersey) and Jonathan Tilly (Stanford University). The membranes were washed at 50° for 1.5 hr, with three changes of 2× standard saline citrate, 0.1% sodium dodecyl sulfate, and were exposed to Kodak X-OMAT film overnight with a single intensifying screen. Positive colonies were purified through successive rounds of screening. *Xba*I fragments containing the entire inserts from these clones were subcloned into compatible sites of pBluescript (Stratagene). Two independently isolated cDNA clones were sequenced on both strands using double-stranded template from ordered sets of exonuclease III/mung bean nuclease-prepared nested deletions and reagents supplied in a Sequenase kit (United States Biochemicals).

COS-7 cell transfections and radioligand binding assays. Cell culture media and sera were from GIBCO-BRL. Angiotensin peptides were from Sigma. These were dissolved in 10 mM acetic acid, stored at −20° as 5 nmol/μl aliquots, based upon the manufacturer's analysis of peptide purity, content, and mass, and used within 12 months of purchase. DuP753 and PD123177 were gifts from DuPont-Merck, Inc., and CGP42112A was a gift from Ciba-Geigy Inc. (Basel, Switzerland). Carrier-free Na^{125}I (2175 Ci/mmol) was purchased from Amersham. ^{125}I-Angiotensin II (2000 Ci/mmol) was synthesized from angiotensin II using the chloramine T method. Monoiodinated peptide was separated from other reaction products by high performance liquid chromatography on a Vidak 0.46- × 25-cm, C-18, 5-μm column, using a linear 2–50% acetonitrile gradient in water with 0.1% trifluoroacetic acid in both phases, over 45 min, at a flow rate of 1 ml/min. The monoiodinated peptide peak eluted routinely at 25–27% acetonitrile. Protein determinations were by the method of Bradford, using a Bio-Rad kit and γ-globulin as standard. Passages 11–20 of COS-7 cells (CRL 1651; American Type Culture Collection, Rockville, MD) were used for transfections. These were subcultured in a 5% CO_2 atmosphere at 37° in DMEM/PS with 10% fetal bovine serum. For transfections, cells were plated on 10-cm dishes in DMEM/PS with 10% NuSerum (Collaborative Research Inc.). Plasmid DNA (20 μg) was mixed in 4 ml of medium and then mixed with 40 μl of 50 mg/ml DEAE-dextran and 4 μl of 100 mM chloroquine phosphate. This mixture was added to a single 10-cm dish, which was returned to the CO_2 incubator for 3.5–4 hr. After this, the cells were rinsed with 4 ml of 10% dimethylsulfoxide in Hanks' balanced salt solution, pH 7.4, with Ca^{2+} and Mg^{2+}, and the dishes were replenished with DMEM/PS plus 10% fetal bovine serum for 48–72 hr. After this, the cell monolayers were rinsed with ice-cold phosphate-buffered saline, pH 7.4, and collected by scraping with a rubber policeman. Cell pellets were resuspended in 30 volumes of ice-cold 50 mM Tris·HCl, pH 8.0, and homogenized with a Tekmar Tissuemizer. After centrifugation at 30,000 × *g* in a Beckman J-2 high speed centrifuge, the particulate was homogenized and recentrifuged as described above. Membrane aliquots were stored as pellets in microfuge tubes at −70° until used for binding assays.

For binding assays, 5–10 μg of membrane protein were resuspended in a buffer containing 25 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$, and 0.1% bovine serum albumin, in a final assay volume of 0.5 ml (5 μl of 100-fold concentrated competitor, 475 μl of membrane solution, and 20 μl of radioligand). Total and nonspecific binding points were measured in triplicate and duplicate, respectively, for saturation binding assays and in duplicate for competition binding assays. These were incubated at 20° for 90 min, followed by aspiration over 0.5% polyethyleneimine-treated GF/B filter strips and two 4-ml washes with 50 mM Tris·HCl, pH 8.0, using a Brandel cell harvester. The samples were counted for 1 or 2 min in a Beckman 4000 γ counter with an efficiency of 74%.

Inositol phosphate assays. COS-7 cells were transfected as described above. Within 24 hr, the cells from a single 10-cm transfected dish were dispersed with trypsin and split into 12 wells on a 12- × 20-mm plate. After attaching to the dishes, the cells were incubated for 36–48 hr with 1.5 μCi (0.05 nmol/ml) of *myo*-[^{3}H]inositol (American Radiolabeled Chemicals, Inc., St. Louis, MO). After this, the dishes were rinsed three times with 5 mM LiCl in HEPES-buffered Hanks' balanced salt solution, pH 7.4, that had been prewarmed to 37°. The dishes were then incubated for 60 min at 37° without or with added ligands. The medium was aspirated and 1.0 ml of ice-cold 20 mM formic

acid in water was added to each dish to stop the reaction. The plates were frozen at −20° and thawed twice before chromatography. The lysate was overlaid onto freshly prepared 0.5-ml Dowex AG 1X8 columns (formate form, 200–400 mesh; Bio-Rad). After 10-ml washes each of distilled water and 5 mM sodium borate/60 mM sodium formate solution were discarded, total inositol phosphates were eluted in 4 ml of 0.1 M formic acid, 2 M ammonium formate. Scintillant was added to the eluates, and samples were counted in a Beckman 2500 scintillation counter for 3–5 min, at an efficiency of 34%.

Results and Discussion

Several strongly hybridizing clones were identified during primary screening, at moderate stringency, of a mixed random oligo(dT)-primed turkey adrenal gland cDNA library. Several weakly hybridizing clones ranging in size from 2.2 to 3.5 kb also survived serial purification through succesive rounds of screening. None of these, however, expressed detectable angiotensin-binding activity when transfected into COS-7 cells. Complete sequence analysis of two of these failed to reveal striking homologies to AT receptors or to known sequences in database searches. Simultaneously with the primary library screen and under the same washing conditions, hybridization of the ^{32}P-labeled turkey adrenal PCR fragment was performed on a nylon membrane from a Northern blot of electrophoretically fractionated rat and turkey adrenal polyadenylated RNA. The probe hybridized to several transcripts in turkey adrenal mRNA that had been described previously (21) but did not hybridize to rat adrenal mRNA (data not shown). This experiment, however, was not repeated. Nevertheless, this suggests that, under these conditions, isolation of rat adrenal cDNA clones encoding homologous AT receptor sequences using the turkey probe would be serendipitous.

Purified clones were tested for expression of ^{125}I-angiotensin II-binding activity by transfection and expression in COS-7 cells. Four strongly hybridizing clones, termed pTAT1a, pTAT10a, pTAT9a, and pTAT2′ and ranging in size from 1.9 to 2.2 kb, had similar diagnostic restriction enzyme patterns and each demonstrated enhanced ^{125}I-angiotensin II-binding activity when transfected into COS-7 cells. Clones pTAT9a (2.2 kb) and pTAT2′ (2.0 kb) were sequenced completely on both strands and shown to encode identical proteins but differed in their extent of 5′ and 3′ untranslated sequence. However, clone pTAT2′ gave comparatively robust levels of binding activity in transfected COS-7 cells, relative to clone pTAT9a, and was characterized further. This clone contains a 2046-bp cDNA insert with 71% overall nucleotide homology to the rat vascular AT_{1a} receptor within a 1077-bp open reading frame (Fig. 1). This open reading frame begins with a strong candidate for an in-frame initiation codon that is downstream of a termination codon and is presumed to be the translation initiation site on this basis and because of the similarity of this region to that of the cloned mammalian AT1 receptor isoforms. The open reading frame predicts a 359-amino acid protein of calculated molecular mass 41,250 that is ~75% homologous to mammalian AT_1 receptors. Computer-assisted hydropathic modeling reveals seven regions of hydrophobic amino acids, consistent with the structures found both for mammalian AT_1 receptors and for other receptors coupled to GTP-binding proteins (22, 23). The predicted protein contains four potential sites for asparagine-linked glycosylation on putative extracellular domains, i.e., Asn^4 near the amino terminus and Asn^{177}, Asn^{187}, and Asn^{188} on the second putative extracellular loop.

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-275                    CCCACGTGACCTGAGCAGCGCAGCCCGGCGCCGTGCCAG
-236  AGCCGACGGGGCTGAGTCGCCCGCCGTCGGCGTGCCCGTGCGGCGGGGCTACGGCAGTG
-177  ATGCCCTCCGCCGAGCCAGGCACAGAAAATGGGTAATAATTGAAGACTACCTCCCAGCA
-118  GGAAAGTGAGATCAGATTGGCAACGAATGGCATGATTGAAGACAAAAAGCCAGCTTCAG
 -59  CACAAGGTTTGATCTTCACATTGTTTCCTTGAAACAAGGCCACAGCAGAAGAGATCAAG

   1  ATG GTC CCA AAC TAT TCT ACT GAA GAA ACT GTT AAG AGA ATT CAC
      M   V   P   N*  Y   S   T   E   E   T   V   K   R   I   H     15
  46  GTC GAC TGT CCT GTT TCA GGA AGG CAC AGT TAC ATC TAC ATT ATG
      V   D   C   P   V   S   G   R   H   S   Y   I   Y   I   M     30
  91  GTT CCA ACT GTT TAC AGT ATC ATC TTC ATC ATA GGC ATA TTT GGG
      V   P   T   V   Y   S   I   I   F   I   I   G   I   F   G     45
 136  AAC AGC CTG GTC GTT ATT GTC ATT TAC TGC TAC ATG AAA TTA AAA
      N   S   L   V   V   I   V   I   Y   C   Y   M   K   L   K     60
 181  ACA GTG GCC AGC ATC TTT CTG CTA AAC CTG GCA CTG GCT GAC TTG
      T   V   A   S   I   F   L   L   N   L   A   L   A   D   L     75
 226  TGT TTT TTA ATA ACT CTG CCA CTC TGG GCA GCC TAC ACG GCC ATG
      C   F   L   I   T   L   P   L   W   A*  A   Y   T   A   M     90
 271  GAA TAC CAG TGG CCT TTT GGC AAC TGT TTA TGC AAG CTA GCA TCA
      E   Y   Q   W   P   F   G   N   C   L   C   K   L   A   S    105
 316  GCA GGA ATA AGT TTC AAT TTG TAT GCC AGT GTG TTC CTA CTC ACG
      A   G   I   S   F   N   L   Y   A   S   V   F   L   L   T    120
 361  TGC CTT AGT ATC GAC CGC TAT CTG GCC ATA GTA CAT CCA GTG AAG
      C   L   S   I   D   R   Y   L   A   I   V   H   P   V   K    135
 406  TCA CGA ATC CGG CGT ACC ATG TTT GTT GCC AGA GTA ACC TGC ATT
      S   R   I   R   R   T   M   F   V   A   R   V   T   C   I    150
 451  GTC ATC TGG CTC CTT GCT GGT GTG GCC AGT TTG CCC GTC ATC ATT
      V   I   W   L   L   A   G   V   A   S   L   P   V   I   I    165
 496  CAT CGT AAT ATA TTT TTT GCA GAG AAC TTG AAC ATG ACA GTC TGT
      H   R   N   I   F   F   A   E   N   L   N*  M   T   V   C    180
 541  GGC TTT CGA TAT GAC AAC AAT AAC ACA ACA CTG AGG GTT GGG TTA
      G   F   R   Y   D   N   N*  N*  T   T   L   R   V   G   L    195
 586  GGT TTA TCC AAG AAT TTA CTG GGA TTT TTG ATC CCT TTT CTT ATC
      G   L   S   K   N   L   L   G   F   L   I   P   F   L   I    210
 631  ATA CTA ACA AGC TAC ACC CTA ATT TGG AAG ACC CTG AAG AAG GCA
      I   L   T   S   Y   T   L   I   W   K   T   L   K   K   A    225
 676  TAT CAA ATT CAA AGA AAT AAG ACC AGA AAT GAT GAC ATT TTT AAG
      Y   Q   I   Q   R   N   K   T   R   N   D   D   I   F   K    240
 721  ATG ATT GTG GCA ATA GTA TTT TTC TTC TTC TTT TCC TGG ATT CCT
      M   I   V   A   I   V   F   F   F   F   F   S   W   I   P    255
 766  CAT CAA GTG TTC ACT TTT CTG GAT GTA TTA ATT CAA TTA CAT GTA
      H   Q   V   F   T   F   L   D   V   L   I   Q   L   H   V    270
 811  ATA ACA GAC TGC AAA ATC ACT GAT ATT GTG GAT ACA GCT ATG CCC
      I   T   D   C   K   I   T   D   I   V   D   T   A   M   P    285
 856  TTT ACT ATT TGC ATC GCT TAC TTT AAT AAT TGT TTG AAT CCT TTT
      F   T   I   C   I   A   Y   F   N   N   C   L   N   P   F    300
 901  TTT TAT GTT TTC TTT GGA AAA AAC TTT AAA AAA TAC TTC CTT CAG
      F   Y   V   F   F   G   K   N   F   K   K   Y   F   L   Q    315
 946  CTA ATA AAA TAC ATT CCA CCA AAT GTC AGC ACA CAT CCA AGT CTC
      L   I   K   Y   I   P   P   N   V   S   T   H   P   S   L    330
 991  ACT ACA AAA ATG AGC TCC CTC TCG TAT CGA CCA CCA GAA AAT ATA
      T   T   K   M   S   S   L   S   Y   R   P   P   E   N   I    345
1036  CGC TTG CCC ACC AAA AAG ACT GCT GGG TCT TTC GAC ACT GAG TGA
      R   L   P   T   K   K   T   A   G   S   F   D   T   E   Umb  359
1081  TGATGCATTTTGCTATATACTTTTTTCCTGAACAAGGTTGTGAACGAAGCAGCATGAAT
1140  GACGAAATCCATCTGCAGTCCCAGACATCGCAGCTTACTGCTTATTACAGAAACATCAA
1199  ATCACCTCAGCAAAATCACAGTACAAAGATTTAGCAGTGCCCTTCTATTTTACATTGTG
1258  CAGAGGACTGCCTGATTTTCATTTTGTCTTACTTTGTTGAAAGCAGCATAAATGATAGG
1317  ACAGGTACATCAGAGTTTGTCAGCATTCTGCCATTTGCTCAAAACTACAGAAGATCTCT
1376  GAACTTAAAGGAAAAGCTTCTGTAAGTGACATGAATTATGAAGAACCTGTTTTGCACAT
1435  GTGAGCCAAGTTTCGCTAAGTTTTGTTTCAGGCCCTGATGGAACTACACTGCTTGGTTT
1494  TGGGTTTGTGGGGTTTTTTTGTTGTTATTTTGTAGGTTGTTTGTTGTTGTTCTTTTTAA
1553  TGAGTCATTTGTCATTGTATTTATAACTTGTTTTTCAAATATATACTTTATCTACAGGC
1612  CCAACTTAAATTGAAATTTAAGGACCATGAAACTGGTCTCATTCTGATTTGTGCTACTA
1671  TTTTCCATTTTTAAATAGCAAAATCTCATAATATATGCAATAAAATGCAGGTTTATTTA
1730  TTAAATATAGAATAAATATATTTTTAGATTTATATTCCTAAA
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Fig. 1. Nucleotide and amino acid sequences of the cDNA insert in clone pTAT2′. The 5′ untranslated nucleotide sequence is negatively numbered from the first base upstream of the initiator codon. Seven domains of hydrophobic amino acid sequence are *underlined*. *, Putative extracellular consensus sites for *N*-linked glycosylation. A potential polyadenylation signal in the 3′ untranslated region is *double-underlined*. This sequence corresponds to an overlapping region of two independently isolated clones that differ only in their most 5′ and 3′ untranslated domains. *Umb*, umber codon.

Three of these are identically placed in the rat AT_1 receptor isoforms. Determination of whether one or more of these sites are glycosylated will require additional experimentation. Additional experiments will also be needed to assess whether glycosylation at any of these residues affects the functional properties of the receptor.

Radioligand binding studies using ^{125}I-angiotensin II were performed to assess the pharmacological properties of clone pTAT2′ after expression in COS-7 cells. No specific binding of radioligand was observed in COS-7 cells transfected with the vector pCDM8 alone (data not shown). Kinetic binding analysis performed in membrane preparations from pTAT2′-trans-

fected cells using 0.1 nM ^{125}I-angiotensin II demonstrated achievement of steady state after 75 min of incubation (data not shown). This was composed of rapidly associating and slowly dissociating interactions, giving a kinetically derived equilibrium dissociation constant of 0.22 nM. These results agree well with the K_d value of 0.172 ± 0.003 nM (three experiments) determined from saturation binding assays (Fig. 2) and with the K_i value of 0.22 nM for angiotensin II determined in competition binding assays. (Fig. 2; Table 1). The B_{max} value of AT receptor expression from pTAT2′ in COS-7 cells was 2.2 pmol/mg of membrane protein for the experiment shown in Fig. 2 and is consistent with a high level of receptor expression in this system.

Competition binding assays demonstrated that clone pTAT2′ encodes an AT receptor that is pharmacologically distinct from both the AT_1 receptors and the AT_2 binding sites of mammals (Fig. 2; Table 1). The compounds tested revealed several differences between the cloned turkey AT receptor and mammalian AT_1 receptors and AT_2 binding sites in both absolute affinity values and rank order of affinities. Other binding studies of cloned rat AT_1 receptor isoforms and of the AT_2 binding site have demonstrated that the affinities of [Sar^1,Ala^8]-AngII and [Sar^1,Ile^8]-AngII are equivalent at mammalian AT binding sites (Table 1) (7, 24, 25). However, [Sar^1,Ile^8]-AngII was 60-fold more potent than [Sar^1,Ala^8]-AngII at the cloned turkey AT receptor. Furthermore, angiotensin II generally is equipotent with or slightly more potent then angiotensin III at mammalian AT_1 receptors and is equipotent with or has slightly less affinity than angiotensin III at AT_2 binding sites. At the turkey AT receptor, however, angiotensin II was 73-fold more potent than angiotensin III. Additionally, unlike any described AT_1 receptor or AT_2 binding site, the affinities of angiotensin III and [Sar^1,Ala^8]-AngII were equivalent at the cloned turkey AT receptor. The affinity of CGP42112A for AT_2 binding sites is higher than that observed for the cloned turkey AT receptor (24). This in turn is a higher affinity than observed for mammalian AT_1 receptors, where the K_i values of CGP42112A are generally ~1000 nM (24, 25). As for AT_1 receptors, the affinity of PD123177 is quite low for the turkey AT receptor. The affinity of Dup753 for the cloned turkey AT receptor is 3 orders of magnitude less than that found for mammalian AT_1 receptor isoforms and is nearer to that often observed for Dup753 at

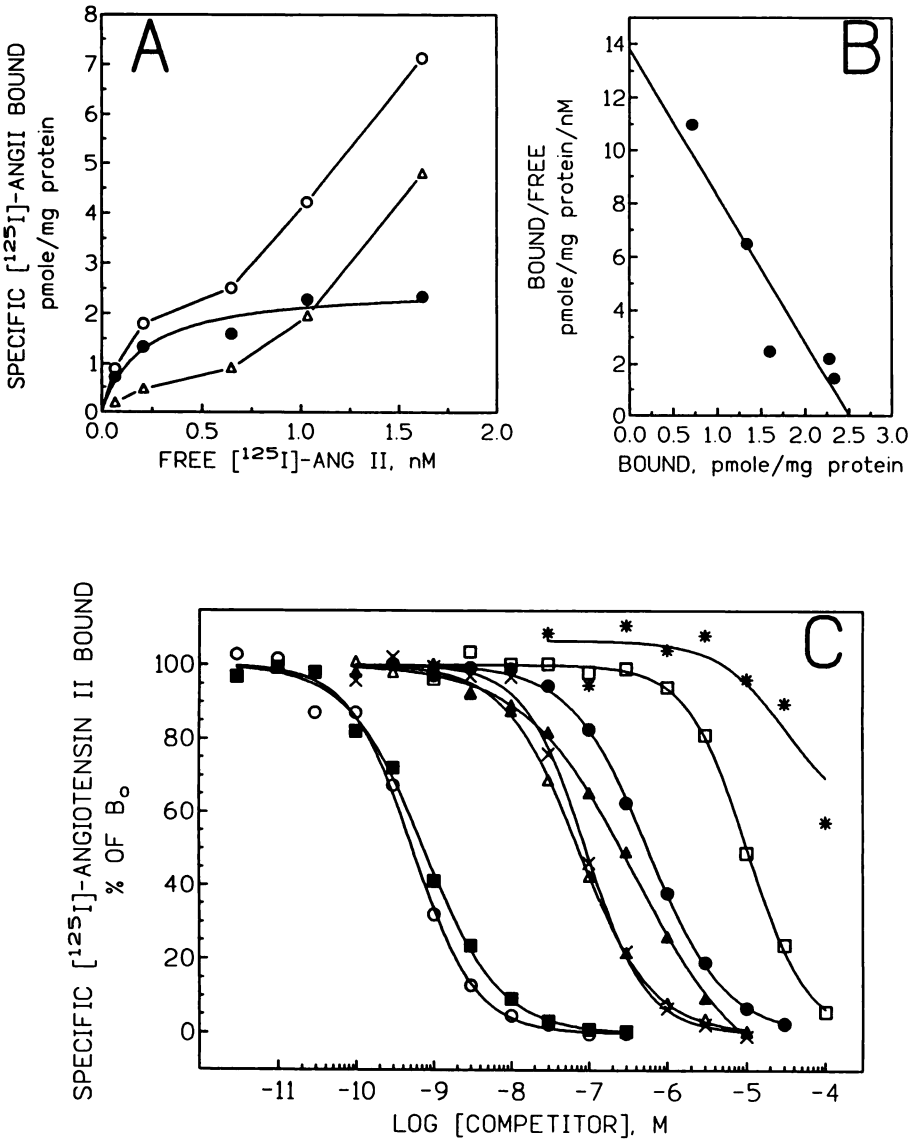

Fig. 2. Representative ^{125}I-angiotensin II binding curves in membranes of COS-7 cells transfected with the turkey AT receptor cDNA clone pTAT2′. A, Saturation binding isotherms. ○, Total binding; △, nonspecific binding in the presence of unlabeled 100 nM [Sar^1,Ile^8]-AngII; ●, specific binding (total minus nonspecific binding). The specific binding curve is a computer-generated, nonlinear regression, best fit of the data points (GraphPad Inplot). B, Rosenthal transformation of the specific binding data. C, Competition binding curves. Each *point* represents the average of duplicate determinations from a single experiment and is expressed as a percentage of specific binding (~10,000 cpm) in the absence of competitor (B_o). Total added radioligand was 0.07 nM. Nonspecific binding (~1500 cpm) was determined for each curve using 100 nM [Sar^1,Ile^8]-AngII. Each *curve* is a computer-generated, nonlinear regression, best fit to the data points using GraphPad Inplot. ●, Angiotensin I; ○, angiotensin II; ▲, angiotensin III; △, [Sar^1,Ala^8]-AngII; ■, [Sar^1,Ile^8]-AngII; □, Dup753; ×, CGP42112A; *, PD123177.

TABLE 1

Competition binding profile of COS-7/pTAT2′ membranes and comparisons with K_i and IC_{50} values for mammalian and nonmammalian AT receptor isoforms

K_i values for the COS-7/pTAT2′ membrane binding assays were derived from the relationship $K_i = IC_{50}/[1 + (F/K_d)]$. IC_{50} values were determined using the nonlinear, single-site, curve-fitting function of GraphPad Inplot. F is the concentration (nM) of free ^{125}I-angiotensin II added, and K_d is the affinity constant of ^{125}I-angiotensin II (0.17 nM) derived from saturation binding assays.

Ligand	COS-7/pTAT2′, K_i[a]	n[b]	Cloned rat AT_{1a}, K_i	Cloned rat AT_{1b}, K_i	AT_2, human uterus, K_i	AT_2, R373 cells, IC_{50}	*Xenopus laevis*, cardiac, IC_{50}	Pekin duck, adrenal, K_i
	nM		nM	nM	nM	nM	nM	nM
Angiotensin I	206 ± 59	3	74	397	ND[c]	130	5.3	72
Angiotensin II	0.22 ± 0.06	3	1.6	1.1	0.66	0.59	2.0	1.3
Angiotensin III	16 ± 4	3	5.4	4.3	0.38	0.49	ND	51
[Sar^{1}Ile^8]-AngII	0.45 ± 0.12	3	0.43	0.18	0.45	ND	0.91	0.6
[Sar^{1}Ala^8]-AngII	29 ± 11	3	0.43	0.62	ND	0.89	19	72
Dup753	5,224 ± 1,266	3	6.3	7.8	100,000	>100,000	>100,000	ND
CGP42112A	29 ± 10	3	987	1,021	0.45	ND	1,200	ND
PD123177	>100,000	3	>100,000	>100,000	ND	130	>100,000	ND
Reference	Present study		9, 25	25	24	7	17	16

[a] Values are mean ± standard error.
[b] n, number of experiments.
[c] ND, ligand affinity value was not determined in the referenced study.

mammalian AT_2 binding sites in other cell lines and tissues that are not shown in Table 1 (8, 24, 26). Thus, clone pTAT2′ expresses an AT receptor isoform whose pharmacological profile is a mixture of those observed for the AT_1 receptor and the AT_2 binding site.

To examine further the low affinity of Dup753 for the cloned turkey AT receptors, its effect on blocking angiotensin II-stimulated inositol phosphate release in transfected COS-7 cells was tested (Fig. 3). Control cells transfected with the plasmid vector pCDM8 alone had basal total inositol phosphate levels

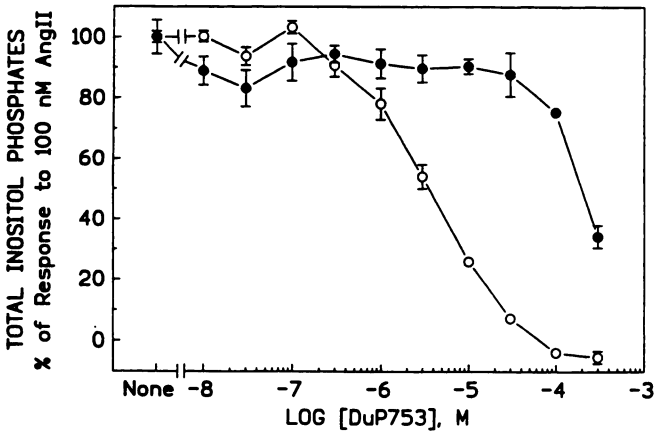

Fig. 3. Angiotensin II-stimulated inositol phosphate production in COS-7 cells transfected with AT receptor clones. Twenty four hours after transfection with 20 μg of either the rat AT_{1a} receptor cDNA (pCa18b) (●) or the turkey adrenal AT receptor cDNA (pTAT2′) (○), cells from each 10-cm dish of transfected cells were split evenly into twelve 20-mm wells in a multiwell dish. These were incubated with 1.5 μCi of *myo*-[^{3}H]inositol for 36 hr and rinsed with 5 mM LiCl in HEPES-buffered Hanks' balanced salt solution. Wells were incubated for 1 hr at 37° with 100 nM angiotensin II in this buffer, in the absence or presence of the indicated concentration of Dup753. Total inositol phosphates were eluted from Dowex/formate columns and counted for 3–5 min in a scintillation counter. Each *point* represents the mean ± standard error of single determinations from three experiments. The total inositol phosphate response to 100 nM angiotensin II alone was 1039 ± 15 cpm and 1340 ± 62 cpm for the pCa18b- and pTAT2′-transfected cells, respectively. Basal total inositol phosphate levels for the two treatments were identical (245 ± 15 cpm, six experiments).

of approximately 200–300 cpm, which were not enhanced after treatment with angiotensin II (50 nM) or angiotensin II in combination with 1 μM Dup753 (data not shown). In cells transfected either with the rat AT_{1a} receptor cDNA pCa18b or with pTAT2′, 100 nM angiotensin II stimulated a 4–6-fold increase in total inositol phosphates above basal levels, with similar EC_{50} values (~1.0 nM; data not shown). The inositol phosphate response to 100 nM angiotensin II was attenuated dose dependently by Dup753 in pCa18b-transfected cells, where >90% of the signal was blocked by 30 μM Dup753. Angiotensin II (100 nM)-stimulated total inositol phosphate production in pTAT2′-transfected COS-7 cells was slightly higher than that observed for AT_{1a} receptor-transfected cells. However, a significant attenuation of this response was not observed at concentrations of Dup753 of <0.3 mM. This further demonstrates the low affinity of Dup753 for the turkey AT receptor. This result also indicates that the cloned turkey AT receptor and rat AT_{1a} receptor share common signaling properties in this system and further supports the notion that clone pTAT2′ encodes a functional AT receptor.

X. laevis cardiac AT receptors have very low affinity for both Dup753 and PD123177 (17), but these receptors have comparatively higher affinity for CGP42112A, a value that is nearer to that observed for AT_1 receptors than to that for AT_2 binding sites. This contrasts with the comparatively higher affinities for Dup753 and CGP42112A found at the cloned turkey adrenal AT receptor. Therefore, the cloned turkey adrenal AT receptor is pharmacologically distinct from the receptor described in amphibian tissues. Nevertheless, the amphibian and cloned turkey receptors appear to possess similar properties in their differential affinities for [Sar^1,Ala^8]-AngII and [Sar^1,Ile^8]-AngII, unlike those observed for mammalian AT_1 receptors or AT_2 binding sites. The affinities of various angiotensin peptide ligands, including [Sar^1,Ala^8]-AngII and [Sar^1,Ile^8]-AngII, have been determined using radioligand binding analysis of Pekin duck adrenal membranes (16). Those data are entirely consistent with the present results, although the affinities of the novel AT receptor ligands were not tested in that study. Therefore, some precedence exists in the literature for avian homologues

of mammalian AT receptors that share some properties with the cloned turkey adrenal AT receptor.

A comparison of the amino acid sequence for the cloned turkey AT receptor with two cloned rat isoforms (termed AT_{1a} and AT_{1b}) is presented in Fig. 4. Interestingly, each isoform is 359 amino acids in length, and the isoforms have predicted extracellular, transmembrane, and intracellular domains of equivalent length. Furthermore, the turkey isoform shares three consensus glycosylation sites with the mammalian isoforms, in addition to possessing a fourth site. This conservation across isoforms from several species may suggest that these asparagine residues are important in AT receptor function. However, a recent study has shown that the wild-type phenotype for ligand binding to the rat AT_{1a} receptor is tolerant to mutations of any one of the three extracellular putative glycosylation sites of the mammalian isoforms (27). Although this study does not demonstrate that these residues are in fact glycosylated, it nonetheless suggests that the extent of AT receptor glycosylation may not be critical for ligand recognition. The turkey isoform differs from those of the rat at a total of 90 residues. The majority of these differences are found outside of presumed membrane-spanning domains. Thirty mismatches are found in the putative extracellular domains and 35 mismatches are found in the proposed intracellular domains. Thus, 25 amino acid differences are in membrane-spanning locations. Of these, eight involve nonconservative amino acid substitutions, defined as differences in charge, polarity, or size of amino acid side chains, whereas the remaining 17 transmembrane domain substitutions are chemically conservative. In contrast, the two cloned rat AT receptor isoforms differ at only 18 amino acid residues. The locations of these are rather evenly split between transmembrane/extracellular and intracellular domains, with only three occurring in transmembrane domains. As noted, the two cloned rat isoforms demonstrate no obvious differences in ligand-binding properties, so it is unlikely that those positions where they differ from each other are critically involved in ligand recognition.

We propose, however, that the amino acid differences between the turkey AT receptor and mammalian AT_1 receptor isoforms account for their differing pharmacological properties with both peptide and nonpeptide ligands. At this time, it is difficult to predict which amino acid substitutions account for the variation in ligand-binding characteristics between the turkey and rat AT receptors. Based upon understanding derived largely from the study of receptors for small biogenic amine molecules, such as acetylcholine and catecholamines, it has been proposed that heptahelical GTP protein-coupled receptors are activated when agonists intercalate within a recessed binding pocket formed by the close association of the seven membrane-spanning regions (28). It is not known, however, whether the relatively bulkier bioactive peptides interact with their receptors through such mechanisms. As a first approximation, we speculate that amino acid residues located either in the transmembrane domains or in the extracellular domains of AT receptors play a role in ligand recognition, yet rat and turkey AT receptors contain a total of 55 substitutions in these regions, which are rather evenly distributed across the length of the protein. Nevertheless, the ability to construct mutant receptors based upon the differences between pharmacologically distinct but structurally related AT receptor isoforms should increase our knowledge about domains of AT receptors essential for both peptide and nonpeptide ligand recognition.

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tAT     MVPNYSTEETVKRIHVDCPVSGRHSYIYIMVPTVYSIIFIIGIFG   45
rAT1a   *AL*S*A*DGI***QD***KA***S**FV*I**L*****VV****
rAT1b   *IL*S*T*DGI***QD***KA***N**FV*I**L*****MV****

tAT     NSLVVIVIYCYMKLKTVASIFLLNLALADLCFLITLPLWAAYTAM   90
rAT1a   *********F*********V*************L******V****
rAT1b   *********F*********V*************L******V****

tAT     EYQWPFGNCLCKLASAGISFNLYASVFLLTCLSIDRYLAIVHPVK  135
rAT1a   **R*****H***I***SV************************M*
rAT1b   **R*****H***I***SV************************M*

tAT     SRIRRTMFVARVTCIVIWLLAGVASLPVIIHRNIFFAENLNMTVC  180
rAT1a   **L****L**K****I***M**L****AV*H**VY*I**T*I***
rAT1b   **L****L**K****I***M**L****AV*Y**VY*I**T*I***

tAT     GFRYDNNNTTLRVGLGLSKNLLGFLIPFLIILTSYTLIWKTLKKA  225
rAT1a   A*H*ESR*S**PI****T******LF**************A****
rAT1b   A*H*ESQ*S**PI****T******VF**************A****

tAT     YQIQRNKTRNDDIFKMIVAIVFFFFFSWIPHQVFTFLDVLIQLHV  270
rAT1a   *E**KNKP******RI*M***L******V***I**********GV
rAT1b   *K**KNTP******RI*M***L******V***I**********GI

tAT     ITDCKITDIVDTAMPFTICIAYFNNCLNPFFYVFFGKNFKKYFLQ  315
rAT1a   *H**K*S********I*************L**G*L**K*******
rAT1b   *R**E*A********I*************L**G*L**K*******

tAT     LIKYIPPNVSTHPSLTTKMSSLSYRPNENIRLPTKKTAGSFDTE  359
rAT1a   *L*****KAKS*SS*S*****T*****SD*MSSSA**P*SC*EV*
rAT1b   *L*****TAKS*AG*S*****T*****SD*MSSSA**S*SF*EV*
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Fig. 4. Alignment of the turkey AT receptor amino acid sequence with those for the rat AT_{1a} and rat AT_{1b} receptor isoforms. *, Amino acid residues identical among the three isoforms. Putative transmembrane domains are *underlined*. *tAT*, turkey AT receptor; *rAT1a*, rat vascular AT_1 receptor; *rAT1b*, rat pituitary/adrenal AT_1 receptor (from unpublished genomic sequence).

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Send reprint requests to: T. J. Murphy, Department of Pharmacology, Emory University School of Medicine, O. Wayne Rollins Research Center, Room 5031, 1510 Clifton Road, Atlanta, GA 30322.