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Angiotensin II Receptor Coupling to Phospholipase D Is Mediated by the $\beta\gamma$ Subunits of Heterotrimeric G Proteins in Vascular Smooth Muscle Cells

MASUKO USHIO-FUKAI, R. WAYNE ALEXANDER, MARJORIE AKERS, P. REID LYONS, BERNARD LASSÈGUE, and KATHY K. GRIENDLING

Department of Medicine, Division of Cardiology, Emory University, Atlanta, Georgia

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ABSTRACT

In cultured vascular smooth muscle cells (VSMCs), activation of phospholipase D (PLD) by angiotensin II (Ang II) represents a major source of sustained generation of second messengers. Understanding the molecular mechanisms controlling activation of this pathway is essential to clarify the complexities of Ang II signaling, but the most proximal mechanisms coupling AT_1 receptors to PLD have not been defined. Here we examine the role of heterotrimeric G proteins in AT_1 receptor-PLD coupling. In alpha-toxin permeabilized VSMCs, GTPγS enhanced Ang II-stimulated PLD activation. In intact cells, Ang II activation of PLD was pertussis toxin-insensitive and was not additive with sodium fluoride, a cell-permeant activator of heterotrimeric G proteins, indicating that AT_1 receptor-PLD coupling requires pertussis toxin-insensitive heterotrimeric G proteins. Ang II-stimulated PLD activity was significantly inhibited in VSMCs electroporated with anti-Gβ antibody (56 $\pm$ 5%) and in cells overexpressing the G$\beta\gamma$-binding region of the carboxyl terminus of beta-adrenergic receptor kinase1 (79 $\pm$ 8%), suggesting a critical role for G$\beta\gamma$ in PLD activation by Ang II. This effect may be mediated by $pp60^{c\text{-}src}$, because in beta-adrenergic receptor kinase1 overexpressing cells, $pp60^{c\text{-}src}$ activation was inhibited, and in normal cells anti-$pp60^{c\text{-}src}$ antibody inhibited Ang II-stimulated PLD activity. Gα_{12} may also contribute to AT_1 receptor-PLD coupling because electroporation of anti-Gα_{12} antibody significantly inhibited PLD activity, whereas anti-Gα_i and G$\alpha_{q/11}$ antibodies had no effect. Furthermore, electroporation of anti-RhoA antibody also attenuated Ang II-induced PLD activation, suggesting a role for small molecular weight G protein RhoA in this response. Thus, we provide evidence here that G$\beta\gamma$ as well as Gα_{12} subunits mediate AT_1 receptor coupling to tonic PLD activation via $pp60^{c\text{-}src}$-dependent mechanisms, and that RhoA is involved in these signaling pathways in rat VSMCs. These results may provide insight into the molecular mechanisms underlying the highly organized, complex, chronic signaling programs associated with vascular smooth muscle growth and remodeling in response to Ang II.

Angiotensin II (Ang II) activates a remarkable spectrum of signaling pathways in vascular smooth muscle cells (VSMCs) upon binding to G protein-coupled AT_1 receptors, including phosphatidylinositol-specific phospholipase C (PLC)-β, PLC-γ, phospholipase A_2, protein kinase C (PKC), calcium release and influx, phosphatidylcholine-specific phospholipase D (PLD), receptor and nonreceptor tyrosine kinases such as c-Src and FAK, mitogen-activated protein (MAP) kinases, and various oxidases (Griendling et al., 1997). This complex series of signaling events is temporally controlled and highly organized. Thus, Ang II activates some enzymes within seconds (e.g., PLC), while the stimulation of others is delayed and persists for more than an hour (Alexander et al., 1985, Griendling et al., 1986). In VSMCs, sustained activation of PLD is a major source of prolonged second messenger generation. Hydrolysis of phosphatidylcholine by PLD results in a robust production of phosphatidic acid (PA) (Lassègue et al., 1991, 1993) and subsequent generation of diacylglycerol by PA phosphohydrolase (Lassègue et al., 1993). PA-derived diacylglycerol contributes to continuous PKC activation, and PA itself is implicated in the activation of the vascular NADH/NADPH oxidase (Griendling et al., 1994) which mediates the hypertrophic effects of Ang II (Griendling et al., 1994, Ushio-Fukai et al., 1996). It is somewhat paradoxical that much of the attention focusing on mechanisms coupled to AT_1 receptor activation has related to the very transient initial stimulation of PLC when it is likely that the great majority of chronic signaling programs associated with growth and remodeling in the cardiovascular system are in fact related to the tonic PLD-mediated responses.

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ABBREVIATIONS: Ang II, angiotensin II; VSMCs, vascular smooth muscle cells; PLC, phospholipase C; PKC, protein kinase C; PLD, phospholipase D; MAP, mitogen-activated protein; PA, phosphatidic acid; PTX, pertussis toxin; βARK1ct, the carboxyl terminus of beta-adrenergic receptor kinase 1; GTPγS, guanosine 5′-[γ-thio]triphosphate; DMEM, Dulbecco's modified Eagle's medium; PMA, phorbol 12-myristate 13-acetate.

Although several reports have indicated a role for the small G proteins ARF and/or Rho in PLD activation in vivo and/or in vitro (Exton, 1997), the most proximal mechanisms by which G protein-coupled receptors couple to PLD have not been well documented. Involvement of heterotrimeric G proteins was suggested by early studies examining the sensitivity of agonist activation of PLD to pertussis toxin (PTX), an agent that prevents receptor coupling to G_i or G_o by ADP ribosylation (Exton, 1996). However, these experiments did not define the exact G proteins which couple the receptor to PLD. Recently, Plonk et al. (1998) reported that overexpression of $G\alpha_{13}$, a member of the PTX-insensitive $G\alpha_{12}$ family, is able to activate PLD in COS-7 cells. The rat AT_1 receptor has been shown in various preparations to be capable of coupling to multiple alpha subunits of heterotrimeric G proteins (G_q, $G_{q/11}$, $G_{i/o}$, and $G_{12/13}$) (Kai et al., 1996, Macrez et al., 1997, Macrez-Leprêtre et al., 1997, Ushio-Fukai et al., 1998), but the precise subunit coupled to the receptor may play an important role in defining the specific complement of signaling pathways and effectors activated in a given system. We and others have previously shown that in VSMCs, coupling of the AT_1 receptor to PLC is mediated by $G\alpha_{q/11}$ and $G\alpha_{12}$ (Timmermans et al., 1993, Kai et al., 1996, Ushio-Fukai et al., 1998), but it is unclear whether these proteins also mediate coupling to PLD, or even if PLD activation is dependent upon heterotrimeric G proteins.

Emerging evidence suggests that G protein-coupled receptor activation of various effectors can also be mediated by Gβγ subunits (Clapham and Neer, 1997). Gβγ regulates K^+ channels (Logothetis et al., 1987), adenylyl cyclase (Inglese et al., 1994), PLC-β (Herrlich et al., 1996), c-Src (Luttrell et al., 1996), MAP kinases (Koch et al., 1994a, Coso et al., 1996), and mediates translocation of the beta-adrenergic receptor kinase (βARK) (Pitcher et al., 1992). Recent findings indicate a critical role for Gβγ in AT_1 receptor-coupled signal transduction. In rat portal vein myocytes, Ang II-induced L-type Ca^{++} channel activation is mediated by Gβγ derived from $G\alpha_{13}$ (Macrez et al., 1997), and in VSMCs, Gβγ associated with $G\alpha_{12}$ and/or $G\alpha_{q/11}$ mediates PLC activation by Ang II (Ushio-Fukai et al., 1998). The involvement of Gβγ in AT_1 receptor-PLD activation has not been investigated. However, a Src family tyrosine kinase, which has been shown to be a downstream effector of the AT_1 receptor (Ishida et al., 1995) and Gβγ (Luttrell et al., 1996), can mediate G protein-dependent PLD activation in other systems (Jiang et al., 1995b), indirectly implicating Gβγ and possibly Src in AT_1 receptor-PLD coupling.

In this study, we clarified the role of heterotrimeric G proteins in AT_1 receptor-PLD coupling, and assessed the possible involvement of Gβγ subunits and c-Src in this response using cultured rat VSMCs that were 1) permeabilized with α-toxin and stimulated with guanosine 5′-[γ-thio] triphosphate (GTPγS), a nonhydrolyzable analog of GTP, 2) electroporated with specific antibodies against G protein subunits, or 3) stably transfected with the Gβγ-binding region of the carboxyl terminus of beta-adrenergic receptor kinase1 (βARK1ct) (Koch et al., 1994b) to sequester free Gβγ. We provide here the first evidence that Gβγ subunits as well as their associated $G\alpha_{12}$ subunits mediate Ang II-induced PLD activation via $pp60^{c\text{-}src}$-dependent mechanisms in VSMCs, and that the small molecular weight G protein RhoA is also involved in these novel signaling cascades. These findings may suggest a novel role for these G proteins in providing selective AT_1 receptor coupling to tonic PLD signaling pathways.

Materials and Methods

Materials. Anti-$G\alpha_i$, anti-$G\alpha_{q/11}$, anti-$G\alpha_{12}$, anti-$G\beta_{common}$, anti-$pp60^{c\text{-}src}$, and anti-RhoA antibodies, protein A/G agarose, and Sam68 (331–433) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). ST638, genistein, and alpha-toxin were purchased from Calbiochem Corp. (San Diego, CA). The ^{125}I-labeled rabbit IgG was obtained from Amersham Pharmacia Biotech (Arlington Heights, IL). The pcDNA3 vector was purchased from Invitrogen (San Diego, CA). GTPγS, bovine serum albumin, and phenylmethanesulfonyl fluoride were obtained from Boehringer Mannheim (Indianapolis, IN). Lipofectin, geneticin, soybean trypsin inhibitor, glutamine, penicillin, streptomycin, Opti-MEM I reduced serum medium, and trypsin/EDTA were purchased from GIBCO BRL (Gaithersburg, MD). The TRI reagent was obtained from Molecular Research Center, Inc. (Cincinnati, OH). The Prime-It II kit was obtained from Stratagene, Inc. (Menasha, WI). Nytran membrane was obtained from Schleicher & Schuell, Inc. (Keene, NH). Monofluor was purchased from National Diagnostics, Inc. (Atlanta, GA). [γ-^{32}P]ATP and [^{3}H]choline chloride (1000 μCi/ml) were obtained from DuPont NEN (Wilmington, DE). Common buffer salts were obtained from Fisher (Pittsburgh, PA). All other chemicals and reagents, including calf serum and Dulbecco's modified Eagle's medium (DMEM) with 25 mM HEPES and 4.5 g/l glucose were obtained from Sigma (St. Louis, MO).

Cell Culture. VSMCs were isolated from male Sprague-Dawley rat thoracic aortae by enzymatic digestion as described previously (Griendling et al., 1991). Cells were grown in DMEM supplemented with 10% calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and were passaged twice a week by harvesting with trypsin/EDTA and seeding into 75-cm^2 flasks. For experiments, cells between passages 6 and 15 were used at confluence.

Stable Transfection of βARK1ct Expression Plasmid. pRK/βARK1ct (Gly^{495}-Leu^{689}) DNA (Koch et al., 1994b), a kind gift from Dr. Robert J. Lefkowitz, was digested with *Eco*RI and *Xba*I and cloned into the eukaryotic expression plasmid pcDNA3. Transcription of pcDNA3/βARK1ct cDNA was under control of the cytomegalovirus immediate-early gene enhancer/promoter. This vector also contains a neomycin-resistance gene, allowing selection of transfected cells with geneticin. Four micrograms of purified pcDNA3 alone or pcDNA3/βARK1ct plasmid in 100 μl of H_2O were gently mixed with Lipofectin solution (100 μl). The DNA/liposome complex was added directly to 40 to 50% confluent VSMCs plated in 60-mm dishes in Opti-MEM I reduced serum medium and incubated for 18 h at 37°C. The medium was then changed to DMEM containing 20% fetal bovine serum. After 48 h, transfected VSMCs were split 1:3 into 100-mm dishes and incubated in DMEM containing 10% fetal bovine serum and 400 μg/ml geneticin. Eight days after selection, geneticin-resistant colonies were isolated using cloning cylinders. Transfected cells were maintained in selection medium until they were plated into 35- or 100-mm dishes for experiments.

RNA Isolation and Northern Blot Analysis. Total RNA was extracted from cells as described previously (Kai et al., 1996). Ten-microgram RNA samples were separated by electrophoresis in 1.0% agarose gels containing 6.6% formaldehyde. RNA was transferred onto a nylon membrane and immobilized by UV cross-linking (Stratalinker; Stratagene, La Jolla, CA). The probe, βARK1ct cDNA derived from *Eco*RI/*Xba*I digestion of pRK-βARK1ct DNA (Koch et al., 1994b), was labeled with [α-^{32}P]dCTP using a random primer labeling kit (Prime-It II). After UV cross-linking, membranes were prehybridized at 68°C for 2 h in QuikHyb solution (Stratagene). The hybridization was performed for 2 h at 68°C with ^{32}P-labeled probe in the same solution. Membranes were washed two times in 1× SSC + 0.1% SDS at 50°C and once in 0.2 × SSC + 0.1% SDS at 55°C. After autoradiography, the relative density of each band was determined using laser densitometry. After transfer to the membrane,

staining of the 28S rRNA band by ethidium bromide was used for normalization.

Measurement of PLD Activity. Assay of PLD activity in intact VSMCs was performed as described previously (Lassègue et al., 1993). Briefly, cells grown in 35-mm dishes were labeled for 24 h with 1 μCi of [^{3}H]choline chloride in 2 ml of culture medium. After washing, cells were incubated at 37°C for 20 min in a buffer of the following composition: [130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 1 mM choline, 1 mM phosphorylcholine, and 20 mM HEPES (buffered to pH 7.4 with Tris base)]. The incubation buffer was replaced with 1 ml of buffer with or without 100 nM Ang II for 20 min. This buffer was then removed and combined with a chloroform/methanol (1:2) cellular extract for determination of total phosphatidylcholine metabolite accumulation. The aqueous phase was further processed for separation of choline and phosphocholine using tetraphenylboron in heptanone. Radioactivity was then quantified by liquid scintillation spectroscopy. We have previously shown that this method faithfully measures PLD activity, as confirmed by phosphatidylethanol formation (Lassègue et al., 1993).

Cell Permeabilization by Alpha-Toxin. Cells labeled for 24 h with 1.0 μCi of [^{3}H]choline chloride were permeabilized with 1250 U/ml of alpha-toxin at 37°C for 30 min in 1 ml of cytosolic buffer of the following composition [25 mM NaCl, 120 mM KCl, 1 mM $MgCl_2$, 2 mM EGTA, 10 mM glucose, 0.005 mM ATP, 1 mM choline, 1 mM phosphorylcholine, and 15 mM HEPES (buffered to pH 7.2 with potassium hydroxide at 37°C)]. After washing with cytosolic buffer without alpha-toxin, the cells were exposed to 1 ml of cytosolic buffer containing $CaCl_2$ (final concentration of 100 nM free calcium) with or without agonist for 20 min. The reaction was stopped, the phases were separated, and the aqueous phase was counted to assess PLD activity as described above.

Electroporation. Cells were electroporated in 35-mm tissue culture dishes using a Petri dish electrode manufactured by BTX (San Diego, CA). The electrode is 35 mm in diameter with a 2-mm gap and is plated with gold. Electroporation was performed in Hanks' balanced salt solution, pH 7.4 [5 mM KCl, 0.3 mM KH_2PO_4, 138 mM NaCl, 4 mM $NaHCO_3$, 0.3 mM $NaHPO_4$, 1.26 mM $CaCl_2$, 0.2H_2O, and 0.82 mM $MgSO_4$] containing antibodies at a concentration of 5 μg/ml. The cells were exposed to 1 pulse at 90 V for 40 ms (square wave) using an ElectroSquarePorator T820 (BTX, San Diego, CA); these conditions were similar to the conditions used for electroporation of VSMCs in 100-mm culture plates (Marrero et al., 1995). The tissue culture dishes were then incubated for 30 min at 37°C (5% CO_2), washed once with DMEM, and further incubated in this medium for 30 min at 37°C. The viability of cells after electroporation was 85%. Radiolabeled rabbit IgG was used to verify the electroporation procedure. VSMCs exposed to radiolabeled rabbit IgG without electroporation incorporated insignificant levels of radioactivity, whereas those undergoing the electroporation procedure showed dramatic uptake (data not shown).

Preparation of Cell Lysates. VSMCs at 80 to 90% confluence in 100-mm dishes were made quiescent by incubation with DMEM containing 0.1% calf serum for 24 h. Cells were stimulated with agonist at 37°C in serum-free DMEM for specified durations. After treatment, cells were washed three times with ice-cold phosphate-buffered saline and placed on ice. Cells were lysed with 500 μl of ice-cold lysis buffer, pH 7.4 [50 mM HEPES, 5 mM EDTA, and 50 mM NaCl], 1% Triton X-100, protease inhibitors (10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin) and phosphatase inhibitors [50 mM sodium fluoride, 1 mM sodium orthovanadate, and 10 mM sodium pyrophosphate]. Solubilized proteins were centrifuged at 14,000*g* in a microfuge (4°C) for 30 min, and supernatants were stored at −80°C. Extracted protein was quantified by the Bradford assay.

Immunoprecipitation and pp60$^{c\text{-}src}$ Immune Complex Kinase Assay. For immunoprecipitation, 400 μg of cell lysates were incubated with rabbit anti-pp60$^{c\text{-}src}$ antibody (1.4 μg) overnight at 4°C, and then incubated with 20 μl of protein A/G agarose for 1.5 h at 4°C with gentle rocking. The beads were washed four times with 500 μl of lysis buffer containing 150 mM NaCl instead of 50 mM NaCl, and two times with 500 μl of kinase buffer [20 mM HEPES (pH 7.6), 10 mM $MgCl_2$]. The kinase reaction was carried out by incubating the beads in 50 μl of kinase buffer containing 10 μCi of [γ-^{32}P]ATP, 50 μM ATP, and 2 μg of Sam68 (331–433), a highly efficient substrate for Src family tyrosine kinases, for 30 min at 30°C. Anti-pp60$^{c\text{-}src}$ immunoprecipitates were subjected to 9% SDS-polyacrylamide electrophoresis and [^{32}P]-labeled Sam68 (331–433) was detected using a phosphorimager and quantified by densitometry using NIH Image 1.61.

Ang II Receptor Binding. Ang II receptor binding was performed as described previously (Socorro et al., 1990). K_d and B_{max} (maximum number of binding sites) were determined by Scatchard analysis.

Statistical Analysis. Results are expressed as mean ± S.E. Statistical significance was assessed by analysis of variance, followed by comparison of group averages by contrast analysis using the SuperANOVA statistical program (Abacus Concepts, Berkeley, CA). A $p < 0.05$ was considered to be statistically significant.

Results

Effect of Ang II on GTPγS-Stimulated PLD Activity in Alpha-Toxin-Permeabilized VSMCs. To determine whether Ang II-induced PLD activation is mediated by coupling to G proteins in VSMCs, we examined the effects of GTPγS on PLD activity in alpha-toxin-permeabilized cells in the presence or absence of Ang II (Fig. 1). Addition of GTPγS in permeabilized cells caused activation of PLD in a concentration-dependent manner. Maximum stimulation was obtained with 200 μM GTPγS (136 ± 5% control, $n = 5$). This effect was antagonized by excess GDP (1 mM) (data not shown). After permeabilization, the stimulatory effect of 100 nM Ang II was decreased from the level observed in intact cells (257 ± 11% control, $n = 5$) to 109 ± 3% control ($n = 5$),

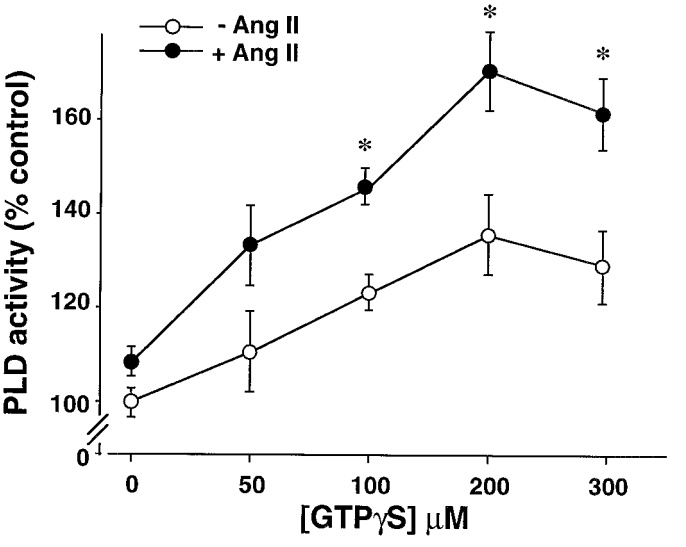

Fig. 1. Effect of Ang II on GTPγS-induced PLD activation in alpha-toxin-permeabilized VSMCs. [^{3}H]choline-labeled VSMCs permeabilized with alpha-toxin (1250 U/ml for 30 min) were stimulated with various concentrations of GTPγS in the absence (open circles) or in the presence (closed circles) of 100 nM Ang II for 20 min. PLD activity was then measured as described in *Materials and Methods*. Data are expressed as the percent increase in PLD activity over that in unstimulated, permeabilized cells. Values are the mean ± S.E. of four to five independent experiments performed in triplicate. *$p < .05$ for PLD activation by GTPγS in the absence, compared with the presence, of Ang II.

presumably due to the leakage of intracellular GTP. However, Ang II enhanced the GTPγS-stimulated PLD activity and increased the maximal response to 200 μM GTPγS to 171 ± 8% (n = 5). This increase was completely inhibited by losartan, an AT_1 receptor antagonist, without affecting the response to GTPγS alone (data not shown). In nonpermeabilized cells, sodium fluoride, a cell-permeant direct activator of heterotrimeric G proteins, stimulated PLD activity in a concentration-dependent manner (5–20 mM)(Fig. 2A). This effect was not additive with Ang II (Fig. 2B), indicating that both Ang II and heterotrimeric G proteins activate PLD through a common pathway. Furthermore, Ang II-induced PLD activation was insensitive to PTX at an exposure time (24 h) and concentration (200 ng/ml) which were sufficient to completely ADP-ribosylate all available substrate in this system (Socorro et al., 1990) (217 ± 2% control, n = 3, in cells with PTX and 201 ± 1% control, n = 3, in cells without PTX). Thus, these data suggest that Ang II-induced PLD activation is mediated by a heterotrimeric, PTX-insensitive G protein.

Role of Gβγ Subunits in Ang II-Stimulated PLD Activation. We have recently shown that Gβγ subunits are involved in Ang II stimulation of PLC activity (Ushio-Fukai et al., 1998). To determine whether Gβγ subunits also mediate Ang II activation of PLD, we intracellularly applied a specific antibody targeting Gβ subunits into VSMCs using electroporation. The electroporation of specific antibodies against cellular proteins has been shown to be an effective technique for interrupting Ang II-induced signal transduction cascades in cultured VSMCs (Marrero et al., 1995, Ushio-Fukai et al., 1998). As shown in Fig. 3, electroporation in the presence of rabbit IgG caused a small decrease in Ang II-stimulated PLD activity (9%) compared to mock electroporation. PLD activity in response to 100 nM Ang II in cells electroporated in the absence of antibody (mock electroporation) was increased by 118 ± 4% (n = 14), whereas that in nonelectroporated cells was increased by 157 ± 11% (n = 5). As shown in Fig. 3, anti-Gβ antibody partially, but significantly blocked Ang II-induced PLD activation (56 ± 5% inhibition, n = 8, $p < .05$). This incomplete inhibition is not due to insufficient amounts of antibody, because doubling the antibody concentration did not cause any further attenuation of the response (anti-β + anti-β, Fig. 3). The effectiveness of anti-Gβ antibody was abolished when it was boiled (100°C for 30 min) before electroporation, confirming that active antibody was required for the observed effect. These data suggest that Gβγ may mediate PLD activation.

To confirm further the role of Gβγ in Ang II-induced PLD activation, we overexpressed a specific Gβγ scavenger, βARK1ct (Koch et al., 1994b), in VSMCs. Control cells were transfected with vector only. The efficacy of βARK1ct cDNA transfection was evaluated by Northern analysis. We isolated 28 clones of geneticin-resistant βARK1ct-transfected cells; however, only four clones showed expression of βARK1ct mRNA. We selected the two highest expressors for further study (Fig. 4A). As shown in Fig. 4B, PLD activation by Ang II was significantly inhibited in both lines of βARK1ct-overexpressing cells (clone 1, 34 ± 9% increase, n = 6; clone 2, 57 ± 8% increase, n = 6) compared with that in vector-transfected cells (167 ± 11% increase, n = 6). In contrast, PLD activation by the protein kinase C activator, phorbol 12-myristate 13-acetate, was unaffected by overexpression of βARK1ct, indicating that the enzymatic activity of PLD is intact in these cells. We verified by measuring equilibrium binding of [^{3}H]-Ang II that AT_1 receptor expression was not different in vector-transfected cells (B_{max} = 632 fmol/mg protein) and in βARK1ct-overexpressing cells (clone 1, B_{max} = 750 fmol/mg protein; clone 2, B_{max} = 679 fmol/mg protein). These data strongly suggest that Gβγ subunits represent one mechanism mediating AT_1 receptor activation of PLD.

Effects of Tyrosine Kinase Inhibitors and Electroporated Anti-pp60$^{c\text{-}src}$ Antibody on Ang II-Stimulated PLD Activation. Because it has been reported that both agonist-bound AT_1 receptor (Ishida et al., 1995) and Gβγ (Luttrell et al., 1996) can activate the tyrosine kinase

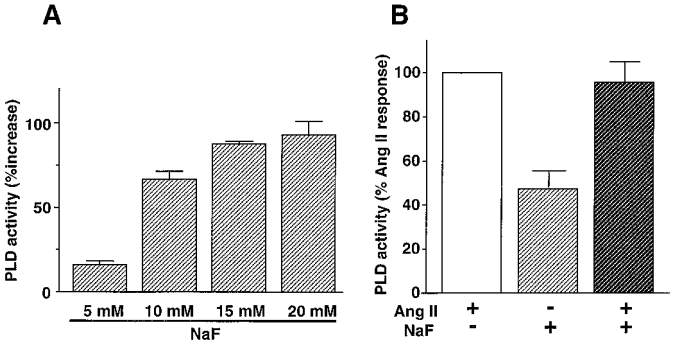

Fig. 2. Effect of sodium fluoride on Ang II-stimulated PLD activation in intact VSMCs. A, [^{3}H]choline-labeled VSMCs were stimulated with the indicated concentrations of NaF for 20 min, and PLD activity was measured. Data are expressed as the percent increase in PLD activity over that in unstimulated cells. B, [^{3}H]choline-labeled cells were stimulated by 100 nM Ang II or by 15 mM NaF with or without Ang II for 20 min. One hundred nanomolar Ang II is the maximum concentration and 15 mM NaF is a submaximum concentration for activating PLD in VSMCs. The Ang II-induced increase in PLD activity was taken as 100%. Values are the mean ± S.E. of three independent experiments performed in triplicate.

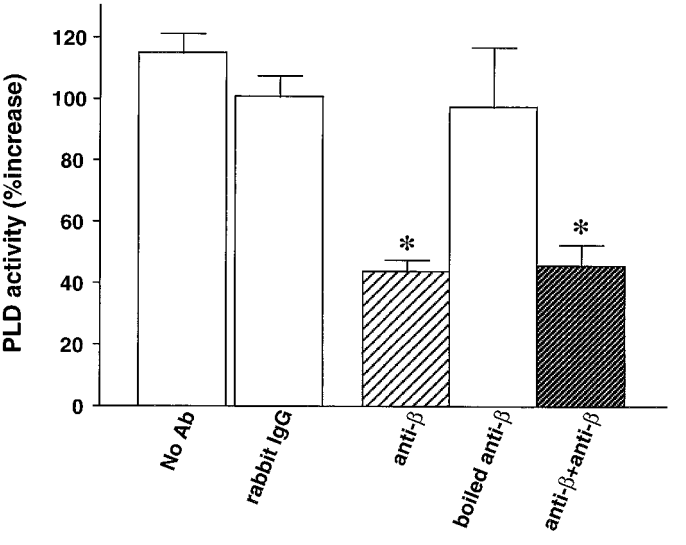

Fig. 3. Effect of electroporation of anti-Gβ antibody on Ang II-stimulated PLD activation. [^{3}H]choline-labeled VSMCs were electroporated in the presence of rabbit IgG or anti-$G\beta_{common}$ antibody (5 μg/ml for all columns except anti-Gβ + anti-Gβ, which had 10 μg/ml) and then stimulated with 100 nM Ang II for 20 min. Rabbit IgG was used as a negative control. Columns represent the percent increase in PLD activity by Ang II over that in unstimulated cells. Values are the mean ± S.E. of eight independent experiments performed in triplicate. *$p < .05$ for PLD activation by Ang II in cells electroporated with rabbit IgG versus anti-Gβ antibody.

pp60$^{c\text{-}src}$, we examined whether pp60$^{c\text{-}src}$ participates in PLD activation by Ang II. As shown in Table 1, the tyrosine kinase inhibitor genistein attenuated Ang II-induced PLD activation in a concentration-dependent manner. Another specific tyrosine kinase inhibitor ST638, which acts as a competitive inhibitor of substrate binding, also significantly inhibited PLD activation by Ang II in VSMCs (Table 1). Additionally, the Ang II response was decreased in cells electroporated with anti-pp60$^{c\text{-}src}$ (26 ± 2% increase, $n = 3$, $p < .05$) compared with that in cells electroporated with rabbit IgG (101 ± 7% increase, $n = 3$) (Fig. 5A). To examine the relationship between G$\beta\gamma$, pp60$^{c\text{-}src}$, and PLD, we measured Ang II-stimulated pp60$^{c\text{-}src}$ activity in βARK1ct-overexpressing cells. As shown in Fig. 5B, the increase of pp60$^{c\text{-}src}$ activity by Ang II was significantly inhibited by overexpression of βARK1ct. These results suggest that pp60$^{c\text{-}src}$ is downstream of G$\beta\gamma$ in AT_1 receptor-PLD coupling.

Role of Gα Subunits in Ang II-Stimulated PLD Activation. The incomplete inhibition of Ang II-induced PLD activation by electroporation of Gβ antibody and overexpression of βARK1ct suggests that an additional coupling mechanism exists. Therefore, we examined the possible involvement of a Gα subunit in AT_1 receptor-PLD coupling. We have previously demonstrated that the Gα proteins G_s, G_i, and $G_{q/11}$ are expressed in rat VSMCs (Kai et al., 1996). Immunoblot analysis in this study confirmed previous results and revealed that Gα_{12} is also expressed in these cells (data not shown). Electroporation of anti-Gα_i antibody had no effect on PLD activation by Ang II; this is consistent with the PTX-insensitivity of Ang II effects. Also, electroporation of anti-G$\alpha_{q/11}$ antibody did not inhibit PLD activation (Fig. 6), even though it significantly blocked PLC activation, as measured by inositol triphosphate production (54 ± 4% inhibition $n = 3$, $p < .05$) (Ushio-Fukai et al., 1998). In contrast, electroporation of antibodies against Gα_{12}, a PTX-insensitive heterotrimeric G protein, significantly inhibited the Ang II response (51 ± 2% inhibition, $n = 5$, $p < .05$)(Fig. 6). As with Gβ antibody, doubling the antibody concentration did not cause any further attenuation of the response (anti-α_{12} + anti-α_{12}, Fig. 6). The effectiveness of anti-Gα_{12} antibody was abolished by boiling (100°C for 30 min), confirming that active antibody was required for the observed effect. These observations suggest that Gα_{12} is also involved in AT_1 receptor coupling to PLD activation.

Involvement of RhoA in Ang II-Stimulated PLD Activation. Because the small molecular weight G protein RhoA has been shown to be involved in PLD activation (Exton, 1997), we also examined the effect of electroporation of anti-RhoA antibody on PLD activation by Ang II. Anti-RhoA antibody significantly inhibited Ang II-stimulated PLD activity by 58 ± 4% (Ang II + rabbit IgG, 117 ± 3% control; Ang II+Anti-RhoA, 62 ± 6% control, $n = 3$, $p < .05$). These observations suggest that RhoA may be part of the biochemical pathway leading to receptor-mediated PLD activation.

Discussion

In VSMCs, activation of PLD represents a major source of sustained generation of second messengers that are involved in the long-term cellular response to Ang II. Because of the potential importance of this pathway, understanding the molecular mechanisms controlling its activation is essential to clarify the complexities of Ang II signaling and their role in growth and remodeling of the cardiovascular system. Accumulating evidence suggest that PLD activation is regulated by small G proteins ARF and/or Rho (Exton, 1997); however, the most proximal mechanisms by which G protein-coupled receptors couple to PLD have not been well defined. In this study, we provide direct evidence that both G$\beta\gamma$ and Gα_{12} subunits of heterotrimeric G proteins play a crucial role in AT_1 receptor-PLD coupling in rat VSMCs via c-Src and RhoA-dependent mechanisms.

Earlier studies investigated the role of G proteins by examining the GTPγS-dependent PLD activity in plasma membranes and in cell-free systems (Olson et al., 1991, Houle et al., 1995). Although these experiments provided convincing

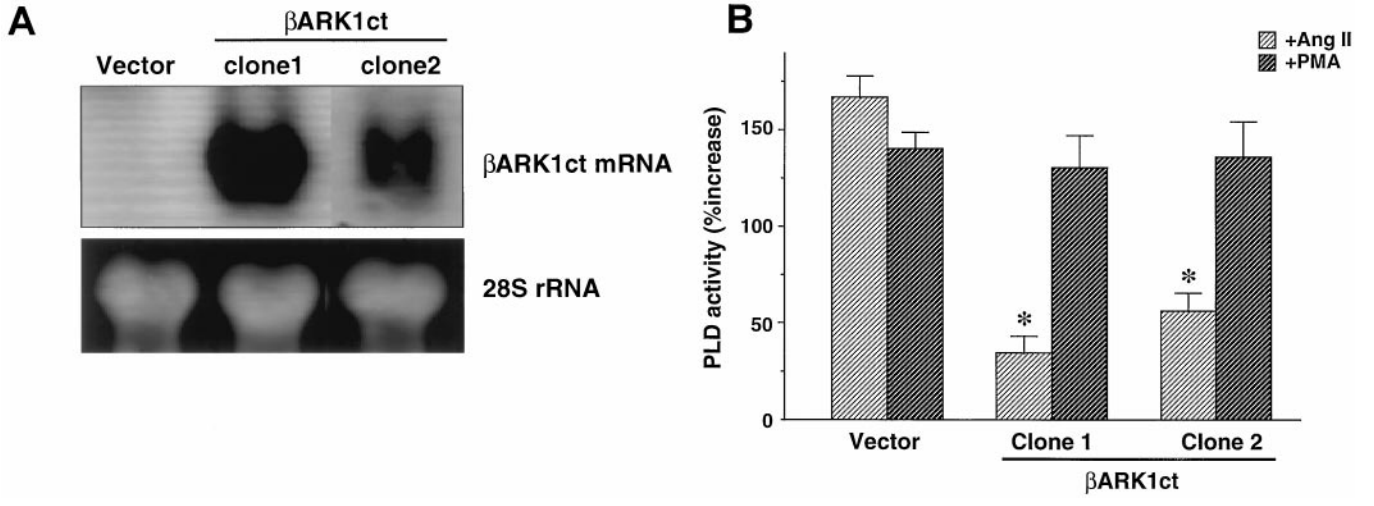

Fig. 4. Effect of overexpression of the carboxyl terminus of βARK1 on PLD activation. A, Northern blot analysis of βARK1 carboxyl terminus (βARK1ct) mRNA in VSMCs stably transfected with plasmid DNA encoding βARK1ct. Representative autoradiogram in a vector-transfected clone and two of the selected βARK1ct-transfected clones (top). The size of the βARK1ct mRNA band is 880 nucleotides. The bottom panel shows the 28S ribosomal RNA band stained with ethidium bromide. B, PLD activation in vector-transfected cells and βARK1ct-overexpressing cells. [^{3}H]choline-labeled VSMCs were stimulated with 100 nM Ang II or 100 nM phorbol 12-myristate 13-acetate (PMA) for 20 min. Columns represent the percent increase in PLD activity by Ang II and PMA over that in unstimulated cells. Values are the mean ± S.E. of four (for Ang II) or three (for PMA) independent experiments performed in triplicate. *$p < .05$ for PLD activation by Ang II in βARK1ct-overexpressing cells versus vector-transfected cells.

evidence that small G proteins are involved in PLD activation, it has been difficult to definitively demonstrate a role for heterotrimeric G proteins. Our data show that in cultured rat VSMCs permeabilized with alpha-toxin, PLD activation by Ang II is enhanced in the presence of GTPγS. Furthermore, in intact cells, PLD is activated by sodium fluoride, a cell-permanent activator of *heterotrimeric* G proteins. This effect is not additive with the effect of Ang II, suggesting that both agonists activate PLD through a common mechanism. Taken together, these findings strongly suggest that Ang II-mediated PLD activation is dependent upon AT_1 receptor coupling to a heterotrimeric G protein.

Growing evidence suggests that Gβγ subunits play an important role in the signal transduction of various G protein-coupled receptors. Gβγ has been shown to mediate agonist-induced activation of adenylate cyclase II (Inglese et al., 1994), PLC-β (Herrlich et al., 1996), MAP kinase (Koch et al., 1994a), and c-Jun kinase (Coso et al., 1996). However, the role of Gβγ in hormone-stimulated PLD activation has not been elucidated. In this study, we show that Ang II-stimulated PLD activity is significantly inhibited by electroporation of anti-Gβ antibody and by overexpression of βARK1ct, which acts as a Gβγ antagonist (Koch et al., 1993) (Figs. 3 and 4). This suggests that Gβγ is a potential signal transducer for coupling AT_1 receptors to PLD in VSMCs. Thus, PLD can be added to the growing list of effectors mediated by Gβγ (Clapham and Neer, 1997). Consistent with these results, a critical role for Gβγ in Ang II signaling has been previously demonstrated using a similar approach. Ang II-induced L-type Ca^{++} channel opening and PLC activation are inhibited by microinjection or electroporation of anti-Gβ antibody and by βARK1ct overexpression (Macrez et al., 1997, Ushio-Fukai et al., 1998). Originally, Gβγ was proposed to act as a transducer specific for PTX-sensitive G protein-dependent responses (Koch et al., 1994a). We have previously shown that Gβγ subunits derived from $G\alpha_{q/11}$ and/or $G\alpha_{12}$, both of which belong to PTX-insensitive heterotrimeric G protein families, mediate AT_1 receptor-PLC coupling, and here we show that Gβγ-mediated PLD activation by Ang II is also PTX-insensitive in VSMCs. Furthermore, Stehno-Bittel et al. (1995) showed that Gβγ participates in PTX-*insensitive* activation of PLC in *Xenopus laevis* oocytes, and Coso et al. (1996) reported that Gβγ is involved in c-Jun kinase activation by $G\alpha_q$-coupled m1 muscarinic receptors in COS-7 cells. Thus, Gβγ appears to represent a common signal transducer for both PTX-sensitive and -insensitive signaling pathways in certain cell types.

The mechanisms by which Gβγ subunits mediate AT_1 receptor coupling to PLD was also assessed in this study. Gβγ-mediated signaling has been shown to be associated with tyrosine kinase pathways. Indeed, Gβγ binds to the

TABLE 1

Effect of tyrosine kinase inhibitors on Ang II-induced PLD activation

[^{3}H]choline-labeled VSMCs were pretreated with inhibitors for 30 min and then stimulated with Ang II (100 nM) for 20 min in the continued presence of inhibitor. Data are expressed as percent inhibition of the response to Ang II in the absence of inhibitors. Values are mean ± S.E. of three independent experiments performed in triplicate.

Inhibitor	% Inhibition
Genistein	
30 μM	25 ± 3
100 μM	61 ± 6
ST638	
30 μM	38 ± 3
100 μM	51 ± 4

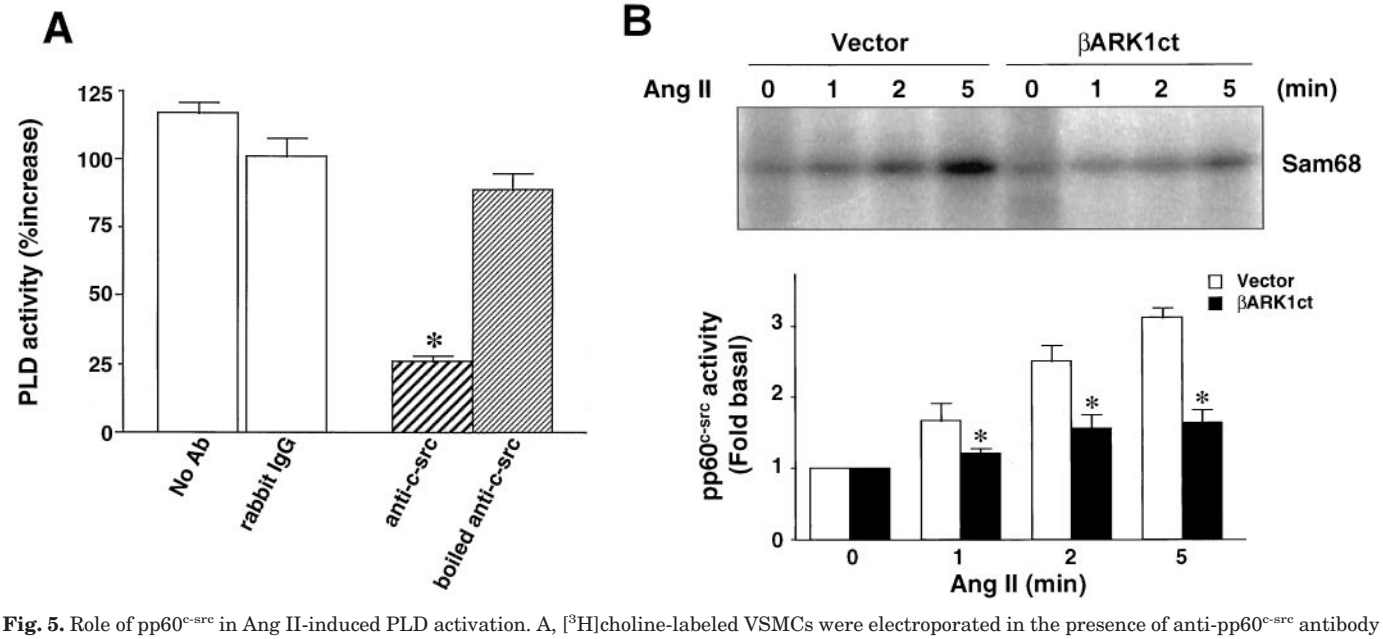

Fig. 5. Role of pp60$^{c\text{-}src}$ in Ang II-induced PLD activation. A, [^{3}H]choline-labeled VSMCs were electroporated in the presence of anti-pp60$^{c\text{-}src}$ antibody (5 μg/ml) and then stimulated with 100 nM Ang II for 20 min. Rabbit IgG was used as a negative control. *$p < .05$ for PLD activation by Ang II in cells electroporated with rabbit IgG- versus anti-pp60$^{c\text{-}src}$ antibody. Columns represent the percent increase in PLD activity by Ang II over that in unstimulated cells. Values are the mean ± S.E. of three independent experiments performed in triplicate. B, effect of overexpression of βARK1ct on Ang II-stimulated pp60$^{c\text{-}src}$ activity. VSMCs were treated with 100 nM Ang II for the indicated times. Lysates were prepared as described in *Materials and Methods* and immunoprecipitated with anti-pp60$^{c\text{-}src}$ antibody. pp60$^{c\text{-}src}$ activity was monitored by following phosphorylation of the Src-specific substrate Sam68 (331–433). Upper panel is a representative image of Sam68 (331–433) phosphorylation by Ang II. Lower panel represents averaged data quantified by densitometry of images, expressed as fold increase in phosphorylation, in which the phosphorylation observed in cells at time 0 was defined as 1.0 (control). Values are the means ± S.E. for three independent experiments. *$p < .05$ for pp60$^{c\text{-}src}$ activation by Ang II in βARK1ct-overexpressing cells versus vector-transfected cells.

pleckstrin homology domain of several tyrosine kinases (Inglese et al., 1995) and activates pp60$^{c\text{-}src}$ (Luttrell et al., 1996). Because Ang II also activates pp60$^{c\text{-}src}$ (Ishida et al., 1995), we hypothesized that G$\beta\gamma$-mediated PLD activation might occur through this tyrosine kinase. The inhibition of AT_1 receptor-stimulated PLD activity by the tyrosine kinase inhibitors, genistein and ST638, and by electroporation of anti-pp60$^{c\text{-}src}$, provides evidence for the involvement of pp60$^{c\text{-}src}$ in this signaling pathway. Consistent with our results, tyrosine kinases have been shown to regulate PLD in other systems (Meacci et al., 1995). Jiang et al. (1995a) reported that overexpression of v-Src leads to increased G protein-dependent PLD activity. Furthermore, we found that pp60$^{c\text{-}src}$ activation by Ang II is dramatically attenuated by βARK1ct overexpression (Fig. 5), suggesting that pp60$^{c\text{-}src}$ is a downstream target of G$\beta\gamma$ in Ang II signaling. Taken together, these findings strongly indicate that the released G$\beta\gamma$ following stimulation of AT_1 receptors may activate PLD through the tyrosine kinase pp60$^{c\text{-}src}$. However, we cannot completely rule out other possible mechanisms. For example, G$\beta\gamma$ may activate PLD secondary to the stimulation of PLC by G$\beta\gamma$ (Stehno-Bittel et al., 1995, Clapham and Neer, 1997, Ushio-Fukai et al., 1998), because PLD activation occurs subsequent to PLC in Ang II-stimulated rat aortic smooth muscle cells (Griendling et al., 1986, Lassègue et al., 1991). This is somewhat unlikely because electroporation of anti-G$\alpha_{q/11}$ antibody which inhibits PLC activation (Ushio-Fukai et al., 1998) fails to block the PLD response (Fig. 6). Another possibility is that G$\beta\gamma$ may activate PLD by stimulation of Ca^{++} channels (Macrez et al., 1997) because Ang II-stimulated PLD activity is largely dependent on extracellular Ca^{++} influx (Lassègue et al., 1993). Finally, G$\beta\gamma$ may activate PLD by binding directly to the ras-related small G proteins Rho (Harhammer et al., 1996) or ARF (Colombo et al., 1995), both of which have been shown to regulate PLD activity directly or indirectly (Exton, 1997). Nonetheless, our data are most consistent with an AT_1 receptor-G$\beta\gamma$-pp60$^{c\text{-}src}$-mediated activation of PLD.

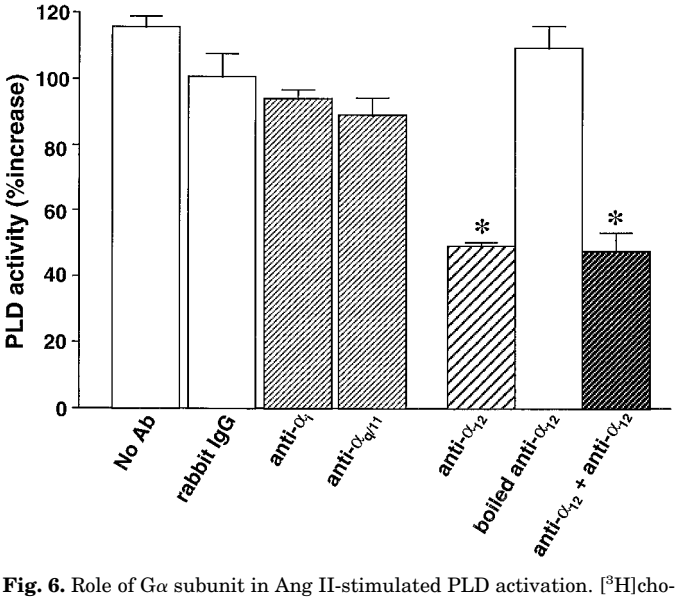

Fig. 6. Role of Gα subunit in Ang II-stimulated PLD activation. [^{3}H]choline-labeled VSMCs were electroporated in the presence of anti-Gα_i, G$\alpha_{q/11}$, or Gα_{12} antibodies (5 μg/ml for all columns except anti-Gα_{12}+anti-Gα_{12}, which had 10 μg/ml) and then stimulated with 100 nM Ang II for 20 min. Rabbit IgG was used as a negative control. Columns represent the percent increase in PLD activity by Ang II in the presence or absence of antibody over that in unstimulated cells. Values are the mean ± S.E. of four to six independent experiments performed in triplicate. *$p < .05$ for PLD activation by Ang II in cells electroporated with rabbit IgG versus anti-Gα antibody.

Because G$\beta\gamma$ is necessary, but not sufficient, for PLD activation (Figs. 3 and 4), we examined whether the Gα subunit is also involved in AT_1 receptor-PLD coupling. Our results suggest that AT_1 receptors activate PLD in part via coupling to Gα_{12}, based on the observation that electroporation of anti-Gα_{12}, but not anti-Gα_i and -G$\alpha_{q/11}$, antibodies significantly inhibited Ang II-induced PLD activation (Fig. 6). We have verified that Gα_i, G$\alpha_{q/11}$, and Gα_{12} are ubiquitously expressed in rat VSMCs by immunoblot analysis (Kai et al., 1996) (data not shown). Thus, our data strongly suggest that Ang II-induced PLD activation is achieved exclusively through selective coupling to Gα_{12}, in contrast to AT_1 receptor coupling to PLC, which utilizes both G$\alpha_{q/11}$ and Gα_{12} (Ushio-Fukai et al., 1998). The lack of involvement of G$\alpha_{q/11}$ in AT_1 receptor-PLD coupling is further supported by the observation that a long-term treatment with vasopressin, which selectively downregulates G$\alpha_{q/11}$ by 90% and inhibits PLC activation (Kai et al., 1996), does not affect Ang II stimulation of PLD (unpublished observations, MUF, MA and KKG). Coupling of the AT_1 receptor to the Gα_{12} family of heterotrimeric G proteins has been previously reported by several groups (Macrez-Leprêtre et al., 1997, Ushio-Fukai et al., 1998).

A dual role for heterotrimeric G proteins and the small molecular weight G protein Rho in agonist-induced PLD activation has been proposed. Plonk et al. (1998) found that overexpression of Gα_{13} activates PLD by a pathway requiring Rho family GTPase (Exton, 1996). Our data suggest that receptor-stimulated PLD activity may require not only heterotrimeric G proteins, but also RhoA, since electroporation of anti-RhoA antibody significantly inhibited Ang II-induced PLD activation. Recently, Kozasa et al. (1998) and Hart et al. (1998) showed that a newly isolated protein, p115 RhoGEF, can serve as a direct link between Rho and G$\alpha_{12/13}$. Thus, activation of Gα_{13} stimulated the guanine nucleotide exchange activity of RhoGEF, leading to activation of Rho. Because Rho has been shown to play a role in activation of PLD1, this coupling mechanism provides an attractive hypothesis to link the receptor, the heterotrimeric G protein, Rho, and PLD. Although VSMCs contain both PLD1 and PLD2, as assessed by Northern blot analysis and reverse transcription-polymerase chain reaction (B.L., M.U.F., and K.K.G., unpublished observations), these observations suggest that PLD1 may be the functional AT_1 receptor-coupled isozyme.

Gα and G$\beta\gamma$ subunits may play a bifunctional role in PLD activation by providing specific coupling and enhancement of the response. Thus, Gα_{12} is an excellent substrate for PKC in vivo and in vitro (Kozasa and Gilman, 1996). Phosphorylation of Gα_{12} by PKC blocks its interaction with G$\beta\gamma$, creating a pool of free G$\beta\gamma$ (Kozasa and Gilman, 1996). Because PKC activation by Ang II is a consequence of PLD stimulation in VSMCs (Lassègue et al., 1993), it is possible that the activated AT_1 receptor couples to G$\alpha_{12}\beta\gamma$ heterotrimers promoting GTPase activity and releasing G$\beta\gamma$ to activate PLD, and thereby stimulating PKC. This activated PKC may play a positive feedback role by phosphorylating Gα_{12}, thus pre-

venting Gβγ reassociation (Kozasa and Gilman, 1996) and potentiating Gβγ-mediated signaling that can continue to activate PLD. Consistent with this scenario is the observation that agonist-induced PLD activation is dependent upon continued PKC activity (Exton, 1997). This hypothesis may explain why the PLD response is sustained during continuous AT_1 receptor stimulation (Griendling et al., 1986).

In summary, the present study demonstrates that 1) Ang II-induced PLD activation is dependent upon AT_1 receptor coupling to a heterotrimeric G protein, 2) Gβγ subunits mediate Ang II-induced PLD activation, possibly through tyrosine kinase $pp60^{c\text{-}src}$- and RhoA-dependent mechanisms, and 3) $G\alpha_{12}$ may provide selectivity for AT_1 receptor-PLD coupling. These observations provide insight into the molecular mechanisms underlying the complex chronic signaling programs associated with vascular smooth muscle growth and remodeling in response to Ang II.

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Send reprint requests to: Masuko Ushio-Fukai, Ph.D., Division of Cardiology, Emory University School of Medicine, 1639 Pierce Drive, Rm. 319, Atlanta, GA, 30322. E-mail address: mfukai@emory.edu