Angiotensin II-induced Association of Phospholipase Cγ1 with the G-protein-coupled AT₁ Receptor*

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An early event in signaling by the G-protein-coupled angiotensin II (Ang II) AT₁ receptor in vascular smooth muscle cells is the tyrosine phosphorylation and activation of phospholipase Cγ1 (PLCγ1). In the present study, we show that stimulation of this event by Ang II in vascular smooth muscle cells is accompanied by binding of PLCγ1 to the AT₁ receptor in an Ang II- and tyrosine phosphorylation-dependent manner. The PLCγ1-AT₁ receptor interaction appears to depend on phosphorylation of tyrosine 319 in a YIPP motif in the C-terminal intracellular domain of the AT₁ receptor and binding of the phosphorylated receptor by the most C-terminal of two Src homology 2 domains in PLCγ1. PLCγ1 thus binds to the same site in the receptor previously identified for binding by the SHP-2 phosphotyrosine phosphatase–JAK2 tyrosine kinase complex. A single site in the C-terminal tail of the AT₁ receptor can, therefore, be bound in a ligand-dependent manner by two different downstream effector proteins. These data demonstrate that G-protein-coupled receptors can physically associate with intracellular proteins other than G proteins, creating membrane-delimited signal transduction complexes similar to those observed for classic growth factor receptors.

Growth factor receptors belong to a family of receptors that contain an extracellular ligand binding domain, a single transmembrane portion, and a large intracellular tyrosine kinase catalytic domain. Ligand-induced receptor autophosphorylation promotes the interaction of the intracellular domains of the receptors with a number of downstream effector proteins or enzymes. Typically, these proteins contain one or more domains known as Src homology 2 (SH2)1 domains. Among these SH2 domain-containing proteins are phosphoinositide-specific phospholipase Cγ (PLCγ), the 85-kDa subunit of phosphatidylinositol 3-kinase, GTPase-activating proteins, growth factor receptor binding protein 2, the phosphotyrosine phosphatase SHP-2, and members of the nonreceptor Src family of tyrosine kinases (1, 2). Autophosphorylation of growth factor receptors occurs on defined tyrosine residues. These phosphorylated residues function to initiate cellular signaling cascades by acting as high affinity binding sites for the SH2 domains of various effector proteins. The selectivity of the receptor-effector interaction is determined, not only by the phosphorylated tyrosine residue in the receptor but also by the three amino acids C-terminal to the phosphorylated tyrosine and by the structure of the SH2 domain of the interacting protein. For example, one of the identified sites for binding of the SH2 domains of PLCγ1 to the platelet-derived growth factor α and β receptors is a YIPP motif present in the receptors at residues 1018–1021 and 1021–1024, respectively. Phosphorylation of tyrosines 1018 and 1021 in these motifs promotes binding of PLCγ1 to the platelet-derived growth factor receptor and tyrosine phosphophosphorylation and activation of the enzyme (3, 4).

Another family of cell surface receptors are the G-protein-coupled receptors that contain seven membrane-spanning α-helices. These receptors lack intrinsic tyrosine kinase activity. However, we have previously shown that the G-protein-coupled angiotensin II (Ang II) AT₁ receptor in vascular smooth muscle cells (VSMC) activates the inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol-generating enzyme, PLCγ1, in a manner similar to that observed for growth factor receptors. PLCγ1 is transiently tyrosine-phosphorylated in Ang II-stimulated VSMC with a time course that parallels that of IP₃ formation (5). Tyrosine phosphorylation of PLCγ1 appears to lie downstream from activation of the c-Src tyrosine kinase because electroporation of neutralizing anti-c-Src antibodies into VSMC virtually eliminates Ang II-induced tyrosine phosphorylation of PLCγ1 and blocks Ang II stimulation of IP₃ production (6). Furthermore, other G-protein-coupled receptors, including those for platelet activating factor, thrombin, and ATP, have also been shown to signal through the tyrosine phosphorylation and activation of PLCγ1 (7–9). In none of these instances, however, is it known whether PLCγ1 phosphorylation and activation involves physical association of the SH2 domains of the enzyme with the receptor.

AT₁ post-receptor signaling in VSMC also involves activation of the janus kinase (JAK) signal transducers and activators of transcription (STAT) pathway. Ang II stimulation of the AT₁ receptor activates the JAK/STAT pathway by inducing rapid

[3,4-; JAK, janus kinase; STAT, signal transducers and activators of transcription; GST, glutathione-S-transferase.]

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† The abbreviations used are: SH2, Src homology 2; PLC, phospholipase C; Ang II, angiotensin II; VSMC, vascular smooth muscle cells; IP₃, inositol 1,4,5-trisphosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; PP1, 4-amino-5-(4-methylphenyl)-7-ct-butylpyrazolo-

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tyrosine phosphorylation, activation, and association of JAK2 with the receptor (10). JAK2-receptor association appears to depend on a YIPP motif in the C-terminal intracellular domain of the AT1 receptor that is identical to the PLCγ1 SH2 domain binding site identified in the platelet-derived growth factor receptor (11). Because JAK2 does not contain any SH2 domains, the finding that JAK2 associates with this motif in the AT1 receptor was initially puzzling. Recently, however, we have found that JAK2 associates with the receptor as a consequence of the SH2 domain-containing SHP-2 phosphotyrosine phosphatase acting as an adaptor or linker protein for JAK2 association.2 In the present study, we have examined whether Ang II-induced tyrosine phosphorylation and activation of PLCγ1 in VSMC involves binding of PLCγ1 to the AT1 receptor in an Ang II- and tyrosine phosphorylation-dependent manner. In addition, we have identified the interacting domains in the two proteins.

**EXPERIMENTAL PROCEDURES**

**Materials—**Anti-AT1 receptor polyclonal antibodies (N-10 and 306) and glutathione S-transferase (GST)-PLCγ1 fusion proteins (sc-4019, sc-4051, sc-4052, sc-4053, and sc-4054) were purchased from Santa Cruz Biotechnology Inc. Anti-PLCγ1 monoclonal antibody (clone D-7-3) and anti-phosphotyrosine monoclonal antibody (clone 4G10) were obtained from Upstate Biotechnology. Anti-SHP-2 monoclonal antibody was purchased from Transduction Laboratories. Purified human recombinant c-Src enzyme and PP1 came from Calbiochem. Affi-Gel 10, Muta-Gen Phagemid kit, and detergent-compatible protein assay kit were purchased from Bio-Rad. [3H]PIP2, GST-agarose, and monoclonal anti-GST antibody were obtained from Amersham Pharmacia Biotech. All other chemicals were purchased from Sigma.

**Cell Culture—**VSMC from 200–300 g male Sprague-Dawley rat aortas were cultured to near confluency at 37°C under 5% CO2 in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum and supplemented with antibiotics (5, 6). Cells were growth-arrested by incubation in serum-free Dulbecco’s modified Eagle medium for 36–48 h before Ang II exposure.

**Immunoprecipitation and Immunoblotting—**VSMC were stimulated with Ang II (10−7 M) for various times, and cells were lysed and subjected to immunoprecipitation with anti-AT1 receptor antibody as described previously (10). Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose by electroblotting, and probed with anti-PLCγ1 or anti-phosphotyrosine antibody as described previously (5, 6).

**Preparation of VSMC Cell Lysates—**Growth-arrested VSMC were stimulated with Ang II (10−7 M) for various times, washed two times with ice-cold phosphate-buffered saline containing 1 mM Na2VO4 and then lysed in 1.0 ml of lysis buffer (25 mM Tris-HCl, pH 7.6, 0.15 M NaCl, 1% Triton X-100, 10% glycerol, 50 mM NaF, 1 mM NaVO3, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin). Cells were scraped off the plates and gently sonicated. Lysates were cleared by centrifugation at 7,500 × g for 15 min, and the protein concentration of the cleared lysates was determined by the Bio-Rad detergent-compatible protein assay. In some experiments, SHP-2 was quantitatively removed (as confirmed by immunoblotting) from VSMC lysates by immunoprecipitation with anti-SHP-2 antibody before use of the lysates in vitro binding assays.

**Preparation of DNA Constructs Encoding GST-AT1 Receptor Fusion Proteins—**A 168-base pair fragment of the Ca18b cDNA encoding the two proteins.

**RESULTS AND DISCUSSION**

To determine whether PLCγ1 associates with the AT1 receptor in a ligand- and tyrosine phosphorylation-dependent manner, we utilized a rabbit polyclonal anti-AT1 receptor antibody directed against the C-terminal 54 amino acid residues (306–359) of the rat AT1 receptor (12). Cultured VSMC were stimulated with Ang II (10−7 M) for various times, cells were lysed, and the AT1 receptor was immunoprecipitated from the lysates with anti-AT1 receptor antibody. Immunoprecipitated proteins were separated by gel electrophoresis, transferred to nitrocellulose, and immunoblotted with anti-PLCγ1 antibody. As shown in Fig. 1, Ang II induced a rapid and transient association of PLCγ1 (140 kDa) with the AT1 receptor that was maximal within 30 s to 1 min. The time course of Ang II-stimulated PLCγ1-AT1 receptor association is thus similar to that reported previously for Ang II-stimulated PLCγ1 tyrosine phosphorylation and activation (5). Identical results were also obtained when the experiments were repeated using a different rabbit polyclonal anti-AT1 receptor antibody that recognizes residues 15–24 in the N terminus of the rat AT1 receptor (data not shown). However, in negative control experiments using rabbit preimmune serum or an irrelevant rabbit polyclonal anti-GST antibody, no PLCγ1 was immunoprecipitated for any of the time points. To investigate whether phosphorylation by an Src family tyrosine kinase is required for the Ang II-induced association of PLCγ1 with the AT1 receptor, we also carried out immunoprecipitation experiments in which cells were pre-treated with the Src family kinase-selective inhibitor, PP1 (10−6 M for 30 min) before Ang II stimulation. PP1, which has been shown previously to be highly selective for Src family kinases relative to other known tyrosine kinases (15), completely prevented AT1 receptor-PLCγ1 association (data not shown), suggesting that tyrosine phosphorylation of either

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PLCγ1 or the receptor (or both) by a Src family tyrosine kinase may be required for the association. This possibility appears plausible because both PLCγ1 and the AT1 receptor have been shown previously to be excellent substrates for Src kinases in vitro (13, 16). Furthermore, PLCγ1 has been shown to form a complex with c-Src in several other cell types (7, 17, 18). An alternative explanation for the inhibitory effect of PP1 is that Src kinase activity may be required for a phosphorylation event that is upstream from either PLCγ1 or AT1 receptor phosphorylation in a tyrosine phosphorylation cascade.

To confirm the results of the communoprecipitation experiments and to determine whether PLCγ1 binds to the AT1 receptor C-terminal intracellular domain, we utilized a GST-AT1 fusion protein (GST-AT1-(306–359)) containing the C-terminal 54 amino acids of the rat AT1 receptor. The GST-AT1 fusion and GST alone were expressed in E. coli and purified to homogeneity on a glutathione-agarose affinity column. VSMC were treated with Ang II (10−7 M) for various times, and cell lysates were prepared and used in in vitro binding assays with the GST-AT1-(306–359) fusion protein prebound to agarose beads. In control experiments, lysates were also incubated with GST alone prebound to agarose beads. After a 2-h incubation at 4 °C, the beads were washed extensively in buffer containing 1 M NaCl, and bound proteins were eluted. The amount of PLCγ1 eluted (and therefore bound by the fusion protein) was then quantitated by immunoblotting with anti-PLCγ1 antibody. As shown in Fig. 3, lysates from Ang II-treated VSMC, cells were either treated or not treated with Ang II (10−7 M for 30 s), and lysates were prepared and then incubated with the GST-AT1-(306–359) fusion protein prebound to agarose beads. Experiments were also carried out in which cells were pretreated with PP1 (10−6 M for 30 min) prior to Ang II stimulation. After a 2-h incubation at 4 °C, the beads were washed extensively, and bound proteins were eluted. The relative phosphotyrosine content of the eluted fusion protein was then assessed by immunoblotting with anti-phosphotyrosine antibody. As shown in Fig. 3, lysates from Ang II-treated VSMC induced the tyrosine phosphorylation of the GST-AT1 fusion protein. Ang II-stimulated phosphorylation, however, was completely blocked when cells were pretreated with PP1. Furthermore, phosphorylation was restricted to the AT1 receptor portion of the fusion protein, as no tyrosine phosphorylation of the GST alone negative control was detected (data not shown).

Recently we have shown that JAK2 association with the AT1 receptor involves the SH2 domain-containing SHP-2 phosphotyrosine phosphatase acting as an adaptor protein for JAK2 association. This conclusion is based on in vitro binding assays with Ang II-treated VSMC lysates and the GST-AT1-(306–359) fusion protein in which lysates were quantitatively depleted of SHP-2 by immunoprecipitation with anti-SHP-2 antibody before determining the extent of JAK2 binding to the fusion protein. Immunodepletion of lysates with anti-SHP-2 completely blocks JAK2 association with the GST-AT1-(306–359) fusion protein.3 In the present study, we have tested whether quantitative depletion of SHP-2 from VSMC lysates also alters PLCγ1 binding to the GST-AT1-(306–359) fusion protein. Lysates were prepared from Ang II-treated (10−7 M for 30 s) VSMC and then depleted of SHP-2 by immunoprecipitation with anti-SHP-2 antibody. Quantitative depletion of SHP-2 from lysates was confirmed by immunoblotting with anti-SHP-2 antibody. Nondepleted (control) lysates were also prepared. Immunodepleted and nondepleted lysates were then used in in vitro binding assays with the GST-AT1-(306–359) fusion protein prebound to beads. PLCγ1 binding to the fusion protein was then quantitated by immunoblotting with anti-PLCγ1 antibody as before. As shown in Fig. 4, PLCγ1 bound to the fusion protein to approximately the same extent whether from SHP-2-depleted or nondepleted lysates. No binding was detected for the GST alone negative control. Therefore, we conclude that PLCγ1 association with the AT1 receptor, unlike that of the JAK2 tyrosine kinase, does not depend on SHP-2 acting as an adaptor protein for PLCγ1 binding.
The hypothesis that c-Src or other Src family tyrosine kinase modulates the PLC-γ1-AT1 receptor association is supported further by the results of binding competition experiments with a GST-AT1-(306–359) fusion protein phosphorylated in vitro by c-Src. In these experiments, the GST-AT1-(306–359) fusion protein was first covalently linked to an agarose matrix and then allowed to bind PLC-γ1 in VSMC lysates prepared from cells exposed to Ang II (10^{-7} M for 30 s). In addition, the purified free GST fusion protein was either treated or not treated with purified recombinant c-Src and MgATP to obtain phosphorylated and nonphosphorylated forms of the protein. Tyrosine phosphorylation of the fusion protein by c-Src in vitro was confirmed by immunoblotting of anti-GST immunoprecipitates with anti-phosphotyrosine antibody. Free nonphosphorylated and phosphorylated forms of the GST-AT1-(306–359) fusion protein were then used to compete with the immobilized GST fusion protein for binding of PLC-γ1. The amount of PLC-γ1 remaining bound to the immobilized fusion protein after incubation with the competitor proteins was quantitated by immunoblotting of glutathione-elicited proteins with anti-PLC-γ1 antibody. As shown in Fig. 5, no competition was observed with the nonphosphorylated protein. However, increasing concentrations of free tyrosine-phosphorylated GST-AT1 receptor fusion protein effectively competed with the GST-AT1 receptor fusion protein agarose matrix for PLC-γ1 binding, suggesting that direct phosphorylation of the AT1 receptor C-terminal tail by c-Src may increase its binding affinity for PLC-γ1.

Inhibition of PLC-γ1 binding to the immobilized GST-AT1 receptor fusion protein by the free phosphorylated but not the nonphosphorylated fusion protein could also be due to an indirect, allosteric interference rather than to competition for the binding site. Therefore, to more directly demonstrate a role for receptor phosphorylation in PLC-γ1 binding to the AT1 receptor, we carried out in vitro binding assays with GST-AT1-(306–359) fusion proteins that were either phosphorylated or not phosphorylated in vitro by c-Src. The AT1 receptor C-terminal cytoplasmic tail (residues 306–359) contains tyrosine residues at positions 312, 319, and 339. To determine whether phosphorylation of one or more of these residues is required for binding of PLC-γ1, we individually mutated each tyrosine residue in the GST-AT1-(306–359) fusion protein to a phenylalanine. Wild-type GST-AT1-(306–359), GST-AT1-(306–359) (Tyr-312→Phe), GST-AT1-(306–359) (Tyr-319→Phe), and GST-AT1-(306–359) (Tyr-339→Phe) fusion proteins prebound to agarose beads was either phosphorylated or not phosphorylated in vitro by purified recombinant human c-Src. GST-AT1 fusion proteins containing mutated tyrosines (at 312, 319, and 339) were also phosphorylated in vitro by c-Src. Nonphosphorylated and phosphorylated proteins prebound to beads were washed extensively with buffer containing 1 M NaCl before use in in vitro binding assays with lysates from untreated VSMC. After a 2-h incubation at 4 °C, beads were again washed extensively with buffer containing 1 M NaCl, and bound proteins were eluted. Binding of PLC-γ1 to the fusion proteins was quantitated by immunoblotting with anti-PLC-γ1 antibody. Similar results were obtained in two separate experiments.
In order for tyrosine phosphorylation of the AT$_1$ receptor C-terminal intracellular domain to have a role in mediating PLCγ1 binding to the receptor in VSMC, it must occur rapidly (within 30 s) in response to Ang II stimulation. To investigate whether Ang II induces rapid tyrosine phosphorylation of the AT$_1$ receptor in VSMC, untreated cells or cells treated with Ang II ($10^{-7} \text{m}$ for 30 s) were lysed and immunoprecipitated with anti-AT$_1$ receptor antibody. Immunoprecipitates were then immunoblotted with anti-phosphotyrosine antibody. Experiments were also carried out in which VSMC were pretreated with either the tyrosine phosphatase inhibitor, sodium orthovanadate ($10^{-4} \text{m}$ for 30 min), or PP1 ($10^{-6} \text{m}$ for 30 min). Results shown in Fig. 7 demonstrate that Ang II induces a rapid and significant increase in the phosphotyrosine content of the AT$_1$ receptor in VSMC. Pretreatment with sodium orthovanadate increased the phosphotyrosine content of the receptor even in the absence of Ang II stimulation. In contrast, pretreatment with PP1 completely abolished the Ang II-induced tyrosine phosphorylation of the receptor, suggesting a requirement for Src family kinase activity in receptor phosphorylation.

To further map the region of the AT$_1$ receptor C-terminal tail that interacts with PLCγ1, we expressed a series to GST-AT$_1$ fusion proteins containing various deletional or point mutations in the AT$_1$ portion of the fusion protein. Proteins were expressed in *E. coli* and purified by affinity chromatography on glutathione-agarose (Table I). Each mutant protein was then individually tested for its ability to bind PLCγ1 in lysates from Ang II-treated ($10^{-7} \text{m}$ for 30 s) VSMC. Binding was detected by immunoblotting with anti-PLCγ1 as described earlier. Fusion proteins of the AT$_1$ receptor containing residues 306–359, 306–348, 306–329, and 318–359 were each bound by PLCγ1. In contrast, fusion proteins containing AT$_1$ receptor residues 336–359, 323–359, and 306–318 were not bound by PLCγ1 (Fig. 8A). Deletional analysis thus identifies residues located between positions 318 and 323 as being essential for PLCγ1 binding. The YIPP motif in the AT$_1$ receptor C-terminal tail, which has been shown previously to bind the JAK/SHP-2 complex, is located at positions 318–322. Thus it is likely that this motif also functions as a binding site for PLCγ1 and that, as shown also in Fig. 6, phosphorylation of tyrosine 319 within the motif enhances PLCγ1 binding in a manner similar to that shown previously for the platelet-derived growth factor α and β receptors. This conclusion is also supported by the results of *in vitro* binding assays using VSMC lysates from Ang II-treated cells ($10^{-7} \text{m}$ for 30 s) and the GST-AT$_1$-(306–359) fusion proteins in which the tyrosine residues at positions 312, 319, and 339 of the AT$_1$ receptor were each individually mutated to phenylalanines. Assays were carried out with Ang II-treated ($10^{-7} \text{m}$ for 30 s) VSMC lysates (which as shown earlier contain activated Src family tyrosine kinases that can phosphorylate the fusion proteins) and either wild-type GST-AT$_1$-(306–359), GST-AT$_1$-(306–359) (Tyr-312 → Phe), GST-AT$_1$-(306–359) (Tyr-319 → Phe), or GST-AT$_1$-(306–359) (Tyr-339 → Phe) fusion proteins. As shown in Fig. 8B, PLCγ1 from VSMC lysates bound to each of the fusion proteins with the exception of GST-AT$_1$-(306–359) (Tyr-319 → Phe), again indicating an essential role for tyrosine 319 in PLCγ1 binding.

The importance of tyrosine 319 in PLCγ1 binding to the AT$_1$ receptor and in activation of the PLCγ1 enzyme was further confirmed in *in vitro* binding assays in which PLCγ1 binding to the receptor was quantitated by PLC activity. Ang II-treated VSMC lysates ($10^{-7} \text{m}$ for 30 s) were incubated with wild-type GST-AT$_1$-(306–359), GST-AT$_1$-(329–359), GST-AT$_1$-(306–359) (Tyr-319 → Phe), GST-AT$_1$-(306–359) (Tyr-312 → Phe), and GST-AT$_1$-(306–359) (Tyr-339 → Phe) fusion proteins prebound to agarose beads. Beads were washed extensively, and proteins were eluted with reduced glutathione. Eluates were then assayed for PLC activity using [$^3$H]PIP$_2$-containing liposomes as substrate. As shown in Fig. 9, a deletional mutant (323–359) lacking the YIPP motif and the point mutant (Tyr-319 → Phe) lacking tyrosine 319 bound very little PLC activity, whereas other mutants lacking tyrosines 312 (Tyr-312 → Phe) and 339 (Tyr-339 → Phe) bound significantly more PLC activity, equivalent to that bound by the wild-type fusion protein.

Full-length PLCγ1 contains two SH2 domains and a single SH3 domain. To determine which of these domains, if any, are required for interaction of PLCγ1 with the AT$_1$ receptor, we also carried out *in vitro* binding assays with commercially available GST fusion proteins containing the various SH2 and SH3 domains of PLCγ1 (Fig. 10A). VSMC were exposed to Ang II ($10^{-7} \text{m}$) for 0, 0.5, and 1 min and then lysed. Lysates were incubated with GST-PLCγ1 fusion proteins prebound to agarose beads. Beads were washed extensively, and bound proteins were eluted with reduced glutathione. The amount of AT$_1$ receptor eluted was then quantitated by immunoblotting with

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**Table I**

| GST-AT$_1$ receptor fusion proteins |  |
|------------------------------------|--|
| GST-AT$_1$-(306–359)               | GKKFKK$^{312}$Y FLQLLK$^{311}$YIPP KAKSHSSLSKSTMGLS$^{339}$Y RPSDMN$^{359}$SSAKKPSACFEVE |
| GST-AT$_1$-(306–348)               | GKKFKK$^{312}$Y FLQLLK$^{311}$YIPP KAKSHSSLSKSTMGLS$^{339}$Y RPSDMN$^{359}$SS |
| GST-AT$_1$-(306–329)               | GKKFKK$^{312}$Y FLQLLK$^{311}$YIPP KAKSHSSLSKSTMGLS$^{339}$Y RPSDMN$^{359}$S |
| GST-AT$_1$-(318–359)               | GKKFKK$^{312}$Y FLQLLK$^{311}$YIPP KAKSHSSLSKSTMGLS$^{339}$Y RPSDMN$^{359}$ |
| GST-AT$_1$-(336–359)               | GKKFKK$^{312}$Y FLQLLK$^{311}$YIPP KAKSHSSLSKSTMGLS$^{339}$Y RPSDMN$^{359}$ |
| GST-AT$_1$-(318–336)               | GKKFKK$^{312}$Y FLQLLK$^{311}$YIPP KAKSHSSLSKSTMGLS$^{339}$Y RPSDMN$^{359}$ |
| GST-AT$_1$-(329–359)               | GKKFKK$^{312}$Y FLQLLK$^{311}$YIPP KAKSHSSLSKSTMGLS$^{339}$Y RPSDMN$^{359}$ |
| GST-AT$_1$-(306–318)               | GKKFKK$^{312}$Y FLQLLK$^{311}$YIPP KAKSHSSLSKSTMGLS$^{339}$Y RPSDMN$^{359}$ |
| GST-AT$_1$-(306–359) (Tyr-312→Phe) | GKKFKK$^{312}$Y FLQLLK$^{311}$YIPP KAKSHSSLSKSTMGLS$^{339}$Y RPSDMN$^{359}$SSAKKPSACFEVE |
| GST-AT$_1$-(306–359) (Tyr-339→Phe) | GKKFKK$^{312}$Y FLQLLK$^{311}$YIPP KAKSHSSLSKSTMGLS$^{339}$Y RPSDMN$^{359}$SSAKKPSACFEVE |
| GST-AT$_1$-(306–359) (Tyr-319→Phe) | GKKFKK$^{312}$Y FLQLLK$^{311}$YIPP KAKSHSSLSKSTMGLS$^{339}$Y RPSDMN$^{359}$SSAKKPSACFEVE |
three separate experiments. VSMC were treated with Ang II (10^{-7} M for 30 s) and cell lysates were prepared and used in *in vitro* binding assays with GST-AT1, fusion proteins containing various deletional or point mutations in the AT_1 receptor C-terminal intracellular domain. Lysates were incubated with each of the different fusion proteins (prebound to agarose beads) for 2 h. Beads were then washed extensively with buffer containing 1 M NaCl, and bound proteins were eluted. The amount of PLCγ1 eluted (and therefore bound by a given fusion protein) was quantitated by immunoblotting with anti-PLCγ1 antibody. Similar results were obtained in three separate experiments. Y, Tyr; F, Phe.

In summary, the results of the present study show for the first time that PLCγ1 binds to the G-protein-coupled AT_1 receptor in an Ang II- and tyrosine phosphorylation-dependent manner. The PLCγ1-AT_1 receptor interaction appears to depend on phosphorylation of tyrosine 319 in a YIPP motif in the C-terminal intracellular domain of the AT_1 receptor and binding of the phosphorylated receptor by the most C-terminal of two SH2 domains in PLCγ1. PLCγ1 thus binds to the same site in the receptor previously identified for binding of the SHP-2 phosphotyrosine phosphatase JAK2 tyrosine kinase complex. A single site in the C-terminal tail of the receptor can, therefore, be bound in a ligand-dependent manner by two different downstream effectors proteins. The data presented here further demonstrates that G-protein-coupled receptors can physically associate with intracellular proteins other than G proteins, creating membrane-delimited signal transduction complexes similar to those observed for classic growth factor receptors.

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