Molecular Cloning of a Novel Angiotensin II Receptor Isoform Involved in Phosphotyrosine Phosphatase Inhibition*

(Received for publication, August 6, 1993, and in revised form, September 8, 1993)

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There are two major isoforms of the angiotensin II receptor, type 1 (AT1) and type 2 (AT2). AT1 is distinguished from AT, with respect to its ligand selectivity, its insensitivity to non-hydrolyzable GTP analogues, and its as yet unidentified biological functions. In the present study we have expression-cloned AT2 cDNA from a cDNA library of a rat pheochromocytoma cell line (PC12w). Rat AT2 cDNA encodes a 383-amino acid protein that has seven transmembrane domains. AT1 is the closest in homology to AT2 but with only a 32% identity of amino acid sequence. Stably expressed in COS-7 cells, the receptor showed selective binding to AT2-specific ligands PD123319 and CGP42112A but not to the AT1-specific ligand, losartan. Northern blot analysis revealed that the mRNA of rat AT2 was expressed not only in PC12w cells but also in the adrenal glands and in the inferior olive of the brain, both of which are known to contain AT2 type binding sites. The expressed AT2 receptor mediated angiotensin II-induced inhibition of protein tyrosine phosphatase, an action that was dependent on a pertussis toxin-sensitive G-protein-coupled mechanism in COS-7 cells. The AT2-specific ligand CGP42112A was an agonist rather than antagonist in the inhibition of phosphotyrosine phosphatase. AT2 did not cause a decrease in cGMP in PC12w or COS-7 cells expressing AT1 stably. These results indicate that the AT2 receptor is structurally and functionally different from AT1 and suggest novel functional roles of the renin-angiotensin system in cross-talk with phosphotyrosine signaling by modulating protein phosphotyrosine levels.

Ang II plays an important role in cardiovascular regulation, fluid volume homeostasis and neuroendocrine regulation, and cellular growth (1–3). The highly diverse nature of its functions suggests the presence of multiple isoforms of its receptor. Recent pharmacological studies with isoform-specific antagonists uncovered the presence of two major receptor isoforms, AT1 and AT2 (4–6). Studies with the AT1-specific antagonist losartan (4) and the cloned and expressed AT2 (7–9) indicated that AT1 mediates many of the biological responses hitherto attributed to Ang II (1–3). However, little is known on the structure and the signaling mechanism of the second major receptor isoform, AT2. Its abundance in the mesenchymal tissues of a developing fetus (10, 11), uterus (16), adrenal medulla (4), pheochromocytoma (12), and specific brain regions (13, 14) suggests a hitherto unidentified role of the type 2 receptor, AT2, in neuronal and developmental function. Here we report cloning of the AT2 cDNA from a rat pheochromocytoma cell line, deduction of its amino acid sequence, and observation of a novel signaling mechanism, by which Ang II inhibits the protein phosphotyrosine phosphatase (PTP) activity in COS-7 cells permanently transfected with a plasmid harboring the AT2 cDNA and in rat pheochromocytoma PC12w cell membranes.

MATERIALS AND METHODS
cDNA libraries were prepared from poly(A)+ RNA (5 μg) isolated from rat PC12w cells. After ligating the cDNA with a BamHI adapter, size fractionation by ultracentrifugation for 3 h at 100,000 rpm in a concentration gradient of potassium acetate (5–20%), and insertion into pCDNAI (Invitrogen), the library was used for transforming Escherichia coli (MC 1061/p3) by electroporation in a Gene Pulser (Bio-Rad). Plasmid DNA (5 μg) from pools of about 300 independent clones grown in 20-ml liquid culture were used to transfect 106 COS-7 cells by electroporation. The cells were cultured on a plastic Cell Disk (Sumitomo) for 48–72 h, then incubated with 0.5 μM CGP42112A in Dulbecco’s modified Eagle’s medium containing 0.2% BSA for 2 h at ambient temperature and autoradiographed with Kodak X-Omat x-ray film for 5 days at –70°C. From one positive pool, a single positive clone was selected by the method described elsewhere (7).

The base sequence of the cDNA insert was determined with a SacI and Xhol fragment of the AT2 cDNA after subcloning it into pBluescript KS(+). Truncated cDNAs prepared with Erase A Base System (Promega) were sequenced in both sense and antisense directions with Sequenase version 2.0 (U.S. Biochemical Corp.) by the dideoxynucleotide chain termination method (15).

The PTP activity of plasma membranes was determined as release of 32P from 32P-phosphorylated Raytide (OncoGene Science) in 15 min at 37°C, according to Streuli et al. (16) after preincubation with or without Ang II for 30 min at an ambient temperature in buffer A containing 25 μM GTP p-Nitrophenyl phosphate (pNPP) was also used as an alternative substrate for the determination of the PTP activity (10).
Cyclic GMP was determined by radioimmunoassay as described previously (18). Cross-linking of 125I-sarile to the Ang II receptor was performed with BS3 as published (19).

For Northern blot analysis, 2 μg of poly(A)+ RNA (1 μg from fetal tissues) were separated in 1% agarose gel. The RNA was transferred to GeneScreen (DuPont NEN), and the filters were hybridized with a labeled ApaI-XbaI fragment of AT1 cDNA in 80% methanol phosphate buffer (pH 7.0) containing 40% formamide, 5 × SSC, 2 × Denhardt’s solution, 0.8% SDS, 10% dextran sulfate, and 100 μg/ml denatured salmon sperm DNA for 20 h at 42 °C. The filters were washed with 2 × SSC, 1% SDS at room temperature twice and then shaken at 55 °C with 0.2 × SSC, 0.1% SDS for 30 min twice.

CGF42112A was obtained from Neosystem (Strasbourg, France); losartan was a gift from DuPont-Merck; PD123319 was from Warner-Lambert-Parke Davis; peptides were purchased from Peninsula Laboratories, and BS3 from Pierce Chemical Co. Other chemicals were from Sigma; [α-32P]dCTP (3000 Ci/mmol) was purchased from DuPont NEN; 125I-CGP42112A and 125I-sarile were prepared as described previously (8). PC12w cells were a gift from Drs. K. H. Kim and R. C. Spoth of Washington State University.

RESULTS AND DISCUSSION

We isolated a cDNA clone for AT2 from a cDNA library (45,000 clones) derived from PC12w cells using the mammalian expression vector pCDNA1. One hundred fifty pools of about 300 independent clones were transfected into COS-7 cells, which had no detectable endogenous Ang II binding sites, and the cells were screened for the expression of the AT2 receptor activity by autoradiography using as a probe 125I-labeled CGF42112A, a potent AT2-specific ligand. Fig. 1a shows the complete sequence of rat AT2 cDNA comprising 2,868 nucleotides. The initiation codon (positions 140–142) was preceded by CAAT (Kozak’s rule (20)), and the open reading frame was terminated by an in-frame termination codon (positions 1,228–1,230). The base sequence encoded 363 amino acid residues corresponding to a theoretical molecular weight of 41,303. The COS-7 cells transfected with the AT2 clone produced a ligand binding protein of ~80 kDa as determined by SDS-polyacrylamide gel electrophoresis and autoradiography of the receptor cross-linked with 125I-sarile using BS3. The molecular mass of the expressed receptor was reduced to around 40 kDa upon treatment with N-glycosidase (data not shown). The size of the deglycosylated protein was in good agreement with the length of the open reading frame of the cDNA. A hydropathy analysis revealed that there are seven hydrophobic domains without a signal sequence. Notable features of the receptor molecule are: five potential N-glycosylation sites located exclusively in the N-terminal hydrophilic domain, the presence of the sequence Asp141-Arg142-Tyr143 highly conserved among seven-transmembrane domain receptors in the N-terminal region of the second cytosolic loop, and a short third cytosolic loop (Fig. 1).

Homology search using GenBank and Swiss Protein data bases revealed that AT2 is a new protein and that AT2 has only 32% amino acid identity with rat AT1 (7,9) and little homology with the related molecule recorded. This contrasts with the 96% identity between the AT1 subtypes, AT1A and AT1B (23–26). Thus, AT2 seems to be a novel and discrete member of the seven-transmembrane receptor superfamily.

Scatchard analyses (n = 3 for each ligand) revealed that the cloned AT2 expressed in COS-7 cells has dissociation constants of 0.22 ± 0.08 and 0.11 ± 0.01 nM for the isoform-nonselective Ang II receptor antagonist 125I-sarile and the AT2 receptor specific ligand 125I-CGP42112A, respectively (Fig. 2a). The stably transfected cells expressed 1.69 ± 0.18 pmol of binding sites per mg of membrane protein (n = 3). Competitive binding experiments showed that the 125I-sarile binding was inhibited by PD123319, a nonpeptidic AT2-specific ligand, with a Kd of 1.7 ± 0.2 nM but not by losartan, an AT1-specific antagonist (Kd > 1000 nM) (Fig. 2b). The cloned AT2 showed a slightly higher binding affinity for Ang III (IC50 = 0.98 ± 0.05 nM) than Ang II (IC50 = 1.4 ± 0.1 nM), with negligible affinity for Ang I (IC50 > 100 nM). This feature of the cloned AT2 is in good agreement with that reported for the AT2 receptor in PC12w cells (12).

Consistent with the previous binding studies (4, 6, 13, 14, 27–29), preincubation with GTPγS did not reduce the binding activity of the cloned AT2 to 125I-Ang II (Fig. 2c). A similar observation was reported for the seven-transmembrane domain dopamine D3 receptor (31). Thus, the lack of the effect of GTPγS on the binding activity, though unusual, does not necessarily indicate the absence of G-protein coupling as further discussed below.

No biological functions for the type 2 receptor AT2 have been rigorously established. Although it is generally agreed that AT2 does not elevate cytosolic [Ca2+] as does AT1 (28, 29, 32), controversy exists as to whether the stimulation of AT2 by Ang II results in a reduction in intracellular cGMP. Thus, we addressed these questions using COS-7 cells stably transfected with AT2 cDNA and PC12w cells. Ang II (10−7 M) did not alter cGMP in these cells with or without pretreatment with 100 nM ANP (Table I), in agreement with many but not all existing observations. The
FIG. 2. Binding and biological properties of COS-7 cells transfected with the rat angiotensin II type 2 receptor cDNA. a, binding isotherms and Scatchard plots (inset) for binding of angiotensin II-related peptides to transfected COS-7 cells. The dissociation constants for the binding to [125I]-sarile (●), a nonselective antagonist, and [125I]-CGP42112A (○), an AT2-specific antagonist, were determined by linear regression analysis (n = 3). b, competitive inhibition of [125I]-sarile (0.5 nM) binding by angiotensin I1 (■), angiotensin II (○), angiotensin III (▲), PD123319 (△), and losartan (○). c. The results are expressed as mean values of three experiments. Standard errors (not shown to avoid complication) are within 5% of each value. d, binding of [125I]-Ang II to plasma membranes of the permanent transfectant of rat AT2 in the presence of various concentrations of GTPyS.

TABLE I

Effect of Ang II on cGMP in PC12w cells and permanently AT2-transfected COS-7 cells

|                  | cGMP (fmol/10⁶ cells) |
|------------------|-----------------------|
|                  | Control | Ang II | Control | Ang II |
| PC12w            |         |        |         |        |
|                  | 9.450 ± 403.2 | 9.316 ± 200.4 | 25.9 ± 1.92 | 25.3 ± 3.23 |
| Transfected COS-7 cells | 69.3 ± 11.9 | 75.3 ± 5.23 | 5.78 ± 0.61 | 7.31 ± 1.50 |

PTP activity in plasma membranes, as determined with 32P-phosphorylated Raytide, was significantly inhibited by Ang II in a concentration-dependent manner (Fig. 3a) with a Kᵣ of around 0.1 nM, which agrees well with the binding affinity for Ang II (Fig. 2b). Bottari and his associates (35) postulated a PTP activation by Ang II through AT2 on particulate guanylyl cyclase as the cause of decline in cGMP. Sumner et al. (34) saw a decline in cGMP in Ang II-treated rat neuronal cells in primary culture, with a mixed population of Ang II receptors. They explained it by postulating activation of calmodulin by

AT2. Possible isoforms of AT2 could explain these discrepancies.

Besides Ang II and Ang III, the AT2-specific ligand CGP42112A (10⁻⁷ M) was found to inhibit the PTP activity by 31.2 ± 4.3% (n = 8), 14.9 ± 2.8% (n = 8), and 8.1 ± 4.9% (n = 10) in the plasma membranes of PC12w cells, rat fetal skin (16 embryonic days), and COS-7 cells stably transfected with the AT2 cDNA, respectively. Thus, CGP42112A, originally thought to be an AT2 antagonist, can now be considered as an AT2 agonist in agreement with recent reports (36). The inhibition of PTP by Ang II was further confirmed with another PTP substrate pNP (Fig. 3c). The inhibition by Ang II was abolished by PD123319, an AT2-specific antagonist, but not by Dup 753, an AT1-specific antagonist, indicating that this inhibition is mediated by an AT2 isoform of the Ang II receptor. Most (80–90%) of the PTP activity was inhibited by a vanadate/molybdate mixture (100 µM each). The inhibition by Ang II was limited to the vanadate/molybdate-sensitive phosphatase activity. Besides the permanent transfectants (COS-7), a similar inhibition of the PTP activity by Ang II was seen in PC12w cells (Fig. 3d). This observation indicates that the results obtained with the permanent transfectants were neither an artifact arising from genetic manipulation nor a peculiarity of COS-7 cells. To determine the mechanism of the inhibition of PTP, we investigated the effect of pertussis toxin. The Ang II-induced inhibition of the PTP activity was eliminated by the pretreatment of the membrane preparation with pertussis toxin (10 µg/ml) in the presence of NAD (10 µM), as shown by solid bars in Fig. 3b, indicating that AT2 regulates the PTP activity through a G-protein (G₁₁ or G₁₂)-mediated mechanism. Although the pertussis toxin sensitivity and the absence of GTPγS-induced shift to a low affinity form appear contradictory to each other, a similar observation was reported with the dopamine D3 receptor as discussed above (30).

The inhibition of the PTP activity was only partial even at a saturating concentration of Ang II. It appears that the AT2-
mediated inhibition may be limited to a certain specific PTP(s). Thus, the second messenger of this system is a PTP(s) that can be inhibited by a pertussis toxin-sensitive G-protein. Recently activation of PTP by somatostatin (17) and dopamine (37) through their seven-transmembrane domain receptors has been reported. It appears that both the activation and inhibition of some PTPs are regulated by the receptor-linked G-proteins. The positive and negative regulation of PTP can provide a possible means for cross-talk between signaling pathways mediated by phosphotyrosine and the G-protein-mediated seven-transmembrane domain receptors.

As shown by Northern blots in Fig. 4a, AT2 mRNA is expressed in various tissues such as the adrenal medulla and inferior olive of the brain (Fig. 4a). The specific localization of AT2 mRNA is consistent with in situ binding studies by autoradiography (4, 10, 13, 14). Furthermore, prominent expression of AT2 mRNA in rat fetal mesenchymal tissues, such as skin and tongue (Fig. 4b), is interesting in view of the postulated developmental roles of Ang II (10). The fetal brain also expresses AT2 mRNA (Fig. 4c). However, it is not yet clear how the PTP inhibition by AT2 is related to physiological regulation of various tissues.

Acknowledgments—We thank Drs. H. K. Kim and R. C. Speth for providing PC12w cells, T. Fitzgerald and E. Price for technical assistance, and T. Stack for preparing the manuscript.

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FIG. 4. Northern blot analysis of rat AT2 mRNA. a, comparison of poly(A)+ RNA from PC12w (PC), rat adrenal gland (AG), inferior olive (IO), heart (Ht), kidney (Kid), liver (Liv), b, comparison of poly(A)+ RNA from the skin and tongue of fetal and neonatal rats. c, comparison of poly(A)+ RNA from whole brain of 15-, 17-, 19-, and 21-day-old fetal and 2-day-old neonatal rats. Molecular size standards in kilobases are indicated to the left. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control probe. E denotes embryonic days; d denotes days after birth; and w denotes weeks after birth.