A Domain for G Protein Coupling in Carboxyl-terminal Tail of Rat Angiotensin II Receptor Type 1A*

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To delineate domains essential for Gq protein coupling in the C-terminal region (C-tail) of rat angiotensin II (Ang II) receptor type 1A (AT1A), we modified the putative cytosolic regions of the receptor by truncation or alanine substitution and determined resultant changes in the guanosine 5′-3-O-(thio)triphosphate (GTPγS) effect on Ang II binding and inositol trisphosphate production by the agonist. Independently, we studied the effect of synthetic C-terminal peptides (P-5) and its alanine substitution analogs on [35S]GTPγS binding to Gq. Effects of GTPγS on Ang II binding (shift to a low affinity form) and inositol trisphosphate production in the deletional mutant receptor 1-317 AT1A was similar to wild type AT1A, whereas in shorter C-terminal deletion mutants 1-309, 1-311, 1-312, 1-313 AT1A, and substitutional mutants Y312A, F313A, and L314A these activities were markedly reduced. The binding of [35S]GTPγS to Gq was promoted by the synthetic C-terminal peptide P-5 but not when mutated at Tyr312, Phe313, or Leu314. Results indicate that Tyr312, Phe313, and Leu314 in cytosolic carboxyl-terminal region of rat AT1A are essential for coupling and activation of Gq.

Angiotensin II (Ang II) receptor type 1A (AT1A) is a seven-transmembrane, G protein-coupled receptor (1, 2). Ang II activates Gq, Gi, and Gs proteins through the AT1A receptor (3–5). The GTP·Gα complex stimulates phospholipase C (PLC) resulting in inositol trisphosphate (InsP3) generation (6, 7).

Not a single consensus structure has been yet identified within the G protein-coupled superfamily that uniquely defines the G protein binding function. Mutagenesis studies on adrenergic receptors and rhodopsin indicate that the C-terminal domains of the third intracellular loop (ICL3) and the N-terminal region of the cytosolic tail are essential for coupling to G proteins (8–10). By contrast, the regions of the thyrotropin receptor essential for intracellular signal transduction appear to be the first intracellular loop (ICL1) and the C-terminal regions of the second intracellular loop (ICL2) and the third intracellular loop (ICL3) (11). Four isoforms of prostaglandin E receptor subtype EP3, which differ only at their C-terminal tails and are produced by alternative splicing, couple to different G proteins. Thus the C-terminal tail of EP3 determines G protein specificity (12).

Studies by Wang et al. (13) using chimeras of human AT1 and AT2 suggested that the N-terminal portion of ICL3 was essential for Gq coupling. We reported observations suggesting that the acidic-arginine-aromatic (DRY) triplet of ICL2, the C-terminal portion of ICL2, the C-terminal region of ICL3, and the cytosolic C-terminal tail region were involved in G protein coupling. Our data from transient transfection of the AT1A receptor in COS7 cells showed that the last 50 amino acid residues (beyond Phe309) were also important for Gq coupling (14). Thomas et al. (15) reported that truncation of the last 45 amino acid residues of the rat AT1A beyond Leu314 was not important for efficient coupling to the G protein. Thus, we focused on the amino acid sequence between Lys310 and Leu314 and their primary and ligand binding. We also synthesized nine peptides based on the amino acid sequence of the carboxyl region and examined their Gq activation with the aim of defining a region in the C-tail essential for the coupling to Gq in rat AT1A.

MATERIALS AND METHODS

Mutagenesis—The entire coding region of rat kidney AT1A was cloned into EcoRI site of a plasmid pUC19 (16). A KpnI-EcoRI fragment was subcloned into polylinker sites of the plasmid vector pBluescript II KS+, and single-stranded DNA was prepared using helper phage R 408 (Stratagene). Site-directed mutagenesis was performed by the procedure of Kunkel (17). Sites of truncation and substitution are shown in Fig. 1. The mutated DNA sequences were confirmed by Sanger’s dideoxynucleotide sequencing method (18). The mutated AT1A cDNA was excised with enzymes BamHI and XhoI and introduced into the expression vector pCDNA1.

Stable Expression of Wild Type AT1A and Its Mutants in CHO-K1 Cells—Forty µg of plasmid constructs containing the wild type or mutated rat AT1A cDNA were co-transfected with 1 µg of pSV-G1-Neo (Green Cross Corp.) into 5 × 106 of Chinese hamster ovary (CHO-K1) cells in 500 µl of phosphate buffer using a gene pulser (Bio-Rad). Native CHO-K1 cells do not express Ang II receptor.

Transfected CHO-K1 cells were cultured for 2 days in 10-cm dishes in Ham’s F12 medium (Life Technologies, Inc.) containing 10% fetal calf serum. Then the culture medium was changed to selection medium containing 400 µg/ml Geneticin (G418, Life Technologies, Inc.). When individual colonies emerged into 10–14 days after the transfection, 60 sufficiently separated colonies were isolated and inoculated into 200 µl of selection medium in 96-well plates. Each of these colonies was scaled up independently to 24-well plates, and the binding assay was performed using [125I]-Ang II (NEN Life Science Products). The binding assay was...
Binding Assay—AT\textsubscript{1A}-expressing CHO-K1 cells were grown in Ham's F12 medium with 10% fetal calf serum in 24-well plates. They were cultured in the selection medium in 96-well plates. Two or three weeks later, each of these clones was scaled up independently to 24-well plates, and binding assay was performed again. The clone expressing the highest specific binding of \([\text{125I-Sar1,Ile8}]\text{Ang II}\) was selected.

Effect of GTP\textsubscript{S} on Ang II Binding—Transfected CHO-K1 cells were grown in 10-cm dishes, washed with Hank's balanced salt solution, scraped, and collected by centrifugation at 1500 \(\times g\) for 5 min. The plasma membrane fraction was prepared by a published method (19). Membranes obtained were suspended at a protein concentration of 250 \(\mu\text{g/m}\)l in 50 mM Tris buffer (pH 7.4) containing 200 mM NaCl, 10 mM MgCl\textsubscript{2}, 1 mM EDTA, 0.1% BSA, and 100 \(\mu\text{g/m}\)l phenylmethanesulfonyl fluoride and used as the membrane preparation.

Dose response was determined as follows. Suspended membranes were incubated with 0.1 mM \([\text{125I-Sar1,Ile8}]\text{Ang II}\) at 25 °C for 60 min in the presence of varying concentrations of GTP\textsubscript{S}. For studying the time course of ligand binding, membranes were incubated with 0.1 mM \([\text{125I-Sar1,Ile8}]\text{Ang II}\) for 60 min at 37 °C to attain binding equilibrium and unlabeled Ang II (1 \(\mu\text{M}\)) or both of them were added to the mixture and incubated for another 60 min. The membrane-bound radioactivity was separated from the free radioligand by filtration over glass filters (GF/B) using a cell harvester (Millipore). Radioactivity was measured in a gamma counter.

Synthetic Peptides and Heterotrimeric G\textsubscript{q}—The amino acid sequences of the peptides used in this study are shown in Fig. 1. They were synthesized by the solid-phase method and purified to 95–99% homogeneity by high performance liquid chromatography using a Nucleosil 5 C18 column eluted with a linear concentration gradient (0–60%) of CH\textsubscript{3}CN containing 0.1% trifluoroacetic acid. The lyophilized synthetic peptide was dissolved in water. Heterotrimeric forms of G\textsubscript{q} proteins from bovine liver were purified to homogeneity as published (20).

GTP\textsubscript{S} Binding Assay—[\text{35S}GTP\textsubscript{S}] binding to 10 nM purified heterotrimeric G\textsubscript{q} promoted by synthetic peptides was measured in 25 mM HEPES-NaOH buffer (pH 7.4) containing 120 \(\mu\text{M}\) MgCl\textsubscript{2}, 100 \(\mu\text{M}\) EDTA, and 100 nM [\text{35S}GTP\textsubscript{S}] in the absence of phospholipids as described by Okamoto et al. (9). Briefly, G\textsubscript{q} was incubated at 37 °C for 10 min in the absence (control) or presence of a synthetic peptide (100 \(\mu\text{M}\)). The incubation was terminated by addition of 10 volumes of ice-cold stopping buffer containing 100 mM Tris-HCl (pH 8.0), 25 mM MgCl\textsubscript{2}, 100 mM
TABLE I

Binding affinity of [3H]Ang II binding for rat AT1A wild type receptor and mutant receptors

|                    | Kd (nM) | Bmax (fmol/mg protein) |
|--------------------|---------|------------------------|
| Wild type          | 1.6 ± 0.1 | 18.8 ± 1.4             |
| Mut 310-del        | 2.4 ± 0.2 | 16.0 ± 1.1             |
| Mut 312-del        | 2.4 ± 0.2 | 17.8 ± 1.6             |
| Mut 313-del        | 2.0 ± 0.3 | 15.2 ± 1.8             |
| Mut 314-del        | 1.8 ± 0.2 | 15.6 ± 1.6             |
| Mut 318-del        | 2.5 ± 0.3 | 17.8 ± 1.8             |
| Mut Y312A          | 2.4 ± 0.4 | 16.6 ± 1.8             |
| Mut F313A          | 2.6 ± 0.4 | 15.5 ± 1.9             |
| Mut L314A          | 2.8 ± 0.3 | 14.8 ± 1.2             |
| Mut K310,311Q      | 2.7 ± 0.3 | 15.3 ± 1.4             |

NaCl, and 20 μM GTP. After a 50-μl aliquot of the reaction mixture was rapidly filtered through a nitrocellulose filter (pore size, 0.45 μm) and washed three times with the stopping buffer, the filter was counted in a liquid scintillation counter. The maximal binding of [35S]GTPγS to Gα was measured in the presence of 1 μM GTPγS and 25 mM MgCl2 at room temperature by the method of Northup et al. (21) as a positive control.

Statistical Analysis—The results of experiments with the synthetic peptide study was examined by unpaired Student’s t test. p values less than 0.05 were considered significant.

RESULTS

Binding Affinity of Mutant Receptors—As shown in Table I the dissociation constants (Kd) and Bmax values of the wild type AT1A and its mutants determined by Scatchard analysis were similar, indicating that the mutants possessed similar ligand binding affinity and sites of comparable magnitude. In this study [3H]-Sar1, Ile8]Ang II was used as ligand. Scatchard plots indicated single high affinity sites. When [35S]-Ang II was used as ligand results indicated similar single high affinity sites. The possible presence of low affinity sites was practically undetectable.

Effects of a Stable GTP Analog—As shown in Fig. 2, the binding of [35S]-Ang II to wild type AT1A, Mut 318-del, and Mut K310,311Q receptors were dose-dependently decreased by GTPγS, whereas the effect of GTPγS (shift from high to low affinity state to a low affinity form) was practically abolished in Mut 310-del, Mut 312-del, Mut 313-del, Mut 314-del, Mut Y312A, Mut F313A, and Mut L314A receptors.

Time-related changes in dissociation of [35S]-Ang II from the wild type and mutated receptors are shown in Fig. 3. The binding of [35S]-Ang II to the receptors in membrane preparations reached a plateau in 60 min. In wild type AT1A and all of its mutants, the receptor-bound [35S]-Ang II was displaced by 1 μM unlabeled Ang II to similar extents (the range of half-life time of dissociation was 19.5 to 21.4 min). GTPγS markedly shortened the half-life time of the spontaneous dissociation in the wild type AT1A, and Mut 318-del, and Mut K310,311Q receptors (3.5 to 4.6 min), whereas the binding of [35S]-Ang II remained unchanged for 60 min in Mut 310-del, Mut 312-del, Mut 313-del, and Mut 314-del. Moreover, although the half-life times in the wild type AT1A and Mut 318-del were shortened in the presence of both Ang II and GTPγS (0.9 to 1.2 min), those in other deletion mutants were similar to the half-life time in the presence of Ang II alone (17.0 to 20.1 min).

InsP₃ Formation—Binding of Ang II to AT1A activates a PLC via Gq resulting in stimulation of InsP₃ formation. Thus, increased InsP₃ formation by Ang II can be considered to indicate effective coupling to Gq of the mutants. In unmutated AT1A, InsP₃ production was significantly increased from 2.52 ± 0.05 pmol/dish of unstimulated control to 16.55 ± 1.88 pmol/dish at 10 s after Ang II stimulation. Similar results were obtained in Mut 318-del and Mut K310,311Q. By contrast, in Mut 310-del, Mut 312-del, Mut 313-del, Mut 314-del, Mut Y312A, Mut F313A, and Mut L314A responses of InsP₃ to Ang II stimulation were abolished (Fig. 4).

Effects of Synthetic Peptides on G Protein Activation—Gq was incubated for 10 min in the presence of [35S]GTPγS with peptides representing domains in the cytoplasmic segments of native AT1A (P-1 to P-5) or mutated peptides of P-5. As shown in Fig. 5, Peptides P-3 and P-5 activated Gq as well as positive control. The Gq-activating function was attenuated to 25% relative to intact P-5 in Mut P-5 (Tyr312, Phe313, and Leu314 were replaced by alanine). The uptake of [35S]GTPγS was significantly lower in Mut Y, Mut F (p < 0.01) and Mut L (p < 0.05) than in P-5.

DISCUSSION

The cytoplasmic C-terminal (C-tail) region was shown to play an essential role in agonist-induced receptor internalization (15). However, its role in the G protein-coupled phospholipase activation has been controversial. Now in three independent approaches using five deletion mutants, four alanine substitution mutants, and synthetic peptides with native and mutated amino acid sequences corresponding to an N-terminal region of C-tail, we were able to identify the tripeptide region Tyr312-Ile8-Val313 as essential for effective coupling to Gq.
Phe313-Leu314 (Fig. 5) as a domain essential for Gq activation.

Different experimental approaches produce results leading to different and sometimes contradicting conclusions. Wang et al. (13) using chimeric human AT1 with a grafted AT2 C-tail that shows Gq activation concluded that the major determinant of Gq coupling specificity is in the ICL-3, and the C-tail has little role in the activation of PLC. However, we had shown that deletion beyond Phe309 (Mut 310-del) abolished the Gq-coupled inositol 1,4,5-trisphosphate formation (14). Since both of these modifications could introduce additional factors such as conformational changes or the effect of ICL3 not directly related to the action of deleted or replaced residues, multiple approaches had to be taken. Loss of Gq coupling in Mut 310-del and complete recovery of the Gq activation in Mut 318-del narrowed the Gq coupling domain to residues 310–317 (Figs. 1 and 4) (14). The observation of a robust activity with Mut 315-del by Thomas et al. (15) further narrowed it to a region between residues 310 and 314. Almost complete loss of the activity with Mut 312-del, 313-del, 314-del, and single residue alanine mutation Y312A, F313A, and L314A and the preservation of a full PLC activity with the double mutant K310Q,K311Q indicated that Tyr312-Phe313-Leu314 is the essential domain required for PLC activation. Its essential role in Gq coupling was also determined by loss of the well known GTPγS-induced shift to a low affinity state for agonist binding in these mutants (Figs. 2 and 3). Further evidence for the essential role of the tripeptide sequence for the G protein coupling was obtained by a third and completely independent approach in which peptides with the amino acid sequences of the native and alanine-substituted C-tail (residues 307–320) were allowed to interact with purified heterotrimeric Gq, and binding to [35S]GTPγS was examined. Again, alanine substitution of Tyr312-Phe313-Leu314 singly or three together significantly reduced GTPγS binding. It is interesting to note that, whereas the triple mutant Mut P-5 lost

![Fig. 3. Time course study on the dissociation of 125I-Ang II binding in the presence of excessive concentration of Ang II and/or GTPγS in the wild type AT1A (a) and its mutants (b, Mut 310-del; c, Mut 312-del; d, Mut 313-del; e, Mut 314-del; and f, Mut 318-del). Membrane preparations were preincubated in the presence of 0.1 nM 125I-Ang II for 60 min, and then Ang II (1 μM) (open circles), GTPγS (10 μM) (open triangles), or both of them (solid triangles) were added. Controls (closed circles) did not contain GTPγS or cold Ang II. Each value represents the mean ± S.D. obtained from three separate experiments at 37 °C](http://www.jbc.org/).
and the C-tail peptide (P-5) activated purified Gq just as well as with the amino acid sequence of the N-terminal region of ICL3 versus 0.05 peptide 100 m at room temperature in the absence (GTP for Gs activation (10).

The present finding that the synthetic 16-mer peptide P-3 (Fig. 5) supports the observation of Wang et al. (13) that in chimeras of AT1 and AT2, ICL3 plays dominant roles in Gq coupling. Shirai et al. (25) showed the same ICL3 domain activates G13, G22, and Gp by using the synthetic peptide P-3. These results indicate that AT1A may use and require the tripeptide sequence of C-tail in collaboration with ICL3 in Gq activation. Interesting information revealed by the activation of Gq proteins by these peptides are that the same peptides (P-3 and P-5) are capable of activating Gq, G22, and Gp. Mechanisms by which a receptor selects the type of G proteins are yet to be clarified. On the other hand, peptides with unrelated sequences like P-1, P-2, and P-4 which did not show the activation may be considered as controls and indicate that activation by P-3 and P-5 is specific to their sequences.

$B_{max}$ values of mutated receptors expressed in each cell line were at levels comparable to that of the wild type. Hence, the decrease in InsP3 formation in Mut 310-del, Mut 312-del, Mut 313-del, Mut 314-del, Mut Y312A, Mut F313A, and Mut L314A should be due to the receptor mutation rather than a decrease in expression of each mutant receptor. Our previous study using substitutional mutations of basic polar amino acid residues in ICL2 and ICL3 indicated that ICL2 and the C-terminal domain of ICL3 would be important for Gq coupling (14). These mutations targeted at domains with dense electrical charges probably caused nonspecific conformational changes and led to erroneous results that could be misinterpreted.

Tyr292 in transmembrane domain 7 was reported to be essential for G protein coupling (26). The conserved sequence NPLFY at the bottom of transmembrane domain 7 was shown to contribute to both agonist binding and signal transduction (24). Thus, the junctional area of AT1 between transmembrane domain 7 and C-tail seems to play an important role in receptor signaling. This area of AT1 also contains the sequence KKFKK that was shown to be an unusual Gi activator domain of insulin growth factor II receptor (9). However, in AT1 mutation to Lys-Lys-Glu-Gln310-Gln311 did not have any effect on Gq coupling. This observation helped our work in narrowing the Gq activating domain to Tyr312-Phe313-Leu314.

In summary, the present study presents evidence that a Gq coupling site in the type IA angiotensin receptor AT1A should reside between residues 312 and 318 in the C-terminal tail, and the specific sequence Tyr312-Phe313-Leu314 is essential for coupling and activation of the Gq protein.

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