Functional Reconstitution of the Angiotensin II Type 2 Receptor and Gi Activation

Jakob Lerche Hansen, Guy Servant, Thomas J. Baranski, Toshiro Fujita, Taroh Iiri, Stig Haunsø, Søren P. Sheikh

Abstract—On the basis of the patterns of conserved amino acid sequence, the angiotensin II type 2 (AT$_2$) receptor belongs to the family of serpentine receptors, which relay signals from extracellular stimuli to heterotrimeric G proteins. However, the AT$_2$ receptor signal transduction mechanisms are poorly understood. We have measured AT$_2$-triggered activation of purified heterotrimeric proteins in urea-extracted membranes from cultured COS-7 cells expressing the recombinant receptor. This procedure removes contaminating GTP-binding proteins without inactivating the serpentine receptor. Binding studies using [$^{125}$I]angiotensin (Ang) II revealed a single binding site with a $K_d = 0.45$ and a capacity of 627 fmol/mg protein in the extracted membranes. The AT$_2$ receptor caused a rapid activation of $\alpha_i$ and $\alpha_o$ but not of $\alpha_s$ as measured by radioactive guanosine 5'-3-O-(thio)triphosphate (GTP$_{\gamma}$S) binding. Activation required the presence of activated receptors, $\beta_y$, and $\alpha$ subunits. As a first step aimed at developing an in vitro assay to examine AT$_2$ receptor pharmacology, we tested a battery of Ang II–related ligands for their ability to promote AT$_1$ or AT$_2$ receptor–catalyzed Gi activation. Two proteolytic fragments of Ang II, Ang III and Ang1–7, also promoted activation of $\alpha_i$ through the AT$_2$ receptor. Furthermore, we found that [Sar$^1$,Ala$^8$]Ang II is an antagonist for both AT$_1$ and AT$_2$ receptors and that CPG42112 behaves as a partial agonist for the AT$_2$ receptor. In combination with previous observations, these results show that the AT$_2$ receptor is fully capable of activating Gi and provides a new tool for exploring AT$_2$ receptor pharmacology and interactions with G-protein trimers. (Circ Res. 2000;87:753–759.)

Key Words: AT$_2$ ■ angiotensin II type 2 receptor ■ Gi activation

Angiotensin II (Ang II) is the primary effector of the renin/angiotensin system. This 8-amino acid peptide is a key regulator of blood pressure and body fluid homeostasis and plays a critical role in the pathophysiology of several cardiovascular diseases such as hypertension, hypertrophy, and congestive heart failure. In particular, blocking the AT$_2$ receptor signal transduction mechanisms is poorly understood. We have measured AT$_2$-triggered activation of purified heterotrimeric proteins in urea-extracted membranes from cultured COS-7 cells expressing the recombinant receptor. This procedure removes contaminating GTP-binding proteins without inactivating the serpentine receptor. Binding studies using [$^{125}$I]angiotensin (Ang) II revealed a single binding site with a $K_d = 0.45$ and a capacity of 627 fmol/mg protein in the extracted membranes. The AT$_2$ receptor caused a rapid activation of $\alpha_i$ and $\alpha_o$ but not of $\alpha_s$ as measured by radioactive guanosine 5'-3-O-(thio)triphosphate (GTP$_{\gamma}$S) binding. Activation required the presence of activated receptors, $\beta_y$, and $\alpha$ subunits. As a first step aimed at developing an in vitro assay to examine AT$_2$ receptor pharmacology, we tested a battery of Ang II–related ligands for their ability to promote AT$_1$ or AT$_2$ receptor–catalyzed Gi activation. Two proteolytic fragments of Ang II, Ang III and Ang1–7, also promoted activation of $\alpha_i$ through the AT$_2$ receptor. Furthermore, we found that [Sar$^1$,Ala$^8$]Ang II is an antagonist for both AT$_1$ and AT$_2$ receptors and that CPG42112 behaves as a partial agonist for the AT$_2$ receptor. In combination with previous observations, these results show that the AT$_2$ receptor is fully capable of activating Gi and provides a new tool for exploring AT$_2$ receptor pharmacology and interactions with G-protein trimers. (Circ Res. 2000;87:753–759.)

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(PTX) substrates, αs and α, that stimulate adenyl cyclases, the α, α11, α12, and α13/16 that activate phospholipase C-βs (PLCβ), and the α2/3 family, that can transform different cell lines.

Which signaling proteins or G proteins does the AT2 receptor activate to exert its biological functions? It has proven difficult to ascertain whether AT2 receptors activate heterotrimetric G proteins in vivo. Several reports indicate that the AT2 receptor may couple to Gs. Thus, in rat hippocampal neurons and a few other cell types, blocking αs with PTX or antibodies directed against αs inhibits the AT2 receptor effects. In two studies, a direct interaction between AT2 receptors and αs was inferred from coimmunoprecipitation experiments. In contrast, in neuroblastoma cells and NG108-15 cells bearing AT2 receptors, PTX does not block Ang II effects. Moreover, in PC-12 cells bearing endogenous AT2 receptors and αs was inferred from coimmunoprecipitation experiments. In contrast, in neuroblastoma cells and NG108-15 cells bearing AT2 receptors, PTX does not block Ang II effects. Moreover, in PC-12 cells bearing endogenous AT2 receptors and αs was inferred from coimmunoprecipitation experiments.

To examine whether the AT2 receptor can activate Gs, we have assessed in vitro AT2-Gs interactions in a solution of urea-washed membranes from AT2-transfected COS-7 cells containing purified G-protein components. The AT2 receptor triggered activation of G-protein subunits (αs and βγ), as ascertained by guanosine 5’-3-O-(thio)triphosphate (GTPγS) binding. Ligand-activated AT2 stimulated GTPγS binding to Gs and Gq, but not G, or Gi. We infer from our results that AT2 receptors directly activate αs and αs. The fidelity and degree of resolution of the interaction allow structure function studies and further AT2 receptor pharmacological characterization.

Materials and Methods

Materials
Angiotensin-related peptides [Sar1,Ala8]Ang II, [1–7]Ang II, Ang II, and Ang III were obtained from Bachem. Isoproterenol and Ang IV were obtained from Sigma and parathyroid hormone (PTH) peptide from Bachem. The radiolabeled [125I]Ang II was obtained from Amersham Pharmacia Biotech and [35S]GTPγS from NEN Life Science Products. Losartan, PD123319, and CGP42112 were generous gifts from Dr Gaetan Guillemette (University of Sherbrooke, Canada). The Elk1 luciferase reporter was obtained from Stratagene.

Construction of the R142A-AT2 Receptor
The point mutation was generated by polymerase chain reaction in two steps with Pfu polymerase with the wild-type receptor cDNA as a probe, as described.

Cell Culture and Transfection
COS-7 cells were maintained in DME-H21 medium containing 10% FCS, 2.5 μg/mL Fungizone, and 10 μg/mL gentamycin. Transient transfections with wild-type serpine receptors were performed using a DEAE-dextran/adenovirus method as described.

Membrane Preparation
Membranes from COS-7 cells transfected with cDNAs encoding human AT1, human PTH, or human β2 receptors were prepared as described. Membrane microsomal fractions, obtained by centrifugation, were stripped of GTP-binding proteins as described by incubation in 6 mol/L urea.

G-Protein Purification
Alpha subunits were purified from SF9 cell membranes infected with baculovirus encoding the wild-type protein, His6-tagged γs, and wild-type β2, as described.

Ligand Binding
Binding of [125I]Ang II was determined as described. Binding was initiated by suspending urea-extracted membranes in a mixture of [125I]Ang II (100 nmol/L), cold Ang II, and a buffer consisting of 25 mmol/L Tris-HCl, pH 7.3, 100 mmol/L NaCl, 5 mmol/L MgCl2, 1 mmol/L EDTA, 2 mg/mL BSA, 0.1 mg/mL bacitracin, and 50 μg/mL soybean trypsin inhibitor.

Gα Activation
Exchange of GTPγS for GDP bound to Goα was measured using a modification of a previously described procedure. Briefly, membranes containing receptors (~5 nmol/mL) were preincubated with purified (50 nmol/L) and βγ (100 nmol/L) for 15 minutes on ice in a buffer containing 20 mmol/L Na-HEPES, pH 7.6, 1 mmol/L Tris-HCl, pH 7.6, 100 mmol/L NaCl, 2 mmol/L MgCl2, 1 μmol/L GDP, and 1 mmol/L β-mercaptoethanol. Assays were initiated by addition of agonist and [35S]GTPγS (10,000 cpm per tube) in each reaction at 30°C. Reactions were terminated by adding a stop solution containing 20 mmol/L Tris-HCl, pH 8, 100 mmol/L NaCl, and 10 mmol/L MgCl2 and filtered over nitrocellulose membranes on a vacuum manifold. Radioactivity was quantitated by liquid scintillation in a β counter. Nonspecific binding (binding to the filter in the absence of membranes) was <10% of total binding.

Mitogen-Activated Protein Kinase (MAPK) Phosphorylation
COS-7 cells were cotransfected with plasmids encoding the AT2 receptor, αs, γs, and β2. After transfection, the cells were incubated in DMEM supplemented with 10% FCS overnight followed by 16 hours of serum starvation. Next, the cells were stimulated with 10% FCS for 12 minutes in the presence and absence of Ang II. Cell lysates and quantification of MAPK phosphorylation was performed as described.

Elk1 Activation
Transcriptional activation of Elk1 luciferase expression by MAPK was assessed by cotransfecting pFA2-EIk1, plasmids encoding pFR-luc, βgalactosidase (β-Gal), and the AT2 receptor, αs, β2, and γs expression plasmids in COS-7 cells. After transfection, the cells were incubated in DMEM supplemented with 10% FCS overnight. Next, the cells were serum-starved for ~16 hours followed by a 24-hour incubation with 10% serum in the presence and absence of 100 nmol/L Ang II. Luciferase and β-Gal activities were measured as described by the manufacturer.

Results
To examine the AT2-receptor/G-protein coupling, we used urea-extracted COS-7 cell membranes transiently expressing AT2 receptors and compared the results with those previously reported for other receptors: rhodopsin, gastrin-releasing peptide, PTHR, or β2AR.12,13 The addition of purified G-protein subunits (both αs and βγ) allowed us to examine the selectivity of G-protein activation and assess the potency of Ang II-related receptor ligands.

Ligand Binding Studies
Urea-treated COS-7 cell membranes bearing the wild-type AT2 receptor or a specific mutant R142A (a mutation that
uncouples receptor-G-protein activation\(^{18}\) were analyzed for Ang II binding. Figure 1 depicts representative curves showing the effects of increasing concentrations of Ang II and analogues on \(^{[125i]}\)Ang II binding. The rank order of binding affinities was Ang III > CPA12112A > Ang II > Ang-I-7 > Ang IV. The apparent \(K_d\) values for Ang II were \(\approx 0.45\) and \(\approx 0.78\) nmol/L, and the calculated \(B_{\text{max}}\) was 627 and 280 fmol/mg protein for AT\(_2\) wild-type and R142A-AT\(_2\) receptors, respectively.

**Receptor Activation Assay**

To assess AT\(_2\)-mediated activation of \(\alpha_i\), we measured ligand-dependent binding of radioactive GTP\(_{\gamma}\)S in a mixture containing pure \(\alpha_i\) and \(\beta\gamma\) and urea-washed membranes from COS-7 cells expressing recombinant receptors and NIH3T3 cells bearing native AT\(_2\) receptors. In both of these systems, Ang II induced significant GTP\(_{\gamma}\)S binding only in the presence of receptors \(\alpha_i\) and \(\beta\gamma\) (Figures 2B and 2D). In urea-treated COS-7 membranes, Ang II increased GTP\(_{\gamma}\)S binding 3- to 10-fold in different experiments. At maximal stimulation, radioactive GTP\(_{\gamma}\)S bound to 10% to 30% of the total \(\alpha_i\) present in the assay. The effect of Ang II was complete within 3 minutes (not shown). This shows that the AT\(_2\) receptor can catalyze the exchange of GDP for GTP\(_{\gamma}\)S on \(\alpha_i\). To explore the effects of urea treatment, we compared GTP\(_{\gamma}\)S binding in P2 membranes and urea-washed membranes from COS-7 and NIH3T3 cells (Figure 2). Ang II induced a 7% and 19% increase in GTP\(_{\gamma}\)S binding in COS-7 and NIH3T3 P2 membranes, respectively. Coexpression of \(\alpha_i\) and \(\beta\gamma\) did not improve the Ang II effect (Figure 2). These results show that with native complement of G proteins, there is a trend toward AT\(_2\) activation–enhanced GTP binding, although the signal-to-noise is hampered by the high level of AT\(_2\)-uncoupled GTP-binding proteins. The data also show that urea treatment drastically reduces endogenous GTP binding without inactivating the recombinant serpentine receptors consistent with previous findings.\(^{12}\)

Figure 3 depicts the concentration dependence of the individual components of the system. The kinetics of G-protein activation would be expected to be saturable with each of the components. This prediction was fulfilled: GTP\(_{\gamma}\)S binding was saturable with increasing concentrations of \(\alpha_i\).
The added 

Ang II proteolytic fragment found in plasma. This peptide with a much lower affinity than Ang II. Ang1–7 is another

receptors. AT 1 receptor activated 

AT 1 receptor expressed PTHR. The activated PTHR did not promote GDP exchange for GTP 

receptors. We examined this using membranes 

receptors.18 These experiments show that AT 2 receptors discriminate between G i, G q , and G s in this system. Another important question is whether the added α i might be activated by a serpentine receptor. We examined this using membranes expressing PTHR. The activated PTHR did not promote GDP exchange for GTPγS on α i (Figure 4B). Thus, both the recombinant AT 2 and the purified α i have retained specificity in this system.

AT 2 Receptor Pharmacology

We examined the effect of different Ang II analogues on AT 1 and AT 2 receptor–induced α i activation. Figure 5 shows that in addition to Ang II, one of its proteolytic fragments, Ang III (desAsp1–Ang II), behaved as an agonist on both AT 1 and AT 2 receptors. Ang I–7 selectively activated AT 2 receptors albeit with a much lower affinity than Ang II. Ang I–7 is another Ang II proteolytic fragment found in plasma. This peptide induces bradykinin-mediated hypotensive responses and reduces smooth muscle growth after vascular injury.20,21 Thus, AT 2 receptors could be responsible for these actions. CGP42112A, a pseudo-peptide that binds the AT 2 receptor with high affinity and selectivity, was initially considered an antagonist.7 Our results using this compound alone suggest that CGP42112A is a selective AT 2 agonist. A similar conclusion was previously made.17 However, in the presence of 100 nmol/L Ang II, a high concentration of CGP42112A works antagonistically and thus inhibits the effect of Ang II (Figure 5B). These data suggest that CGP42112A is a partial agonist. Losartan and PD123319, two nonpeptidic Ang II analogues, bind with high affinity and selectivity to AT 1 and AT 2 receptors, respectively.22 Accordingly, losartan selectively blocked AT 1 activity in the described assay (Figure 5). Olmesartan and candesartan also selectively blocked AT 2 receptor activity (data not shown). In almost all reported AT 2–mediated effects, PD123319 is a selective competitive antagonist (Table). In our study, this compound selectively blocked AT 2 receptor activity. In addition, an antagonist [Sar1,Ala8]-Ang II, thought to block both receptors, did so in our assays (Figure 5).

AT 2 Receptor Effects in Intact COS-7 Cells

Given that our assay is a reconstitution of purified G proteins with urea-washed COS-7 membranes, we wanted to analyze whether the transfected AT 2 receptor could activate signaling pathways in intact cells. The AT 2 receptor has been reported to couple to ERK1/2 MAPK phosphorylation. To explore this concept, we investigated MAPK (ERK1/2) phosphorylation by Western blotting and MAPK transcripational activation of Elk1 using Elk1 luciferase reporter plasmid. AT 2 receptor activation induced both MAPK phosphorylation and Elk1 luciferase ex-
pression in the presence of serum compared with the effect of serum alone (Figure 6). AT$_2$ receptor activation did not affect MAPK phosphorylation or activation in the absence of serum (not shown). These data show that AT$_2$ receptor activation in COS-7 cells is functionally coupled to MAPK.

**Discussion**

We have set up a system to assay AT$_2$ receptor activation of G$_i$ to understand the G-protein signaling properties of this receptor. The AT$_2$ receptor has diverse biological effects in cell growth, differentiation, and control of blood vessel tone (Table). However, the molecular events behind these effects have not been clearly defined. In particular, it has not been possible to document G protein–regulated activation of classical second messengers such as cAMP and the phospho-inositide metabolism. Almost all other serpentine receptors regulate at least one of these systems. Can the AT$_2$ receptor activate G proteins? To answer this question, we tested the ability of the AT$_2$ receptor to activate the $\alpha_i$ subunit of G$_i$ in a reconstituted system. In this system, the AT$_2$ receptor activated $\alpha_i$ in much the same way as other receptors activate G proteins. In similar assays, rhodopsin activates transducin, and the gastrin-releasing peptide receptor activates G$_q$. Mutations can produce promiscuous receptors, which activate classes of G proteins not activated by the parent wild-type receptors. Could the described assay simply unmask an inherent promiscuity of AT$_2$ receptors? To address this question, we performed two experiments, the results of which suggested that the assay does exhibit specificity. First, the AT$_2$ receptor was unable to induce GTP$_G$S binding to purified G$_q$ and G$_s$, two different G proteins, and vice versa, the G$_i$ was not activated by PTHR. Data from ligand binding studies, use of PTX, which inactivates $\alpha_i$, and coimmunoprecipitation experiments using anti-G-protein antibodies, support the inference that the AT$_2$ receptor activates G proteins. First, GTP$_G$S reduces AT$_2$ receptor binding affinity in certain cell membranes and in HEK293 cells with overexpressed AT$_2$ receptors. Second, at least three AT$_2$-mediated biological effects are blocked by PTX: a delayed rectified K$^+$

### AT$_2$ Receptor Signal Transduction in Different Cellular Environments

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>AT$_2$ Effect</th>
<th>PTX Sensitivity</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC12W</td>
<td>MKP-1 stimulation → Bcl$_2$ inhibition</td>
<td>+</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>SHPI stimulation → apoptosis induction</td>
<td>ND</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Stimulation of ceramide production</td>
<td>+</td>
<td>...</td>
</tr>
<tr>
<td>VSMCs</td>
<td>MKP-1 stimulation → MAPK inhibition</td>
<td>+</td>
<td>10</td>
</tr>
<tr>
<td>ARVMs and CMECs</td>
<td>MKP-1 stimulation → MAPK inhibition</td>
<td>ND</td>
<td>28</td>
</tr>
<tr>
<td>NIE-115</td>
<td>SHPI-stimulation → MAPK inhibition</td>
<td>–</td>
<td>11</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>Stimulation of cGMP production</td>
<td>ND</td>
<td>27</td>
</tr>
<tr>
<td>Renal proximal tubular epithelia</td>
<td>mPLA2 stimulation → MAPK stimulation</td>
<td>ND</td>
<td>29</td>
</tr>
<tr>
<td>NIH3T3</td>
<td>Growth inhibition</td>
<td>+</td>
<td>9</td>
</tr>
<tr>
<td>Rat hypothalamus neurons</td>
<td>G-protein stimulation → $k_{i, delayed}$ induction</td>
<td>+</td>
<td>8</td>
</tr>
<tr>
<td>COS-7</td>
<td>PTPase inhibition</td>
<td>+</td>
<td>...</td>
</tr>
<tr>
<td>NG108-15</td>
<td>PTPase stimulation → T-type Ca$^{2+}$ current inhibition</td>
<td>–</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>p21 Ras inhibition → MAPK stimulation</td>
<td>ND</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>NMAD Ca$^{2+}$ current inhibition → NO production inhibition</td>
<td>ND</td>
<td>31</td>
</tr>
</tbody>
</table>

Bcl$_2$, B-cell lymphoma 2; PP-2A, Ser/Thr phosphatase 2A; MKP-1, MAP kinase phosphatase-1; (m)PLA2, (membrane-associated) phospholipase A2; AA, arachidonic acid; SHC, Src homologous and collagen protein; GRB2, growth factor receptor binding protein; SOS, son of sevenless; MAPK, mitogen-activated protein kinase; SHPI, SH2 domain–containing phosphatase; PTPase, phosphotyrosine phosphatase; PTX, pertussis toxin; ARVM, adult rat ventricular myocyte; CMEC, cardiac microvascular endothelial cell; VSMC, vascular smooth muscle cell; and ND, not determined.
current in neurons, inhibition of NIH3T3 cell growth, and DNA synthesis in vascular smooth muscle cells. Third, AT₁ receptor protein has been coimmunoprecipitated with α₁ from fetal rat cells and vascular smooth muscle cells, suggesting that a physical association exists. AT₂ receptor activation also promotes PTX-insensitive cellular events (Table). Our results show that the AT₂ receptor can support activation of G₁ and G₅ but not G₂ and G₃.

If the AT₂ receptor activates G₅, why does it not inhibit cAMP production or robustly activate MAPK, effects attributed to activation of α₁ and liberation of βγ? Our results do not unequivocally answer this question; however, possible explanations can be offered. First, in at least three reports, AT₁ receptor stimulation led to MAPK activation. Second, the AT₂ receptor could need concentrations of resident G proteins in a given cell type may modify receptor coupling. It is possible that, in vivo, the AT₂ receptor receptors to Gαi with a low affinity or with a low rate of GDP-GTP exchange. Thus, only cells expressing high levels of AT₁ and Gαi would produce detectable levels of second messengers. Fourth, cell-specific post-translational modifications of the AT₂ receptor could play a role in determining G-protein specificity.

Our simple assay proved useful for the determination of AT₁ receptor pharmacology, something that has been difficult to evaluate because of the lack of reproducible and easily quantifiable effects. We compared different peptidic and nonpeptidic Ang II analogues on AT₂-induced Gαi activation. Furthermore, to validate our observations, we tested the same battery of analogues on membranes bearing AT₁ receptors, for which responses to these ligands are well characterized. We found that Ang II and its pro teaseic fragment Ang III (lacks the amino-terminal Asp) act as agonists on the AT₂ receptor. Similarly, and as expected, they also triggered AT₁-induced Gαi activation.

In one study, Ang1–7, an Ang II pro teaseic fragment in which the carboxyl-terminal residue is cleaved, was reported to induce prostaglandin synthesis in human astrocytes through AT₂ receptors. Moreover, evidence suggests that Ang II pro teaseic fragments other than Ang III and including Ang1–7 may have some biological activities. Accordingly, we found that this peptide selectively activated the AT₂ receptor. Taken together, these results suggest that, in vivo, both Ang II and Ang III could serve as agonists for the AT₂ receptor given that they have similar affinities.

Acknowledgments
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References
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