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Isolation and Expression of a Novel Angiotensin II Receptor from *Xenopus laevis* Heart

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SUMMARY

A *Xenopus laevis* heart cDNA library was screened using the human angiotensin type 1 (AT_1) receptor cDNA coding sequence as a hybridization probe. A cDNA was isolated that encodes a protein of 363 amino acids that shares 63% sequence identity with the human AT_1 receptor. Radioligand binding studies with the cloned receptor expressed in COS cells indicated that it is an angiotensin II receptor that possesses pharmacological properties distinct from those of the two known mammalian receptor subtypes, AT_1 and AT_2. Electrophysiological studies with the recombinant receptor expressed in *X. laevis* oocytes revealed that the amphibian receptor, like the mammalian AT_1 receptor, can functionally couple to a second messenger system, leading to the mobilization of intracellular stores of calcium. However, nonpeptide antagonists selective for the mammalian AT_1 and AT_2 receptors do not block angiotensin II-stimulated functional responses in injected oocytes, which confirms that the amphibian receptor is a pharmacologically unique angiotensin II receptor. Nevertheless, based on conservation of structural features and motifs and similarity in coupling mechanisms, we speculate that the cloned *Xenopus* receptor is the amphibian counterpart of the mammalian AT_1 receptor, having acquired its unique pharmacology as a consequence of evolutionary divergence.

As part of the renin-angiotensin system, the hormone AII plays a pivotal role in controlling blood pressure and electrolyte balance (1). Two forms of cell surface receptors for AII have been identified pharmacologically and classified as distinct AII receptor subtypes, AT_1 and AT_2 (2, 3). The AT_1 receptor is responsible for the vascular effects of AII and, accordingly, is the target of novel therapeutic agents for the treatment of hypertension and congestive heart failure (4). The AT_1 receptor possesses a seven-transmembrane region topology and is coupled through G proteins to effectors including phospholipase C and adenylate cyclase (5). The AT_2 receptor subtype, for which a function has not yet been definitively described (4), does not appear to interact with G proteins (6).

The AT_1 receptor was first isolated from rat and bovine cDNA plasmid libraries by mammalian cell expression cloning (7, 8). Subsequently, we and others isolated the human AT_1 receptor cDNA and its genomic counterpart (9–12). Recently, a second form of the AT_1 receptor was found to exist in both rat and mouse mRNA and genomic DNA (13–18). The two receptor isoforms, named AT_{1A} and AT_{1B} or AT_3 (18), are highly homologous, sharing approximately 95% and 92% protein and nucleotide sequence identity, respectively. Binding studies performed on membranes derived from COS cells tranfected with rodent AT_{1A} and AT_{1B} receptors revealed no major differences in ligand-binding properties (14, 17, 18). Because Southern blot analysis of human and mammalian genomic DNA indicated that the AT_1 receptor gene is represented as a single copy in the genome of all mammals except rodents (11, 19),[1] the significance of the existence of two AT_1 receptor genes in rodents is unknown. Presumably, the two AT_1 receptor gene copies present in rat and mouse genomes originated by gene duplication and subsequent divergence from a common rodent ancestral gene (19).

AII receptor sites have been identified in *Xenopus laevis* follicular oocytes and heart membranes (20, 21). Like the mammalian AT_1 receptors, the amphibian ovarian AII receptors bind peptide agonists and antagonists with nanamolar affinity. Also, the *Xenopus* receptors are functionally coupled to a second messenger system that leads to mobilization of intracellular stores of Ca^{2+} (22). However, pharmacological studies indicated that the amphibian AII receptors are distinct from both the mammalian AT_1 and AT_2 receptors, in that they do not recognize either DuP753 (23) or PD123177 (24), nonpeptide antagonists that specifically bind to AT_1 or AT_2 recep-

[1] D. Bergsma and C. Ellis, unpublished observations.

ABBREVIATIONS: AII, angiotensin II; SIA, [Sar^1,Ile^8]angiotensin II; DPBS, Dulbecco's phosphate-buffered saline; AI, angiotensin I; AIII, angiotensin III; kb, kilobase(s); bp, base pair(s); UTR, untranslated region; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; AT_1, angiotensin type 1 receptor; AT_2, angiotensin type 2 receptor.

tors, respectively. These results suggest that the ligand-binding domain of the *Xenopus* receptor has a unique structure that differentiates between nonpeptide and peptide antagonists (21).

The protein domains of the AT_1 receptor that are essential for ligand binding are unknown. Identification of the binding epitopes for agonists and antagonists not only is critical for understanding the structure and function of the receptor but also may aid in the design of synthetic ligands for therapeutic use. The unique pharmacology of the *Xenopus* receptor offers the opportunity to relatively rapidly localize the binding epitopes for a nonpeptide antagonist by construction of amphibian/mammalian chimeric receptors, with the exchange of nonconserved amino acids contained within corresponding regions of each receptor, and determination of the consequences of the exchange by conventional pharmacological analysis of the recombinant receptors expressed in an appropriate cell system. This hybrid receptor approach was pioneered by Lefkowitz and co-workers (25) in their delineation of domains involved in ligand-binding specificity and effector coupling of α_2- and β_2-adrenoceptors. In this study, as a first step to investigate the structural basis for the molecular interaction of the peptide and nonpeptidic antagonists with the mammalian AT_1 receptor, the *Xenopus* heart AII receptor cDNA was cloned and characterized by binding and functional studies after transient expression in COS cells or *Xenopus* oocytes. Comparison of the amphibian receptor with the mammalian AT_1 receptor protein reveals a number of regions that likely contain nonpeptidic antagonist binding sites.

Materials and Methods

cDNA cloning. Total RNA was prepared from *X. laevis* heart tissue by the guanidinium isothiocyanate method (26), and poly(A)$^+$ RNA was purified by oligo(dT)-cellulose chromatography (27). cDNA was commercially prepared from this mRNA and cloned into λ ZAP II arms (Stratagene, La Jolla, CA). Based on the human AT_1 cDNA sequence (11), oligonucleotide primers corresponding to the amino and carboxyl termini were made and, together with clone phAT_1-3 as template DNA, were used in a polymerase chain reaction (28, 29) to obtain an approximately 1100-bp cDNA probe encoding the human AT_1 receptor that was devoid of 5′ and 3′ UTR sequences. This probe was ^{32}P-radiolabeled using the TAG-IT kit ((BIOS Corp, New Haven, CT) and was used to screen the *Xenopus* heart cDNA library by *in situ* plaque hybridization, as described previously (30). Positive clones were plaque purified, and cDNAs were excised to yield recombinant Bluescript plasmids in *Escherichia coli* XL1-Blue cells, according to the methods recommended by Stratagene. Recombinants were analyzed by restriction mapping and DNA sequencing using a Sequenase kit (United States Biochemical Corporation, Cleveland, OH). Sequences were aligned and compared using computer software designed by the Genetics Computer Group at the University of Wisconsin (Madison, WI). A single clone, designated pBXAT-1, which harbored a 4066-bp insert (including *Eco*RI linkers), was chosen for further analysis.

Expression of the amphibian AII receptor in COS cells. The 2152-bp *Eco*RI-*Hind*III insert of pBXAT_1-1, which harbored 138 bp of 5′ UTR, the entire coding region, and 924 bp of 3′ UTR, was subcloned into the COS cell (31) expression vector pCDN, generating the clone pCDNXAT-1. COS cells were plated onto 100-mm plates so that they would reach between 60 and 80% confluency within 24 hr. Cells were then transfected with 25 μg of DNA as described previously (11). After overnight incubation at 37°, the cells were detached from the plates with trypsin-EDTA and transferred to six-well, 33-mm, tissue culture plates with fresh complete medium. Cells were incubated at 37° for 2 days before receptor binding assays.

Membrane receptor binding assays. Membranes from *X. laevis* heart tissue were prepared by homogenization of the tissue in buffer A (10 mM Tris·HCl, pH 7.4, 5 mM EDTA) and were pelleted by centrifugation at 43,500 × *g* for 20 min. The pellet was washed twice with buffer B (50 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$) and resuspended in the same buffer containing 250 mM sucrose, to give a final protein concentration of 5 mg/ml. Aliquots of membranes were stored at −70° until use. For binding assays, 40–80 μg of membranes were incubated at 25° for 60 min with ^{125}I-SIA (specific activity, 2200 Ci/mmol; New England Nuclear, Boston, MA) (10–240 pM for saturation binding experiments and 120 pM for competition binding experiments) in a total volume of 100 μl of buffer B, in the presence or absence of unlabeled ligands. The binding reaction was terminated by the addition of 3 ml of ice-cold wash buffer (5 mM Tris·HCl, pH 7.4, 150 mM NaCl) and rapid filtration, using a Skatron harvester (Skatron Instruments, Norway), through Skatron filtermats that had been presoaked in 0.2% bovine serum albumin. Nonspecific binding was determined in the presence of 1 μM unlabeled AII. Each experiment was performed with duplicate determinations and was repeated at least two or three times. Data were analyzed by computer-assisted, nonlinear curve fitting, using the Lundon-2 software program (Lundon Software Inc, OH).

Recombinant receptor binding assays. Three days after transfection, cells were washed with DPBS containing 10 mM $MgCl_2$, 0.1% glucose, and 0.2% bovine serum albumin. Binding assays were done for 60 min at 25° in 1.0 ml of DPBS with 40–50 pM ^{125}I-SIA, in the absence or presence of appropriate concentrations of unlabeled competitors. After incubation, the medium was removed, cells were rapidly washed three times with ice-cold DPBS, and cell-associated radioactivity was solubilized with 1.0 N NaOH and counted using a γ counter (80% efficiency). Nonspecific binding was determined in the presence of 1 μM unlabeled AII. Each experiment was performed with duplicate determinations and was repeated at least two or three times. Data were analyzed as described above.

***Xenopus* oocyte electrophysiology.** Mature *X. laevis* females were anesthetized by hypothermia and ovaries were surgically removed. Follicle cells were dispersed, and individual oocytes were released by incubation with 2 mg/ml collagenase (Worthington, NJ) in modified Barth's medium, as described (32). The oocytes were allowed to recover from treatment with overnight incubation at 18° in Barth's medium. For each experimental group, stage V to VI oocytes were selected, follicular membranes were manually removed, and 10–20 individual defolliculated oocytes were injected (Drummond injection apparatus) with 50 nl of water containing 10 ng of *in vitro* transcribed *Xenopus* AII receptor RNA. RNA was synthesized from linearized pBXAT-1 plasmid DNA using a RNA transcription kit obtained from Stratagene Cloning Systems (CA). Injected oocytes were maintained in modified Barth's medium at 18° for 2 days to allow for AII receptor protein expression. Electrophysiology was performed using the voltage-clamp technique, with an oocyte voltage-clamp apparatus (Warner Instruments Corporation, CT). Oocyte membrane potentials were clamped at −60 mV and the Ca^{2+}-activated Cl^- channel activity was recorded in Barth's medium at room temperature, as described (33).

RNA blotting and hybridization. Poly(A)$^+$ RNA extracted from *Xenopus* heart tissue was separated by electrophoresis through 1.5% agarose/formaldehyde gels and blotted onto nitrocellulose (26). The blot was baked for 2 hr at 80°, prehybridized for 4 hr at 42° in hybridization buffer [50% formamide, 5× SSPE (20× SSPE is 3.0 M NaCl, 0.2 M Na_2HPO_4, pH 7.4, 0.02 M EDTA), 5× Denhardt's solution, 0.2% SDS, 100 μg/ml tRNA], hybridized with a ^{32}P-radiolabeled 2152-bp *Eco*RI-*Hind*III *Xenopus* AII receptor cDNA fragment (isolated from pBXAT-1) at 42° in hybridization buffer for 18 hr, washed twice at room temperature with 2× SSC (20× SSC is 3.0 M NaCl, 0.3 M sodium citrate)/0.1% SDS for 20 min each, and washed twice at 68° with 1× SSC/0.1% SDS for 15–30 min. Filters were air dried and exposed to Kodak XAR-5 X-ray film at −70°, with an intensifying screen.

Results and Discussion

Cloning of a cDNA encoding a *X. laevis* AII receptor. The coding region of the human AT_1 receptor, previously cloned in our laboratory (11), was used to probe a *X. laevis* heart cDNA

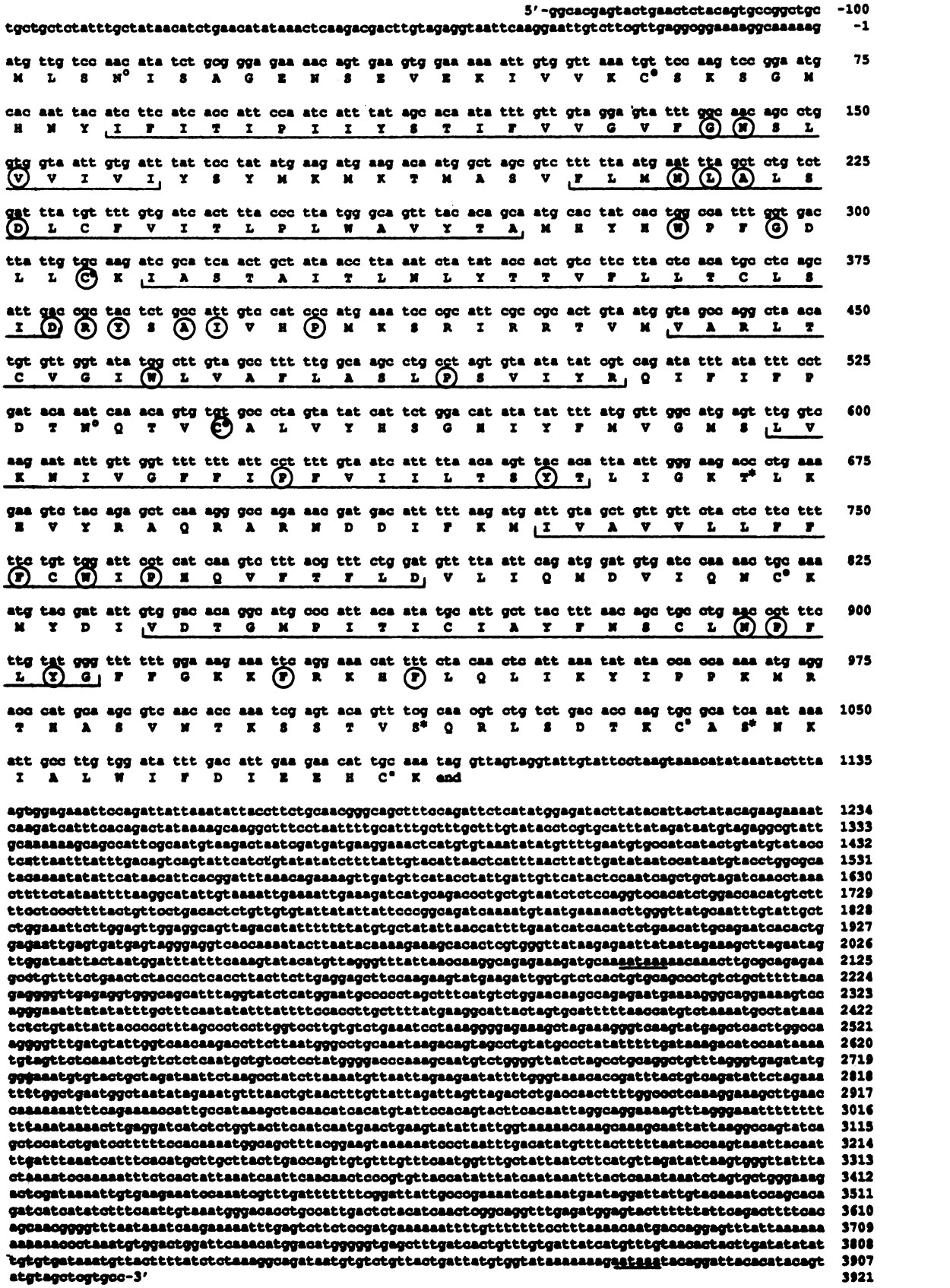

Fig. 1. Nucleotide and deduced amino acid sequences of the *Xenopus* receptor. Amino acids (represented in one-letter code) are indicated *below their respective codons and numbered on the left*, beginning with the initiation methionine codon (*M*). *Numbers on the right*, nucleotide positions; *bracketed amino acid sequences*, putative seven transmembrane domains; *underlined nucleotide sequences*, canonical eukaryotic polyadenylation signals. O, Potential sites for post-translational glycosylation; ●, cysteine residues available for disulfide bridge formation; *, potential phosphorylation sites; ■, possible palmitoylation site. *Circled amino acids*, those that are highly conserved among G protein-coupled receptors.

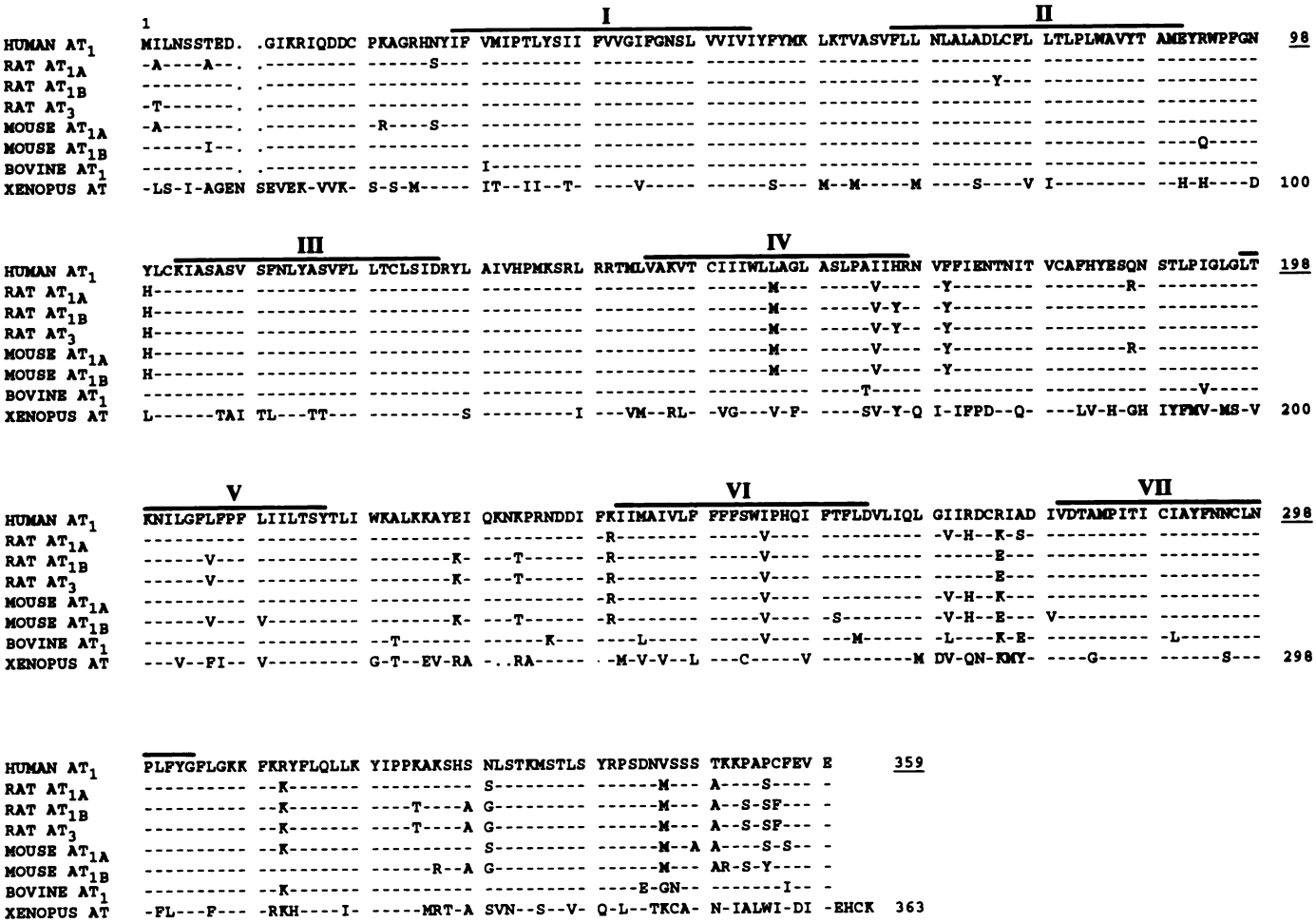

Fig. 2. Amino acid sequence comparison of all known AT_1 receptors. This compares the amphibian receptor, together with all of the AT_1 receptors published to date, with the human AT_1 receptor. The human sequence is numbered beginning with the initiating methionine residue. –, Amino acid identity; amino acid differences are shown in their corresponding positions. *Solid lines above the sequence*, hydrophobic amino acid stretches forming putative seven transmembrane domains.

library under low stringency screening conditions. A number of hybridizing clones were discovered; one clone, which contained the largest cDNA insert, was chosen for complete characterization. The nucleotide sequence of this clone, designated pBXAT-1, and the deduced protein sequence are shown in Fig. 1.

The amphibian cDNA is 4054 bp in length and contains an open reading frame of 1089 bp encoding a protein of 363 amino acid residues. The deduced *Xenopus* protein possesses several features in common with G protein-coupled receptors. Most prominent is the existence of 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 (34). Interspersed within the protein are 29 amino acid residues that are also highly conserved among G protein-coupled receptors. Other notable features of this protein include several serine and threonine residues that may be substrates for possible regulatory phosphorylation (35, 36), two asparagine residues for potential post-translational *N*-glycosylation (37), and two cysteine residues within the carboxyl-terminal region that could be palmitoylated and thus function to anchor the domain to the intracellular membrane, possibly serving some role in receptor coupling to a signal transduction system (38). For example, mutation of a cysteine residue in the carboxyl terminus of the human β_2-adrenoceptor results in a nonpalmitoylated form of the receptor that is incapable of coupling to adenylate cyclase (38).

The deduced *Xenopus* protein contains one cysteine residue in the amino terminus and in each of the three predicted extracellular loops. Two of these residues, at positions 103 and 182, are highly conserved within the G protein receptor superfamily. Structure-function analyses of the hamster β-adrenoceptor and bovine rhodopsin receptors suggest the existence of a disulfide bridge between two cysteines in similar positions that is essential for the formation of the correct tertiary structure of the receptor required for ligand binding (39, 40). A disulfide bond may also form between the two other cysteine residues, at positions 20 and 274. Site-directed mutagenesis studies of the rat AT_1 receptor indicated that all four of the cysteine residues in similar or identical positions are required for ligand binding (41).

Primary sequence comparison of the AII receptors. The deduced *Xenopus* protein, together with all of the mammalian AT_1 receptors published to date, is compared with the human AT_1 receptor protein in Fig. 2. Although four amino

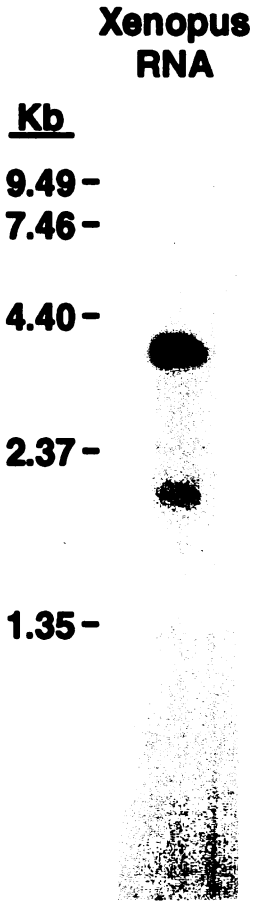

Fig. 3. Northern blot analyses of *Xenopus* heart mRNA. Northern blot analysis was used to examine the size of the cloned *Xenopus* receptor mRNA in heart tissue. One microgram of poly(A)$^+$ RNA isolated from *Xenopus* heart was analyzed with the coding region of the *Xenopus* pBXAT-1 cDNA as a hybridization probe. Molecular weight markers are indicated (in kb).

acids larger, the amphibian protein shares considerable amino acid sequence similarity with the mammalian AT_1 receptors. Overall, it is about 63% identical to the primary sequence of the human AT_1 receptor, which represents a significant level of sequence homology, albeit substantially less than the approximately 92% sequence homology found among the mammalian AT_1 receptor group. Notably, the degree of sequence conservation is not evenly distributed throughout the structural domains of the amphibian protein; the highest level of sequence homology exists within the seven transmembrane regions and three intracellular loops, varying between 63 and 85% identity. Greater sequence divergence resides within the four extracellular regions and the carboxyl-terminal region, which differ 40–70% from corresponding regions of the mammalian receptors.

In additon to the seven-transmembrane region topology, most of the structural features and motifs of the *Xenopus* protein are conserved among the mammalian AT_1 receptors, including potential glycosylation and phosphorylation sites and four cysteine residues proposed to form two disulfide bridges. However, most mammalian AT_1 receptors contain a possible palmitoylation site at the Cys-355 residue rather than the two putative sites at the Cys-346 and Cys-362 residues of the amphibian protein. Also, the mammalian receptors harbor additional potential glycosylation and phosphorylation sites. It remains to be determined whether these or other amino acid differences would result in species differences in possible regulatory palmitoylation or phosphorylation-mediated receptor desensitization.

RNA analysis of *Xenopus* heart tissue. To determine the mRNA transcript size of the cloned *Xenopus* protein, *Xenopus* heart RNA was examined by Northern blot analysis using a 2.2-kb portion of clone pBXAT-1 as a hybridization probe. As shown in Fig. 3, one major band of approximately 4.0 kb and one minor band of about 2.0 kb were homologous to the probe. The size of the upper, more intense, signal is consistent with that of the cloned cDNA (4054 bp); accordingly, the cDNA likely represents the entire mRNA transcipt. A potential polyadenylation signal, AATAAA (42), is located 34 bp upsteam from the 3′ end of the cDNA (Fig. 1), again in agreement with the clone being a full length copy of the mRNA transcript.

The less intense 2.0-kb band may be a product of an alternately processed precursor mRNA; the smaller size could result from a different post-translational splicing event or utilization of a secondary polyadenylation signal. In the context of the latter possibilty, a consensus polyadeylation signal is present at position 2101 bp of the cDNA (Fig. 1), which, if utilized, would produce a RNA trancript of the approximate size observed for the less intense signal. Alternatively, the smaller band may represent a mRNA transcript of a related gene.

Ligand-binding properties. To investigate the ligand-binding properties of the cloned receptor, the cDNA was sub-

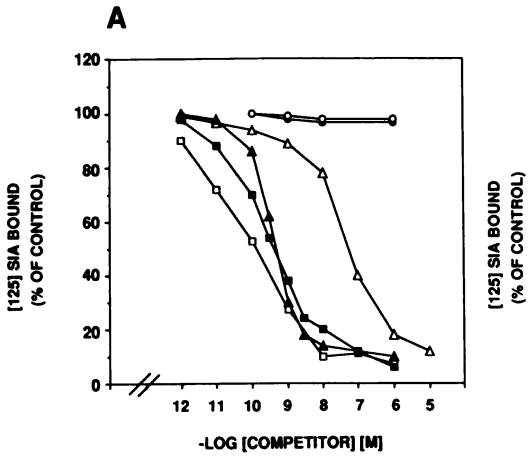

Fig. 4. Competitive inhibition of ^{125}I-SIA binding to transfected COS cells and *X. laevis* heart membranes. The displacement of ^{125}I-SIA binding to recombinant receptors expressed in COS cells (A) or to membranes prepared from heart tissue (B) by unlabeled AII (□), SIA (■), AIII (▲), AI (△), PD123319 (○), and SK&F 108566 (●) is shown. The maximum binding was 16,000–18,000 cpm/0.5 × cells and the nonspecific binding was 200 cpm. All *points* are averages of duplicate determinations of three or four experiments. The unrelated peptide vasopressin (1 μM) displayed no effect on binding (data not shown).

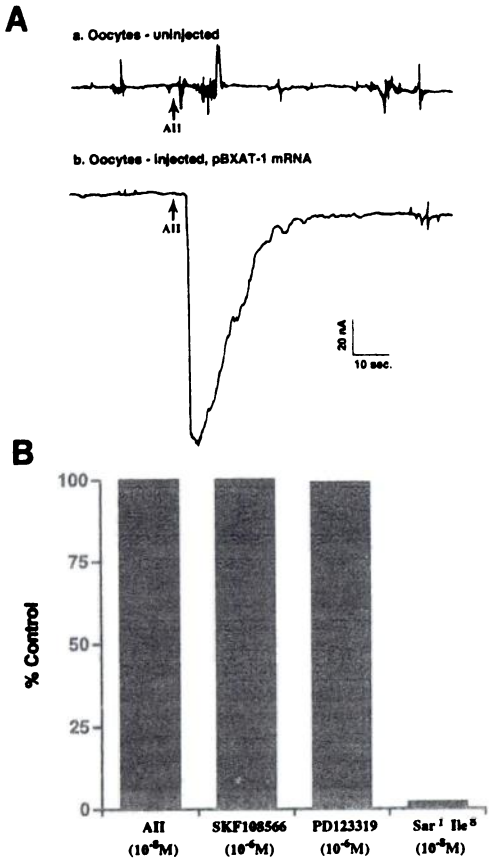

Fig. 5. Functional coupling of the recombinant amphibian AII receptor, as assessed by *Xenopus* oocyte electrophysiology. AII-induced currents were measured in oocytes injected with synthetic amphibian AII receptor mRNA. Defolliculated oocytes did not bind ^{125}I-SIA and, when injected with synthetic human AT_1 mRNA produced *in vitro*, oocytes bound ^{125}I-SIA with a high degree of specificity (data not shown). A, Traces of AII (10 nM)-mediated current (in nA) induced 2 days after oocyte injection with water (*trace a*) or 10 ng of synthetic AII amphibian receptor mRNA (*trace b*). *Arrow*, AII addition. Increased concentrations of AII induced a greater magnitude of current responses (data not shown). B, Defolliculated oocytes were injected with 10 ng of synthetic amphibian AII receptor mRNA. The mean peak response to 10 nM AII (210 ± 50 namps; n = 20) was arbitrarily assigned a value of 100. The levels of inhibition obtained with the addition of the indicated concentrations of AII, SK&F 108566, PD123319, and SIA are shown relative to this value.

cloned into the mammalian cell expression vector pCDN and transfected into COS cells. Fig. 4A shows competition curves for agonists and antagonists competing for ^{125}I-SIA binding to the expressed receptor. The peptides AII, SIA, AIII, and AI displaced ^{125}I-SIA binding in a concentration-dependent fashion, with IC_{50} values of 0.30 ± 0.08, 0.70 ± 0.15, 0.70 ± 0.18, and 62.5 ± 5.0 nM, respectively. In contrast, the expressed recombinant protein did not bind the nonpeptide antagonists SK&F 108566 (43) and PD123319, which are selective for the mammalian AT_1 and AT_2 receptors, respectively. This pharmacological profile is almost identical to that of the *Xenopus* heart AII receptor, as shown in Fig. 4B. The heart receptor likewise did not bind the nonpeptide antagonists but exhibited high nanamolar affinity for the peptide ligands, showing comparable IC_{50} values of 1.0 ± 0.2, 0.40 ± 0.05, 0.80 ± 0.15, and 30 ± 3 for AII, SIA, AIII, and AI, respectively. These common pharmacological characteristics indicate that the cloned receptor encodes the amphibian heart AII receptor. Equally significantly, these results demonstrate that the cloned amphibian receptor is pharmacologically distinct from both the mammalian AT_1 and AT_2 receptor subtypes.

Electrophysiological studies using *Xenopus* oocytes. *Xenopus* oocytes were used to examine the functional coupling of the recombinant heart AII receptor to a second messenger system, as assessed by the voltage-clamp technique. For these experiments, oocytes were defolliculated to remove an endogenous AII receptor that is coupled through gap junctions to inositol-1,4,5-trisphosphate-induced Ca^{2+} mobilization in AII-stimulated oocytes (22). As shown in Fig. 5A, *trace a*, application of AII to uninjected oocytes did not elicit any change in membrane potential. In contrast, oocytes injected with synthetic *Xenopus* heart AII receptor mRNA showed a rapid response, producing a strong Ca^{2+}-dependent Cl^- current when exposed to the same concentration of AII (Fig. 5A, *trace b*). These results indicate that the recombinant receptor is a functional receptor that is capable of coupling to a second messenger system, leading to the mobilization of intracellular stores of Ca^{2+} (presumably induced by inositol trisphosphate).

The electrophysiological response of the injected oocytes to AII application was significantly diminished by the peptide antagonist SIA at very low concentrations (10 nM) (Fig. 5B). In contrast, the nonpeptidic antagonists SK&F 108566 and PD123319 did not inhibit AII-stimulated functional responses even at concentrations as high as 1 μM. These results suggest that the cloned amphibian AII receptor binds the peptide antagonist SIA but does not recognize the nonpeptide antagonists. This property differed markedly from that observed for the human AT_1 receptor examined in identical experiments, where nonpeptidic AT_1 receptor-selective antagonists blocked, whereas an AT_2 receptor-selective antagonist did not inhibit, AII-induced currents in injected oocytes (11). Therefore, the electrophysiological studies indicate that the *Xenopus* AII receptor is an unique AII receptor, which is consistent with the conclusion of the COS cell expression experiments.

Structural model of the amphibian AII receptor. A hypothetical secondary structure model of the amphibian AII receptor is shown in Fig. 6. This model is based on the arrangement of the seven transmembrane domains in a rough circle within the cell plasma membrane, forming a central ligand-binding pocket, as was first proposed for adrenoceptors (25). When compared with the human AT_1 receptor, the greatest level of sequence conservation is contained within the seven transmembrane domains and three intracellular loops. Accordingly, these regions may provide the peptide ligand-binding and second messenger-coupling properties that are common to both receptors. In contrast, the hydrophilic extracellular and carboxyl-terminal domains are significantly more divergent in amino acid sequence. It is possible that the extracellular domains of the human AT_1 receptor contain most if not all of the epitopes essential for the binding of the nonpeptidic ligands and these epitopes are lacking in the amphibian receptor, resulting in its unique pharmacology. In this context, the binding epitopes of a nonpeptidic antagonist of the rat substance P (neurokinin receptor type 1) receptor were localized to the junction of extracellular loops 2 and 3 at the top of transmembrane domains 5 and 6 (44). These epitopes were not important for the binding of the natural peptide ligand substance P. Notably, the junction of extracellular loop 2 and transmembrane domain 5 of the human AT_1 receptor is strikingly differ-

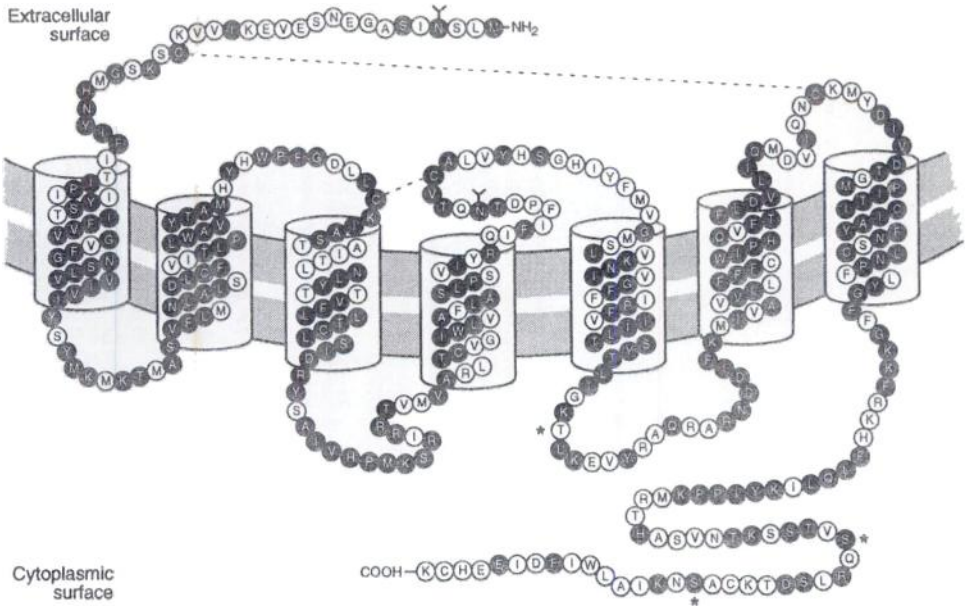

Fig. 6. Secondary structure model for the distribution of the amphibian AII receptor in the plasma membrane. The amino acids in the seven transmembrane domain helices are depicted within cylindrical columns embedded within the plasma membrane. Also shown are the amino acid sequences connecting the successive helices and forming loops in the cytoplasmic and extracellular spaces. *Shaded residues*, amino acid residues conserved between the amphibian and human AII receptors; *Y above the sequence*, potential sites for post-translational glycosylation; *dashed lines*, potential disulfide bridges between extracellular cysteine residues; •, potential phosphorylation sites.

ent from the corresponding region of the *Xenopus* receptor, suggesting the possibility of a similar function of this region.

Conclusions. Pharmacological and functional properties support the conclusion that the isolated *Xenopus* cDNA encodes an AII receptor. The distinctive pharmacology of the amphibian receptor may warrant its classification as a novel AII receptor subtype (i.e., AT_4?), according to the nomenclature developed for AII receptors (3). However, based on amino acid homology, conservation of structural features and motifs, and similarity in coupling to a second messenger system, it is possible that the cloned receptor represents the *Xenopus* AII receptor counterpart of the mammalian AT_1 receptor subtype. Accordingly, the unique pharmacological properties of the mammalian and amphibian receptors result from their amino acid residue dissimilarities, which presumably arose during evolution; the degree of dissimilarity likely is the consequence of the relatively early divergence of the amphibians from a common vertebrate ancestor. Through the generation of mammalian/amphibian chimeric receptors, these differences can be exploited to localize the binding epitope for a nonpeptide antagonist. This will facilitate structure-function studies of the receptor, which may contibute to the strategy for the design of novel compounds for therapeutic use.

Note Added in Proof. During the review of this manuscript we were informed by Dr. K. Sandberg that another AII receptor exists in *X. laevis* (to be published in *Biochem. Biophys. Res. Commun.* **187**:756–762, 1993). This receptor appears to be pharmacologically similar to the AII receptor described in this report, although it shares 88.7% protein sequence identity.

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