ESTABLISHMENT OF CHINESE HAMSTER OVARY CELL LINES STABLY EXPRESSING THE CLONED HUMAN TYPE 1 ANGIOTENSIN II RECEPTOR AND CHARACTERIZATION OF THE EXPRESSED RECEPTOR

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ABSTRACT

We have isolated the human angiotensin II (AII) type 1 receptor (AT1) gene, whose coding region was contained within a single exon and have established Chinese hamster ovary (CHO) cell lines stably expressing this recombinant receptor. The expressed receptor revealed the typical binding characteristics of the AT1 receptor to several AII receptor agonists and antagonists. Treatment of the intact CHO cells with the sulphydryl reducing reagent dithiothreitol predominantly decreased the radioligand binding, indicating that a disulphydryl bond(s) necessary for AII binding is located on extracellular domains of the receptor molecule. Addition of AII to the cells evoked a rapid, transient increase in intracellular Ca2+ concentrations followed by a lower, sustained phase; the initial increase was mediated by inositol triphosphate and the second phase was due to Ca2+ influx through voltage-dependent L-type Ca2+ channels. These results demonstrate that the cloned gene indeed encodes a functional AT1 receptor. Furthermore, the appearance of a single hybridization band on genomic Southern blot analysis performed both at high and low stringency, suggests the existence of one copy of the AT1 receptor in the human genome in contrast to that of the two distinct AT1 receptor genes in the rat.

Angiotensin II (AII), the biologically active component of the renin-angiotensin system, plays an important role in the regulation of blood pressure and volume in the cardiovascular system by AII-evoked diverse actions such as vascular muscle contraction and stimulation of aldosterone secretion from the adrenal cortex (7, 21, 23). These various AII-induced events are initiated by AII binding to its specific receptor on the surface of target cells. This receptor is now classified into two groups designated type 1 (AT1) and type 2 (AT2) receptors based on their biochemical and pharmacological properties (4, 5, 18). Most of the known functions of AII are mediated through the AT1 receptor, whereas the function of the AT2 receptor remains unknown. Recently, cDNAs for the rat, bovine, and human AT1 receptor have been cloned and the AT1 receptor has been shown to belong to the superfamily of GTP-binding protein-coupled receptors (2, 15, 19, 24, 25). Iwai and Inagami (14) have further reported the cloning of a cDNA for another type of the AT1 receptor from a rat adrenal cDNA

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; BSA, bovine serum albumin; Sar, sarcosine; PMSF, phenylmethylsulfonyl fluoride; FCS, fetal calf serum
library and proposed that the previously cloned \textit{AT}_{1} receptor from the kidney be called \textit{AT}_{1A} and that from the adrenal be referred to as \textit{AT}_{1B}. These findings raise an important question concerning the relationship between the diverse functions of \textit{AII} and its receptor subtypes. On the other hand, since \textit{AII} is thought to relate to several cardiovascular diseases including cardiac muscle hypertrophy and hypertension in the human (9, 13), it is also of great importance to characterize the human \textit{AT}_{1} receptor gene and investigate its signal transduction pathways. However, there are no suitable human cell lines expressing sufficient levels of the \textit{AT}_{1} receptor for investigation of these issues. In addition, mammalian cultured cell lines permanently expressing the human recombinant \textit{AT}_{1} receptor have not yet been established, which is essential for the study of detailed intracellular transduction mechanism of the receptor, although Bergsma \textit{et al.} (2) examined the coupling of the cloned human receptor to second messenger systems electrophysiologically using \textit{Xenopus} oocytes.

In the present study, we isolated the human \textit{AT}_{1} receptor gene, whose entire coding region is contained in a single exon, and indicated that it exists as a single copy in the genome. Moreover, we established cell lines stably expressing this receptor by introducing the cloned gene into Chinese hamster ovary (CHO) cells, and examined the signal transduction pathways mediated by the expressed receptor as well as its biochemical and pharmacological properties.

**MATERIALS AND METHODS**

**Materials**

\textsuperscript{125}I-[\textsuperscript{5}Ile\textsuperscript{8}]\textit{AII} (~2,000 Ci/mm mol) and \textsuperscript{[\alpha-\textsuperscript{32}P]}dCTP (~3,000 Ci/mm mol) were obtained from Amersham International plc (Bucks, U.K.). The nonpeptide antagonists Dup753 and PD123319 were provided by Dr R. D. Smith (Du Pont Merck Pharmaceutical, Wilmington, DE, U.S.A.) and Dr J. A. Keiser (Warner-Lambert Co., Ann Arbor, MI, U.S.A.), respectively. Chinese hamster ovary cells (DXV-11 strain), which were dihydropyranolate reductase gene (\textit{dhfr}) deficient, were obtained from Upjohn Company (Kalamazoo, MI, U.S.A.). Angiotensin I, II, III, leupetin, and antipain were purchased from the Peptide Institute (Osaka, Japan). Restriction enzymes and \textit{Taq} DNA polymerase were from Takara Shuzo (Kyoto, Japan). Lambda phage EMBL 3 and fura-2 were from Promega (Madison, WI) and Wako Pure Chemicals (Osaka, Japan), respectively.

**Cell Culture**

Chinese hamster ovary cells were grown in Nutrient mixture F-12 Ham medium (Ham F-12) supplemented with 10% FCS, 4 mM L-glutamine, 100 units/ml penicillin, and 100 \mu g/ml streptomycin. Transfected CHO cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine, the antibiotics listed above, and 10% dialyzed FCS. Cultures were maintained at 37°C in a humidified atmosphere consisting of 95% air, 5% \textit{CO}_2 and routinely passaged by trypsinization.

**Isolation of Human Genomic \textit{AT}_{1} Receptor Clone**

To clone the gene encoding the human \textit{AT}_{1} receptor, we first obtained a cDNA fragment from human primary aldosteronoma by means of the polymerase chain reaction (PCR). The employed primers were based on the nucleotide sequence of the rat \textit{AT}_{1} receptor (15) and the PCR conditions are described below in detail. A human genomic library was constructed in Lambda phage EMBL3 by inserting 15–20 kb fragments of human leucocyte genomic DNA partially digested with Sau3 AI. The library was screened by plaque hybridization with a 760-bp PCR product. Approximately 4 x 10\textsuperscript{5} plaques were plated, transferred to nylon membranes (GeneScreen Plus, Du Pont-New England Nuclear), and the membranes were hybridized at 42°C in solution A (50% formamide, 5 x SSC (1 x SSC=0.15 M NaCl, 15 mM sodium citrate), 5 x Denhardt's solution (1 x Denhardt's solution=0.02% bovine serum albumin (BSA), 0.02% polyvinylpyrrolidone, 0.02% Ficoll), 1% SDS, 100 \mu g/ml denatured salmon sperm DNA, 50 mM sodium phosphate, pH 6.8) using the probe labeled with \textsuperscript{[\alpha-\textsuperscript{32}P]}dCTP by the random primer method. The membranes were then washed twice at room temperature with 2 x SSC, 0.5% SDS for 15 min and at 50°C with 2 x SSC, 0.5% SDS until the background radioactivity was acceptably low. Two positive clones with inserts of about 17 kb, designated \textit{\lambda}MB13 and 20, were isolated from this screening and found to be identical by restriction nuclease mapping. Therefore, clone \textit{\lambda}MB13 was used for subsequent characterization. Clone \textit{\lambda}MB13 was digested with \textit{Hinc} II, electrophoresed, blotted, and hybridized to the labeled PCR product. The result-
ant 3.2-kb positive fragment was subcloned into the multiple cloning sites of the vector pBluescript II KS (+) (Stratagene, La Jolla, CA, U.S.A.) and a partial nucleotide sequence (~2,400 bp) of this fragment around the AT1 receptor sequence was determined. In result, it was found that this isolated gene is identical to the human AT1 receptor gene recently cloned by Furuta et al. (6).

**Preparation of a Probe by Polymerase Chain Reaction**

Two micrograms of poly(A+)-RNA from a human primary aldosteronoma were reverse-transcribed using random hexamer primers as described in a First-strand cDNA synthesis kit (Pharmacia LKB Biotechnology). The cDNA mixtures were amplified by PCR using Tag DNA polymerase with the following two primers: 5'-TCTAGATGGTATCAGAGAATCC-3' (5'-sense primer) and 5'-TCTAGAAAGTGATATCTGGTGGGG-3' (3'-antisense primer). The sequences TCT and TCTA were added to the 5' end of endogenous sequences corresponding to the coding region from 24 to 44 and from 763 to 783 in the rat AT1 receptor cDNA, respectively (15), to generate the Xba I site. Denaturing, annealing, and polymerase reactions were performed 35 times at 94°C for 1 min, 40°C for 2 min, and 72°C for 5 min, respectively, after which the incubation was continued for 15 min at 72°C to complete polymerization.

**Construction of Cell Lines**

The 3.2-kb Hinc II fragment containing the entire coding region of the AT1 receptor was cloned into the Bam HI site of the expression vector pSVD originally constructed for renin expression (22). The resulting plasmid was transfected into dhfr-deficient CHO cells by calcium phosphate coprecipitation. Selection of transfectants was accomplished as described in (22).

**Competition Binding Assay**

Confluent cells in 24-well plastic dishes (~1 × 10⁴ cells/well) were incubated with 50 pM [125I]Sar¹, Ile⁶]AII at 25°C for 2 h in the presence of various concentrations of unlabeled drugs (10⁻¹²-10⁻⁶ M) in a total volume of 1 ml buffer A consisting of Hanks' balanced salt solution (HBSS) (in mM: NaCl, 137; KCl, 5.4; KH₂PO₄, 0.44; Na₂HPO₄, 0.33; MgSO₄, 0.40; MgCl₂, 0.5; CaCl₂, 1.25; NaHCO₃, 4.0) buffered with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.4, containing 0.1% BSA.

**Measurement of [Ca²⁺]**

Intracellular Ca²⁺ concentration ([Ca²⁺]) was determined using the Ca²⁺-sensitive dye fura-2 (10). Cells (~1 × 10⁴ cells) grown on 100-mm diameter dishes were trypsinized. Soybean trypsin inhibitor was added, then the harvested cells were washed twice with buffer B (in mM: NaCl, 140; KCl, 4; MgCl₂, 1; CaCl₂, 1.25; NaH₂PO₄, 1; Hepes, 5; glucose, 11; 0.1% BSA, pH 7.4), and incubated with 4 μM fura-2/AM, dissolved in dimethyl sulfoxide, in the same buffer for 50 min at 20°C. The resultant fura-2-loaded cells were washed twice with buffer B without 0.1% BSA, resuspended in the same buffer, and kept in the dark at room temperature until use. The fluorescence was measured with a CAF-100 spectrofluorometer (Japan Spectroscopy, Tokyo, Japan) with excitation at 340 nm and 380 nm and emission at 500 nm. Cell suspensions in cuvettes were constantly stirred and maintained at 30°C during the measurement of fluorescence.

**Northern and Southern Blot Analyses**

For northern blot analysis, poly(A⁺)-RNA was denatured by glyoxal/dimethylsulfoxide, separated through 1.5% agarose gel, and transferred to a nylon membrane (GeneScreen Plus). The membrane was prehybridized for 2 h at 60°C in solution B (1% SDS, 1 M sodium chloride, 10% dextran sulfate, and 100 μg/ml denatured salmon sperm DNA). Subsequent hybridization was performed overnight at 60°C in the same solution except that the [³²P]labeled PCR product was added as a probe. Thereafter, the membrane was washed twice at room temperature for 5 min with 2× SSC and twice at 60°C for 30 min with 2× SSC/1% SDS. For Southern blot analyses high molecular weight human genomic DNA was digested with Eco RI or Hinc II, then the fragments were size-fractionated on 0.4% agarose gels, and transferred to nylon membranes (GeneScreen Plus). For hybridization at high stringency, membranes were prehybridized at 42°C in solution A, then hybridized at 42°C overnight in the same solution except for the addition of the same probe used for the northern blot. Membranes were washed twice at room temperature for 5 min with 2× SSC followed by two 65°C washes for 30 min with 2× SSC/1% SDS. For hybridiza-
Fig. 1 Competitive inhibition curves for $^{125}$I-[Sar$^1$, Ile$^8$]AII binding to CHO cells. Cells were incubated with $^{125}$I-[Sar$^1$, Ile$^8$]AII in the presence of the indicated concentrations of unlabeled [Sar$^1$, Ile$^8$]AII, AI, AII, AIII, Dup753, and PD123319. Each point represents the mean of duplicate determinations. The plotted data are from a typical experiment.

RESULTS
Pharmacological and Biochemical Properties of the Expressed Human AT$_1$ Receptor

To confirm that the cloned gene indeed encodes a functional AT$_1$ receptor, the 3.2-kb Hinc II fragment containing the entire AT$_1$ receptor coding sequence was inserted into the expression vector pSVD originally constructed for renin expression, then stably expressed in CHO cells. The CHO cells were assessed for their ability to bind several AII receptor agonists and antagonists. As shown in Fig. 1, the binding of $^{125}$I-[Sar$^1$, Ile$^8$]AII, an AII receptor antagonist, to the cells was dose-dependently inhibited by AII, AIII, [Sar$^1$, Ile$^8$] AII, and the AT$_1$ receptor-selective antagonist Dup753 with the following order of potency: [Sar$^1$, Ile$^8$] AII $>$ AII $>$ Dup753 $>$ AIII $>$ AI. In contrast, PD123319, an AT$_2$ receptor-selective antagonist, had no effect on the binding of $^{125}$I-[Sar$^1$, Ile$^8$] AII. This displacement pattern was similar to those for the rat, bovine, and human AT$_1$ receptors which have been transiently expressed in COS-7 cells (2, 19, 24, 25), demonstrating that the cloned gene encodes a functional AII receptor with the pharmacological characteristics of an AT$_1$ receptor. Scatchard plots transformed from the competitive displacement study revealed binding parameters with a dissociation constant ($K_d$) of 0.65 nM and a binding site number ($B_{max}$) of 1.2 $\times$ 10$^5$ sites/cell. This $K_d$ value was also in good agreement with that previously reported for the rat AT$_1$ receptor expressed in COS-7 cells ($K_d=0.68$ nM for $^{125}$I-[Sar$^1$, Ile$^8$] AII) (19).

It is well known that the AT$_1$ receptor is sensitive to sulphydryl reducing reagents and its binding activity is reduced by reagents such as DTT, while the binding of the AT$_2$ receptor is increased by these reagents (3, 18). Therefore, the cells were treated with DTT at 25°C for 15 min before incubation with $^{125}$I-[Sar$^1$, Ile$^8$]AII. As shown in Fig. 2, DTT decreased the radioligand binding in a dose-dependent manner, confirming that the cloned gene encodes the AT$_1$ receptor. Moreover, this result shows that a disulphhydryl band(s) necessary for AII binding is located on extracellular domains
Fig. 2 Dithiothreitol dependence of 125I-[Sar1, Ile8]AII binding. Cells were incubated with the indicated concentrations of DTT at 25°C for 15 min and assayed for 125I-[Sar1, Ile8]AII binding activity as described under Materials and Methods. Results are given as the percent of control binding measured in the absence of DTT. Each point represents the mean of duplicate determinations.

of the receptor molecule.

Generation of Intracellular Second Messengers in Response to AII

A characteristic of the AT1 receptor is its ability to activate the Ca2+/inositol signal transduction pathway (8, 17). To examine the ability of the cloned gene to couple with this pathway, the changes in intracellular Ca2+ concentrations ([Ca2+]i) induced by AII were measured using fura-2 as a Ca2+ indicator. Addition of 10 nM AII to the cells evoked a rapid, transient increase in [Ca2+]i, which peaked at 15 s, followed by a lower, sustained phase (Fig. 3A). This response was markedly attenuated by 1 μM Dup753 (Fig. 3B), but not by 10 μM PD123319 (data not shown). To investigate the effect of extracellular Ca2+ on the AII-induced Ca2+ mobilization, the cells were incubated with 4 mM EGTA prior to addition of AII. This treatment significantly attenuated the initial, transient phase and completely abolished the sustained phase (Fig. 3C).

Fig. 3 The effect of AII on intracellular free Ca2+ ([Ca2+]i). Fura-2-loaded cells were exposed to 10 nM AII in the absence (A) or presence of the indicated reagents (B–D). Dup753 (1 μM) was added to cells with AII at the same time (B). EGTA (4 mM) (C) and nicardipine (1 μM) (D) were applied to bathing solution 5 min and 10 min before stimulation with AII, respectively. Representative tracings are shown and similar results were obtained from three separate experiments.
Fig. 4 Southern and northern blot analyses. Panels A and B and Southern blots of total cellular genomic DNA isolated from human leukocytes or HepG2 cells performed at high (A) and low (B) stringency, respectively. Panel C is a Southern blot of DNA from the λMB13 clone. Each lane contained 10 μg DNA digested with EcoRI or HindIII. Panel D represents a northern blot of poly(A)+RNA obtained from primary aldosteronoma. Other experimental conditions are described in detail under Materials and Methods.

The same result was obtained when the cells were pretreated with 1 μM nicardipine, a blocker of voltage-dependent L-type Ca2+ channels (Fig. 3D). On the other hand, the production of IP3 was also observed in response to AII (data not shown). Together, these observations demonstrated that following binding of AII, the human AT1 receptor stimulates phospholipase C (PLC) with the production of the second messenger IP3, resulting in the [Ca2+]i increase. The data further indicated that the AII-induced transient increase in [Ca2+]i is mainly due to the release of intracellular Ca2+ stores mediated by IP3, whereas the sustained increase completely depends on Ca2+ influx through L-type Ca2+ channels.

Northern and Southern Blot Analyses

Northern blots probed with the 760-bp fragment of the PCR product at high stringency revealed the presence of a 2.3-kb hybridization transcript in poly(A)+RNA obtained from the human primary aldosteronoma (Fig. 4D); the nucleotide sequence of the PCR product is identical to that of the cloned gene. The size of the hybridization band agrees with that reported by Takayanagi et al. (25) and Bergsma et al. (2). Genomic Southern blot analysis at high stringency using the same PCR product as a probe revealed the presence of unique HindII and EcoRI fragments, of 3.2 and 9.0 kb respectively (Fig. 4A), with a hybridization pattern identical to that of the isolated clone (Fig. 4C). This indicated that the insert of λMB13 is an authentic copy of the human genomic locus. Additional signals did not appear on Southern blots even at low stringency (Fig. 4B), suggesting that the human genome has a single copy of the AT1 receptor. These results disagree with the notion that two types of the rat AT1 receptor cDNAs are thought to be derived from separate genes.

DISCUSSION

We have isolated the AT1 receptor gene from a human leukocyte genomic library and found that it was identical to that recently isolated by Furuta et al. (6). Takayanagi et al. (25) and Bergsma et al. (2) cloned a cDNA for the human AT1 receptor and investigated the ligand binding characteristic of the receptor transiently expressed in COS-7 cells. However, they did not examine the coupling of the receptor to second messenger systems using the transfected COS-7 cells. In this study, therefore, we investigated AII-induced signal transduction pathways mediated by the human AT1 receptor as well as its biochemical and pharmacological properties by establishing CHO cells stably expressing the recombinant receptor.
The expressed cloned gene exhibited several typical characteristics of the AT₁ receptor: inhibition of $^{125}$-i[Sar$^1$, Ile$^8$]Ala binding by the AT₁ receptor-selective antagonist Dup753, but not by the AT₂ receptor-selective agent PD123319; decreased radioligand binding by the sulfhydryl reducing reagent DTT; and activation of PLC resulting in an increase in [Ca$^{2+}$]. A number of growth factors and hormones exhibit diverse physiological actions through their receptor subtypes. At present, various AII-induced events are known to be mediated by at least three different signalling pathways: i.e. phosphoinositide hydrolysis by stimulation of PLC resulting in a release of intracellularly stored Ca$^{2+}$ (8, 17, 20); inhibition of adenylate cyclase (11, 16, 20); and activation of voltage-dependent L-type Ca$^{2+}$ channels (1, 12, 20). Together with these data, molecular cloning of the cDNAs for two different types of the AT₁ receptor, AT₁A and AT₁B, in the rat raised the issue of whether a single population of the receptor mediates these pathways or subtypes of the AT₁ receptor couple with the individual pathways. However, that we identified a unique hybridization band on genomic Southern blot analysis even at low stringency, suggests the existence of one copy of the AT₁ receptor gene in the human genome in contrast to the rat genome. Therefore, it is thought that the product of the cloned human AT₁ receptor gene activates all the three signalling pathways. The present study demonstrates at least the coupling of the receptor with the inositol phosphate/Ca$^{2+}$ second messenger pathway. Furthermore, the finding that the abolishment of the AII-induced sustained [Ca$^{2+}$], response by nicardipine, a blocker of L-type Ca$^{2+}$ channels, might indicate the activation of the channels.

Considering that the AT₁ receptor is a target of drug therapy in several diseases such as hypertension and hypertrophy, the established CHO cell lines will become a powerful means to assess the activity of developed drugs as well as to analyze the AII-induced signalling pathways in detail.

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