The AT2 Receptor Selectively Associates with Giα2 and Giα3 in the Rat Fetus*

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The effects of angiotensin II are mediated by a family of seven transmembrane receptors. In the adult, the majority of the receptors are of the AT1 isoform, which is coupled to heterotrimeric G proteins (either Go, or Gi). In contrast, the AT2 receptor is expressed at low levels in the adult but is the major form expressed in the fetal and neonatal animal. Previous results have failed to show G protein coupling of the AT2 receptor in the fetus. We now provide evidence that the AT2 receptor is G protein-coupled. An antibody that binds several Gs subunits immunoselected angiotensin II receptor–Gα complexes. In addition, Gi1α3 antibody, which recognizes Gα1α2, Giα2 and Giα3, also co-immunoselected the AT2 receptor. Anti-Gα1α2 and anti-Gα1α3 antibodies were both able to co-immunoselect AT2 receptor–Gα complexes, but consistent with the lack of Giα1α2 in the fetal extracts, anti-Gα1α2 antibodies did not nor did any other G protein-directed antisera. The finding that AT2 receptor couples to both Gα1α2 and Gα1α3 antibodies raises the possibility that selective interactions between AT2 receptor and different G proteins may result in specific cellular effects mediated by AT2 stimulation.

Two Ang II1 receptor subtypes (AT1 and AT2) have been defined (1, 2). Most known physiological actions of Ang II are mediated by the AT1 receptor (1–4). On the other hand, the AT2 receptor remains an enigma. Cloning of AT2 receptor by our laboratory and others (5–8) has revealed that like the AT1 receptor, the AT2 receptor also possesses a seven transmembrane domain structure, which is similar to that of the heterotrimeric guanine nucleotide binding protein (G protein)-coupled receptors (9). However, the biochemical association of this receptor to this class of proteins has not been demonstrated.

Heterotrimeric G proteins, comprised of α, β, and γ subunits, function as intermediates that couple cell surface receptors to intracellular effectors (10, 11). Much of the specificity of receptor action is dictated by the α subunit to which it is coupled. This subunit can be grouped into four major classes, each comprised of multiple members that are related by sequence homologies (αo (αo and αolf), αi (αi1, αi2 and αi3), αo, αi1, αi2, αo1, αq (αq, α11, α14, α15, and α16) and the α12 (α12 and α13)) (12). Interestingly, many seven-transmembrane receptors can, in fact, couple to several G proteins, increasing the complexity of the systems. Once thought to be only negative regulators of α subunit activity, the β subunits have also been shown to be linked to the activation of various intracellular pathways (10–12).

In radioligand binding and in autoradiographic studies, the AT1 receptor is known to be sensitive to GTP analogs (1, 2, 13), suggesting the coupling to G proteins. Stimulation of Ang II receptor in AT1-rich tissues results in calcium mobilization through Gα11, and modulation of cAMP levels through Gαi (14–16). In contrast to the AT1 receptor, in most tissues examined, binding to the AT2 receptor is not susceptible to GTP analogs (1, 2, 13, 17–19), which has led to the suggestion that the AT2 receptor does not interact with G proteins (13). However, indirect evidence has suggested that AT2 receptor may also couple to G proteins (20, 21). In certain regions of the rat brain, binding to the AT2 receptor was sensitive to GTP analogs and to pertussis toxin treatment (22). Similarly, in PC12W cells, the AT2 receptor-mediated inhibition of phosphorylase phosphatase was reversed by pertussis toxin treatment (23).

More recently, Kang et al. (24) reported that in vivo in cultured rat neurons, AT2 receptor-mediated stimulation of delayed rectifier K+ current (I(K)) could be abolished by intracellular application of an anti-Gα1 antibody.

The above data demonstrating sensitivity (or insensitivity) of radioligand binding to GTP-β-S and pertussis toxin are indirect evidence for G protein coupling (or uncoupling). However, these approaches suffer from several caveats. Although the work of Kang et al. (24) is more direct, the use of cultured cells, which require several weeks of culture in order to develop an AT2 receptor-mediated action, is also problematic. Therefore, the purpose of the present work was to demonstrate a direct biochemical association of AT2 receptor and its coupled G proteins in the rat fetus. The rat fetus was chosen because the AT2 receptor is expressed at extraordinarily high levels in many fetal tissues.

EXPERIMENTAL PROCEDURES

Membrane Preparation—Membrane fractions (100,000 × g pellet) were isolated as described (6, 7) from 18-day Sprague-Dawley rat whole fetal tissue. To solubilize the receptor, the membrane pellets were resuspended in 25 mM sodium phosphate, pH 7.4, 5 mM EDTA, 5 mM EGTA, 400 mM KCl, 25% glycerol, 25 mM MgCl2, plus 1% CHAPS (25). The 100,000 × g supernatant was collect and stored at −70 °C. Radioligand Binding Assay—Membrane (10 μg of protein) or immunoselected fractions were incubated with 2 nM 125I-AngII (2.176 Ci/mM) at room temperature for 60 min in 100 μl of 20 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 0.25% bovine serum albumin. Bound and free ligands were separated by rapid filtration through GF/B Whatman glass fiber filters. For solubilized membranes, the filters were prewashed in 0.5% polyethyleneimine. The filters were washed three times, and the bound radioligand was quantitated. Each point was performed in duplicate. Non-
specific binding was defined as radioactivity bound in the presence of 1 μM unlabeled ligand. For characterization of AT2 receptor subtypes, the selective AT2 receptor antagonist DuP 735 (losartan) and the selective AT1 ligands CGP42112A or PD123319 (each at 1 μM) were employed.

Western Blots—Solubilized membrane samples in SDS-polyacrylamide gel (PAGE) buffer were incubated in boiling water bath for 5 min. Approximately 30 μg of protein was subjected to 10% SDS-PAGE and transferred onto nitrocellulose. Immunoblotting was carried out according to ECL Western blotting protocol (Amersham Life Science Inc.).

Immunoselection of AT2 Receptor-G Protein Complexes—Solubilized AT2 receptor preparations (1 ml of a 1 mg/ml solution) diluted in solubilization buffer containing 0.3% CHAPS were incubated with antibodies or antisera overnight at 4°C with constant shaking (amounts of the antibody or antisera are indicated in the figure legends). Protein A-agarose (60%) was then added and incubated with rocking for an additional 4 h at 4°C. The samples were centrifuged at 14,000 rpm. The supernatant was removed, and the immunoselections were washed three times with 1 ml of ice-cold solution binding buffer containing 0.3% CHAPS. The AT2 receptor in the immunoselection and in the supernatant was detected by radioligand binding assays as described above. In some experiments, 10 mM NaCl or 10 mM NaF plus 10 μM AIF3 were added to the solubilized membrane preparations at the time of the immunoselection.

[35S]GTPγS Binding Assay—[35S]GTPγS binding was measured with a modification of assay described by Johnson and Corbin (26). Immunoselections were mixed with incubation buffer (20 mM Tris-HCl, 25 mM MgCl2, 1 mM EDTA, 100 mM NaCl, and 1 mM dithiothreitol, pH 8) and 5 nM [35S]GTPγS (1,332.0 Ci/mmol) in 100 μL. The binding was initiated by the addition of the immunoselections to the incubation buffer and was carried out for 1 h at 30°C. The reactions were terminated by rapid filtration through GF/B Whatman glass fiber filters. The filters were washed three times with 5 ml of ice-cold buffer (20 mM Tris-HCl, pH 8.0, 25 mM MgCl2, 100 mM NaCl) and then quantitated. Non specific binding was determined in the presence of 1 nM GTP and subtracted from total bound radioactivity.

ADP-ribosylation of Solubilized and Immunoselected Proteins with Pertussis Toxin—Pertussis toxin was preactivated by incubation with 20 mM dithiothreitol, 0.125% SDS and 1 mg/ml BSA for 30 min at 30°C. Solubilized membrane (30 μg) or immunoselections were incubated with pertussis toxin (10 μg/ml) and 5 μCi of [32P]NAD in the presence or in the absence of 100 μM NAD for 1 h at 30°C, in a final volume of 100 μl reaction buffer (pH 7.4, 1 mM EDTA, 10 mM thymidine, 10 mM Hepes, 0.5 mM ATP, 0.1 mM GTP) (27, 28). The proteins were loaded into 10% SDS-PAGE gels. Gels were stained, dried, and autoradiographed at ~70°C for 5–24 h.

Materials—[125I]-Ang II (2, 176 Ci/mmol) was purchased from Robert J. Rock at Washington State University. Dithiothreitol, GTPγS, and CHAPS were from Sigma. Losartan was provided by Ronald Smith of Du Pont Harford Park, PD123319 was provided by Joan Keiser at Parke-Davis. CGP42112A was provided by Marc de Gasparo of Ciba-Geigy. ECL Western blotting detection reagents was from Amersham International Plc. Antisera sources are listed in Table I. (29–33).

Calculations and Statistics—Data are presented as mean values ± S.E. Student’s t test for unpaired observation and one-way analysis of variance were used for statistical evaluation. p < 0.05 was considered significant. Specific binding was calculated by subtracting nonspecific binding measured in the presence of unlabeled ligand from total binding. Saturation analysis were performed using nonlinear regression curve-fitting. Dissociation constant Kd and Bmax were calculated from Scatchard plot using a linear regression program (Statview).

RESULTS

Pharmacological Analysis of AT2 Receptor in Rat Fetus Membrane Preparations—Binding of [125I]-Ang II to membranes prepared from 18-day-old rat fetus was of high affinity, saturable, and linearly proportional to the protein concentrations tested (1–60 μg, data not shown). Scatchard analysis revealed a single class of binding sites (Fig. 1A), yielding a Kd of 1.89 ± 0.11 nM and Bmax of 1.91 ± 0.10 pmol/mg protein (n = 3). Nonspecific binding was determined in the presence of 1 μM CGP42112A and was less than 15% of total binding at the Kd of binding. Binding of [125I]-Ang II to the membrane preparation was competed by AT2 selective ligand CGP42112A (91 ± 3.7% displacement at 1 μM, n = 3) and PD123319 (78 ± 3.1% displacement at 1 μM, n = 3) but was not sensitive to the AT1-selective antagonist DuP 753 (7 ± 2.1% displacement at 1 μM, n = 3). (Fig. 1B).

We next tested the ability of detergents to solubilize the fetal membranes and release AT2 receptors, which retain binding capacity. As shown in Fig. 1C, optimal solubilization of AT2 receptor was achieved with 0.5–1% CHAPS, which yielded 70–60% of specific [125I]-Ang II binding and 50–60% of total protein. Because solubilization of AT2 receptor with 1% CHAPS resulted in less nonspecific binding but higher AT2 receptor binding in immunoselections (data not shown), 1% CHAPS was used in subsequent experiments.

[125I]-Ang II binding to solubilized membrane preparations was specific and saturable, with moderate decrease in the affinity and Bmax (Fig. 1A, Kd = 3.46 ± 0.12 nM, Bmax = 1.33 ± 0.11 pmol/mg protein.). Competition studies with receptor specific ligands (losartan, PD123319, and CGP42112A) and non-subtype selective ligands (Sar-1, Ile-8 Ang II, and Ang II) exhbitied an inhibitory pattern similar to that seen in crude membrane preparation (Fig. 1B) and consistent with the pharmacology of the AT2 receptor.

Presence of G Protein α and β Subunit Immunoreactivity in Solubilized Rat Fetus Membrane Preparation—Prior to an ex-
amination of the coupling of the AT2 receptor to specific G proteins, we first assessed the expression of the various G proteins in the solubilized 18-day-old rat fetus membranes using Western analysis. A majority of the anti-G protein antibodies tested yielded strong signals with appropriate molecular sizes (Fig. 2). Specific immunoreactivity was detected at 40–41 kDa with Gi2 and Gi3 antibodies. Anti-Gq/11 antibody detected proteins of molecular masses of 42–43 kDa. Anti-Gz antibody recognized a major band at 41 kDa. Anti-Gb common recognized a band at approximately 35–36 kDa. A weak band at 39 kDa was detected by anti-Go antibody. However, several different anti-Gi1 antibodies as well as antibodies specific for G12 and G13 failed to detect protein, suggesting the low expression of these proteins in the whole rat fetus. Control immunoblots using IgG were negative (Fig. 2).

Immunoselection of [35S]GTPgS Binding Proteins with Anti-G Protein Antibodies—In order to test the immunoselection procedure, the presence of GTP binding proteins in the immunoselection was examined by measuring the specific binding of [35S]GTPgS. Binding of [35S]GTPgS to both solubilized membrane preparation and to immunoselections was concentration-dependent and specific (data not shown). Under our experimental conditions, nonspecific binding defined in the presence of 1 mM of GTP was less than 10%. As shown in Fig. 3, polyclonal antibodies raised against several G proteins (Gi1–3, Gz, and Gq/11) were tested and shown to immunoselect, in a concentration-dependent manner, GTP binding proteins. Similar numbers of GTP binding sites were immunoselected with each antibody. The percentage of total GTP binding sites in the solubilized membrane that was immunoselected with the antibodies specific for Gi1–3, Gz, and Gq/11 were 1.8, 1.4, and 1.2%, respectively. This must be considered as an underestimate because solubilized membrane has been reported to contain GTP binding activities distinct from Gα proteins (34).
A second method was used to test the immunoselection procedure. Because \( \text{G}_{i1-3} \) are known to be sensitive to pertussis toxin, the presence of pertussis toxin-sensitive G proteins was also investigated. Labeling of both solubilized membrane preparation and SC-262-immunoselected fractions but not IgG-immunoselected fractions with \(^{32}\text{P-NAD} \) in the presence but not in the absence of pertussis toxin resulted in a radiolabeled band corresponding to molecular mass of approximately 40–41 kDa (Fig. 4). This band was not observed when 100 \( \mu \text{M} \) NAD was added, suggesting pertussis toxin-specific reaction of ADP-ribosylation (Fig. 4).

Immunoselection of \( \text{AT}_2 \) Receptor-G Protein Complexes with Anti-G Protein Antisera—We next tested the ability of antibodies raised against the G proteins to co-select angiotensin II binding sites. Initially, we used an anti-\( \text{G}_{\text{common}} \) antibody (SC-388), which interacts with \( \text{G}_{i1-3}, \text{G}_{z11}, \text{G}_{t1} \), and \( \text{G}_{z1} \). The results demonstrate the selection of a low level of \( \text{Ang II} \) binding sites that were not observed with IgG (Fig. 5A). Next, antibodies more selective against specific G proteins were tested. Consistently, anti-\( \text{G}_{i1-3} \) (SC-626) concentration-dependently co-immunoselected the solubilized \( \text{Ang II} \) binding sites (Fig. 5B). Interestingly, this antibody selected a higher amount of \( \text{Ang II} \) binding sites compared with \( \text{G}_{\text{common}} \) antibody. To address specificity, SC-262P, the peptide to which SC-262 was raised completely blocked the ability of this polyclonal antibody to immunoselect the \( \text{Ang II} \) binding sites. SC-262P itself did not interfere with \(^{125}\text{I}-\text{Ang II} \) binding (Fig. 5C). No angiotensin II binding sites were co-immunoselected with control IgG. As shown in Fig. 5D, binding of \(^{125}\text{I}-\text{Ang II} \) to SC-262-immunoselected samples was of high affinity and yielded saturation binding curves comparable with those observed with solubilized membrane preparations. Scatchard analysis revealed that the \( K_d \) of the immunoselected binding sites was comparable with that observed for the binding sites in the solubilized membrane fraction. In contrast, no specific \( \text{Ang II} \) binding sites were detected in immunoselections by anti-\( \text{G}_{i1} \), anti-\( \text{G}_{i2} \), anti-\( \text{G}_{i3} \), anti-\( \text{G}_{z1} \), anti-\( \text{G}_{t1} \), and anti-\( \text{G}_{z1} \) antibodies (Fig. 6), even though anti-\( \text{G}_{i1-3} \) and anti-\( \text{G}_{z1} \) were almost as effective in concentration-dependently immunoselecting GTP binding proteins (Fig. 3).

These \( \text{Ang II} \) binding sites represented \( \text{AT}_2 \) receptors. In experiments where specific binding was defined independently with Sar-1, Ile-8 \( \text{Ang II} \), or CGP42112, the compounds were shown to compete to similar levels (Fig. 7). Moreover, DuP 753 was not able to compete \(^{125}\text{I}-\text{Ang II} \) binding to the immunoselected material. In addition, binding with \(^{125}\text{I}-\text{CGP42112} \) was performed in selective experiments and demonstrated results comparable with those obtained with the \(^{125}\text{I}-\text{Ang II} \) (data not shown).

The above results suggest the close association between the \( \text{AT}_2 \) receptor and \( \text{G}_{i1} \), which may be an actual coupling of the receptor to this G protein. Alternatively, this association may only indicate the presence of these proteins within the same mixed micelles, which are formed during the solubilization step. The latter explanation is unlikely because antibodies specific for several other G proteins failed to immunoselect \( \text{AT}_2 \) binding sites; the proteins in the mixed micelles should be randomly distributed. However, to provide further and more direct data concerning this point, we examined the ability of aluminum fluoride, which is known to disrupt G protein-receptor complexes, to inhibit the co-immunoselection of the \( \text{AT}_2 \) binding sites by the anti-\( \text{G}_{i1} \) antibody. The addition of NaF plus AlF to the immunoselection decreased the ability of the anti-\( \text{G}_{i1} \) antibody to immunoselect \( \text{AT}_2 \) binding sites by 38% (specific binding obtained with anti-\( \text{G}_{i1} \) antibody, 1200 \( \pm 66 \); anti-\( \text{G}_{i1} \) antibody plus NaF plus AlF, 740 \( \pm 98 \)). However, NaCl at the same concentration was without effect. Thus, taken together, the results are consistent with a coupling of the \( \text{AT}_2 \) receptor and \( \text{G}_{i1} \).

To determine which subtype of \( \text{G}_{i1} \) was involved, several anti-\( \text{G}_{i1} \), anti-\( \text{G}_{i2} \), and anti-\( \text{G}_{i3} \)-specific antibodies were tested. An anti-\( \text{G}_{i2} \) antibody (J-883) co-immunoselected \( \text{AT}_2 \) receptor-G\(_{i1} \) complexes but to an extent less than that selected with anti-
**Fig. 5. Immunoselections of AT₂ receptor-Gᵢᵣ complexes by anti-Gᵢᵣ antibodies.** A, solubilized fetal membranes (1 mg/ml) were incubated with anti-Gᵢᵣ common antibody (SC-386) or IgG (10 µg) overnight at 4 °C. Antibodies were then collected with protein A-agarose. The immunoselections were incubated with ¹²⁵I-Ang II (2 nM) for 1 h at room temperature. Nonspecific binding was defined in the presence of 1 µM CGP42112. *, p < 0.05 compared with IgG control. B, solubilized membranes (1 mg/ml) were incubated with anti-Gᵢ₁–³ (SC-262) antibody (closed circles) or IgG (1–20 µg) (open circles) overnight at 4 °C and collected with protein A-agarose. Ang II binding was performed as above in A. Data are presented as means ± S.E., n = 5. C, to determine the specificity of the immunoselection by anti-Gᵢ₁–³ antibody (SC-262), the peptide (SC-262P, 50 µg/ml) to which the antibody SC-262 was generated was included in the immunoselection buffer. Nonspecific binding was determined in the presence of 1 µM CGP42112A. Each bar is presented as specific binding of ¹²⁵I-Ang II (2 nM) and presents the mean ± S.E. for 3 experiments. *, p < 0.05 compared with IgG control. †, p < 0.05 compared within SC-262 group. D, increasing concentrations of ¹²⁵I-Ang II (0.1–5 nM) were incubated for 1 h at room temperature with 10 µg of solubilized membrane preparations (open circles) or AT₂ receptors immunoselected with 10 µg anti-Gᵢ₁–³ antibodies (SC-262) (closed circles). Nonspecific binding was defined by 1 µM CGP42112A. Data are presented as mean of two determinations. E, analysis of the data by Scatchard plot yielding Kᵣ values of 3.7 and 4.2 nM for the solubilized membrane and immunoselected receptor, respectively.

Gᵢ₁–³ (SC-262) (Fig. 8). Similarly, an anti-Gᵢᵣ₂ antibody (NEI-803) also co-immunoselected AT₂ receptor, again to an extent less than that observed with anti-Gᵢᵣ₁–³ (Fig. 8). Several other anti-Gᵢᵣ subtype antibodies yield similar results (Gᵢᵣ₂ (1521), 670 ± 93 cpm; Gᵢᵣ₂ (Asano), 675 ± 83 cpm; Gᵢᵣ₃ (06–270), 504 ± 67 cpm; Gᵢᵣ₃ (371729), 431 ± 84 cpm). We next tested the ability of anti-Gᵢᵣ₂ and anti-Gᵢᵣ₃ antibodies to immunoselect Ang II binding sites in sequential experiments. Previous immunoreaction of the membrane preparation with anti-Gᵢᵣ₃ (NEI-803) antibody did not affect the ability of anti-Gᵢᵣ₂ (J-883) antibody to immunoselect Ang II binding sites. Similarly, previous immunoreaction of the membrane preparation with anti-Gᵢᵣ₂ (J-883) antibody did not affect the ability of anti-Gᵢᵣ₃ (NEI-803) antibody to immunoselect Ang II binding sites. On the other hand, prior treatment with anti-Gᵢᵣ₁–³ (SC-262) antibody abolished the subsequent immunoselection with anti-Gᵢᵣ₁–³ antibody (Table II).

Consistent with the very low levels of Gᵢᵣ₁ observed in the 18-day-old rat fetal membranes by Western analysis, an anti-Gᵢᵣ₁ antibody (I-355) was not capable of co-immunoselecting AT₂ binding sites (Fig. 8). Similar results were obtained with other anti-Gᵢᵣ₁ antibodies (anti-Gᵢᵣ₁ (3646), 277 ± 101 cpm; anti-Gᵢᵣ₁ (371720), 303 ± 103 cpm; anti-Gᵢᵣ₁ (Asano), 182 ± 28 cpm).
The AT₂ receptor is not coupled to G<i>a</i>, G<i>q</i>, G<i>12</i>, or G<i>13</i>. Solubilized rat fetal membranes (1 mg/ml) were incubated with anti-G<i>a</i> (SC-389), anti-G<i>q</i> (SC-387), anti-G<i>12</i> (SC-409), anti-G<i>13</i> (SC-392) antibody or IgG (10 μg for each antibody) overnight at 4°C. Antibodies were then collected with protein A-agarose. The immunoselections were incubated with 125I-Ang II (2 nM) for 1 h at room temperature. Nonspecific binding was defined in the presence of 1 μM CGP42112A. Data are presented as means ± S.E., n = 3. The anti-G<i>a</i> (1–883) and anti-G<i>a</i> (NEI-803) antibodies co-immunoselected AT₂-G<i>a</i> complexes but to an extent less than that selected with anti-G<i>a</i> (SC-262). Several other anti-G<i>a</i> subtype antibodies yield similar results (see text). * <i>p</i> < 0.05 compared with nonimmune serum. † <i>p</i> < 0.05 compared with IgG control.

Ang II acts at multiple sites within the cardiovascular system to assist in the regulation of cardiovascular homeostasis (1, 2). These effects are mediated by high affinity Ang II binding sites, which, at least in the adult, have been shown to be of the AT₁ subtype (1, 2). This receptor is a seven transmembrane-spanning receptor (35) that is coupled primarily to G<i>q</i>, resulting in the activation of phospholipase C and the subsequent release of IP₃ and diacyl glycerol, leading to increased intracellular calcium and activation of protein kinase C (14–16). Alternatively, this receptor is coupled to G<i>i</i>, resulting in the inhibition of adenylyl cyclase activity (14, 15). Activation of the AT₁ receptor induces a variety of actions depending on the tissue, exhibiting specific adrenal, renal, and vascular effects (1–4).

In contrast to the AT₁ receptor, the AT₂ receptor remains a mystery. Initially described on the basis of selective binding to specific ligands, this receptor was found to be expressed at very low levels in the adult and only in selective tissues (1, 2). On the other hand, binding studies revealed that this receptor was highly expressed in embryonic, fetal, and neonatal tissues, which lead to the speculation that this receptor may mediate some undefined action of Ang II in the processes associated with growth, development, or differentiation (18, 36, 37). Ang II has been shown, both in culture as well as in vivo, to induce growth and alter cellular phenotype (39, 40). However, the ability of AT₂-specific antagonists to block these effects of Ang II, at least in the adult, argued against this role for the AT₂ receptor (41, 42). Recently, we have found that the AT₂ receptor mediates an antigrowth effect, counteracting the growth promoting effects mediated by the AT₁ receptor (43). These results were obtained in a variety of models and may depend on the AT₂-mediated decrease in MAP kinase activity.

The discovery that the AT₂ receptor shared the structural characteristics of the serpentine receptor family strongly suggested that this receptor may couple to G proteins (5–8). Indirect evidence, such as the ability of pertussis toxin to block certain actions ascribed to the AT₂ receptor, was consistent with this suggestion (20–23). In the course of our study, we became aware of the work of Kang et al. (24), who demonstrated that the AT₂ receptor mediated an activation of a po-
G Protein Coupling of the AT₂ Receptor

Table II

| Antibody | 125I-Ang II specific bound (cpm) | Antibody | 125I-Ang II specific bound (cpm) |
|----------|---------------------------------|----------|---------------------------------|
| Round I  |                                 | Round II |                                 |
| NI, 10 μl| 139 ± 68                        | NI, 10 μl| 120 ± 50                        |
| Anti-Gi₂ (J-883), 10 μl | 730 ± 97*                      | Anti-Gi₂ (NEI-803), 10 μl | 690 ± 153*                      |
| Anti-Gi₃ (NEI-803), 10 μl | 613 ± 81*                      | Anti-Gi₂ (J-883), 10 μl | 701 ± 176*                      |
| IgG, 10 μg | 128 ± 90                        | IgG, 10 μg | 189 ± 143                       |
| Anti-Gi₂-3 (SC-262), 10 μg | 1261 ± 172*                    | Anti-Gi₂-3 (SC-262), 10 μg | 276 ± 86                       |

* p < 0.05 with respect to the appropriate control.

The direct evidence for the association of the AT₂ receptor and Gi₂ brings up another issue, that of the lack of sensitivity to GTP analogs such as GTPγS. It is known that G proteins cycle between an inactive (GDP bound form) and active (GTP bound) states (10–12). The GDP-bound α subunit associates with βγ subunits to form the heterotrimer, which is able to associate with ligand-free receptors. Receptors coupled to αβγ hetrotrimers usually bind ligand with high affinity. When the receptor is activated by agonist it undergoes a conformational change that is transmitted to the α subunit, promoting the exchange of GDP for GTP. This causes a dissociation of the α subunit both from the receptor and from βγ and frees the α subunit to interact with its appropriate effector. Receptors not associated with αβγ heterotrimers usually exhibit a lower affinity for agonist. Once GTP is cleaved to GDP, the α subunit ceases its effector stimulation, reassociates with βγ, and subsequently can reassociate with the receptor. Substitution of GTP with a nonhydrolyzable analog such as GTPγS or Gpp(NH)p forces the α subunit to remain in its active form. Subsequently, no reassociation with βγ and receptor occurs, and the affinity of the receptor thus remains in a low affinity state. The only evidence for a GTPγS shift in affinity state of the AT₂ receptor has been obtained in radioligand autoradiographic studies or certain brain nuclei (20, 21); all other published studies using whole cells or membrane fractions have been negative (1, 2, 13, 17–19). This inability of GTPγS to shift the AT₂ receptor to a low affinity state has often been cited as evidence that the receptor in not G protein coupled. The inability of GTPγS to induce a low affinity shift of AT₂ receptors in membranes from whole rat fetus may suggest that a majority of the receptors are not G protein coupled. Consistent with this, the co-immunoselection of the receptor using anti-G protein antibodies was inefficient, yielding only a small fraction of the total AT₂ binding sites. Alternatively, the AT₂ receptor may couple to G proteins by a nonconventional mechanism or may couple to a G protein with a low rate of GDP-GTP exchange. Consistent with this latter possibility, in our study, GTPγS did not influence binding to the immunoselected AT₂ receptor nor did it inhibit the immunoselection of the receptor by the G protein antibody, whereas AIF, which binds to the GDP bound G protein, did attenuate the ability of G protein antibody to co-immunoselect AT₂ binding sites.

In conclusion, co-immunoselection AT₂ receptor with anti-G protein antibodies proved useful in identification of direct association of AT₂ receptor and its coupled G proteins. The present study provide the first biochemical evidence that AT₂ receptor is G protein-coupled. The demonstration that AT₂ receptor is able to couple to both Gi₂ and Gi₃ may enhance our understanding of the signal transduction pathways underlying the physiological functions mediated by AT₂ receptor stimulation.

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