Temporal Dispersion of Activation of Phospholipase C-β1 and -γ Isoforms by Angiotensin II in Vascular Smooth Muscle Cells

ROLE OF αq/11, α12, AND βγ G PROTEIN SUBUNITS*

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Activation of phospholipase C (PLC) is one of the earliest events in angiotensin II (Ang II) type 1 (AT1) receptor (R)-mediated signal transduction in vascular smooth muscle cells (VSMCs). The coupling mechanisms of AT1 Rs to PLC, however, are controversial, because both tyrosine phosphorylation of PLC-γ and G protein-dependent PLC-β activation pathways have been reported. The expression of PLC-β1, furthermore, has not been consistently demonstrated in VSMCs. Here we identified the PLC subtypes and subunits of heterotrimeric G proteins involved in AT1 R-PLC coupling using cultured rat VSMCs. Western analysis revealed the expression of PLC-β1, -γ1, and -α1 in VSMCs. Ang II-stimulated inositol trisphosphate (IP3) formation measured at 15 s, which corresponds to the peak response, was significantly inhibited by electroporation of antibodies against PLC-β1, but not by anti-PLC-γ and -α antibodies. Electroporation of anti-Gαq/11 and -Go12 antibodies also showed significant inhibition of the Ang II-induced IP3 generation at 15 s, while anti-Gq and Go12 antibodies were ineffective. Furthermore, in VSMCs electroporated with anti-Go β-antibody and cells stably transfected with the plasmid encoding the Go βγ-binding region of the carboxyl terminus of β-adrenergic receptor kinase 1, the peak Ang II-stimulated PLC activity (at 15 s) was significantly inhibited. The tyrosine kinase inhibitor, genistein, had no effect on the peak response to Ang II stimulation, but significantly inhibited IP3 production after 30 s, a time period which temporally correlated with PLC-γ tyrosine phosphorylation in response to Ang II. Moreover, electroporation of anti-PLC-γ antibody markedly inhibited the IP3 production measured at 30 s, indicating that tyrosine phosphorylation of PLC-γ contributes mainly to the later phase of PLC activation. Thus, these results suggest that: 1) AT1 receptors sequentially couple to PLC-β1 via a heterotrimeric G protein and to PLC-γ via a downstream tyrosine kinase; 2) the initial AT1 receptor-PLC-β1 coupling is mediated by Goq/11βγ and Go12βγ; 3) Go βγ acts as a signal transducer for activation of PLC in VSMCs. The sequential coupling of AT1 receptors to PLC-β1 and PLC-γ, as well as dual coupling of AT1 receptors to distinct Go proteins, suggests a novel mechanism for a temporally controlled, highly organized and convergent Ang II-signaling network in VSMCs.

Angiotensin II (Ang II) plays an important role in controlling both contraction and growth of vascular smooth muscle cells (VSMCs) through complex intracellular signaling events involving pathways classically associated with both G-protein coupled and tyrosine kinase-mediated responses (1). In VSMCs, most of the Ang II effects are mediated by AT1 receptors, which belong to the 7-transmembrane spanning, heterotrimeric G protein-coupled receptor family (2, 3). The rat AT1 receptor has been shown in various preparations to be capable of coupling to various α-subunits (Gq, Goq/11, and Gαo) (4-5), which may provide insights into the potential mechanism by which a single AT1 receptor stimulates various signaling cascades.

Recently, it has become apparent that AT1 receptors also couple to Goq/11βγ, a heterotrimeric G protein whose α-subunit belongs to the nonpertussis toxin-sensitive Go12 family. In rat portal vein myocytes, the Goβγ subunits derived from Go12 apparently mediate Ang II activation of an L-type Ca2+ channel (6, 7). In general, however, the immediate effectors coupled to the Go12 family of G proteins are unknown. Although Go12 transduces thrombin receptor activation of AP-1-mediated gene expression (8), and both Go12 and Go13 activate Jun kinase/stress-activated protein kinase, the most proximal signals remain to be defined.

Ang II binding to AT1 receptors in VSMC causes a distinctly biphasic response, with a rapid and transient activation of phosphatidylinositol-specific PLC to produce inositol trisphosphates (IP3) and diacylglycerol, followed by prolonged activation of phospholipase D (9). IP3 formation is markedly increased within a few seconds, reaches a maximum at 15 s, and then gradually returns to control levels (10). Although activation of PLC is one of the earliest events in Ang II signaling (10), the mechanisms by which AT1 receptors couple to PLC in VSMCs are controversial.

Three families of mammalian PLC isoforms, PLC-β, -γ, and -α, have been described based on their molecular structure and mechanism of regulation (11). PLC-β isoforms have been shown to be activated by Go and Go βγ subunits of the heterotrimeric G proteins, while PLC-γ isoforms are regulated by tyrosine phosphorylation (11). PLC-δ isoforms are smaller (85 kDa) than PLC-β and -γ (150 and 145 kDa, respectively) and their function remains unclear (11). In general, G protein-coupled receptors are assumed to activate PLC-β isoforms by coupling to the heterotrimeric G proteins (11), while growth

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factor receptors are proposed to activate PLC-γ by tyrosine phosphorylation (11). However, in rat VSMCs, Marrero et al. (12) demonstrated that activation of the G protein-coupled AT1 receptor induced tyrosine phosphorylation and activation of PLC-γ, although potential involvement of G proteins was not analyzed. We previously showed that Ang II-stimulated PLC activation is mediated by a pertussis toxin-insensitive G protein (13), at least in part represented by Goq11 (4), in these same cells. We also showed that prolonged incubation with Ang II causes selective down-regulation of Goq11, providing indirect additional evidence that the AT1 receptor interacts with Goq11, in intact VSMCs as demonstrated in vitro (4). Consistent with our reports, AT1 receptors in other systems have been shown to couple to PLC-β via Goq11 (14, 15), although Goq11-mediated activation of PLC-β is only partial (4, 16). In rat and rabbit VSMCs, however, PLC-β1 protein has been difficult to detect (12, 17). In human aortic VSMCs, Schelling et al. (18) showed that both PLC-β1 and PLC-γ are expressed, and that Ang II-PLC signaling is mediated by PLC-β1, but not by PLC-γ. Thus, in VSMCs, it remains unclear which PLC isozymes are expressed, and whether AT1 receptor-PLC activation is mediated by direct coupling to G protein subunits or by stimulation of a downstream tyrosine kinase.

In addition to these uncertainties, it is unclear how tyrosine phosphorylation of PLC-γ, which occurs at 30 s to 1 min (12), could mediate the earliest measurable increase in IP_3_ formation (<5 s) (10) in response to Ang II. Since the most proximal signal transmission by G protein-coupled receptors is likely achieved through the heterotrimeric G protein subunits, Go and Gβγ, both of which have been shown to stimulate PLC-β in other systems (11), we hypothesized that the earliest activation of PLC by AT1 receptors occurs through the coupling to Go or Gβγ, and the later phase of IP_3_ generation involves tyrosine phosphorylation of PLC-γ. Thus, the present study was designed to clarify the role of tyrosine kinases and G proteins in AT1 receptor-PLC coupling, and to identify the PLC subtypes and the subunits of heterotrimeric G proteins involved in their coupling in VSMCs. For this purpose, we measured IP_3_ production by Ang II in cultured rat VSMCs electroporated with specific antibodies against PLC isozymes and G protein subunits, and in cells stably transfected with a plasmid encoding the Gβγ-binding region of the carboxyl terminus of β-adrenergic receptor kinase 1 (βARK1ct) (19) to sequester free Gβγ. We provide evidence for the temporal dispersion of AT1 receptor signals through the sequential activation of PLC-β1 and PLC-γ, and for a role for Goq11 and Goq12 as well as their associated Gβγ subunits in activation of PLC-β1.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tween 20, acrylamide, SDS, nonfat dry milk, low molecular weight protein markers, and goat anti-rabbit IgG-horseradish peroxidase (HRP)-conjugate were purchased from Bio-Rad. Protein A/G Plus-agarose, anti-Gα, anti-Gαq11, anti-Gβγ, anti-Gαs, anti-Gq12, anti-Gq13, and anti-PLC-β antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PLC-γ (IgG), anti-PLC-61 (IgG), and anti-phosphotyrosine (IgG) monoclonal antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal antibody against PLC-β (IgM) and rat pituitary homogenate were obtained from Transduction Laboratories (Lexington, KY). The enhanced chemiluminescence (ECL) Western blotting detection system and goat anti-mouse IgG-HRP-conjugate were obtained from Amersham Life Sciences. PVDF membranes (0.45 μm) and Nytran membranes were purchased from Millipore (Bedford, MA) and Schleicher & Schuell, Inc. (Keene, NH), respectively. The pCDNA3 vector was from Invitrogen (San Diego, CA). Bovine serum albumin and phenylmethanesulfonyl fluoride were from Boehringer Mannheim. Lipofectin, geneticin, soybean trypsin inhibitor, glutamine, penicillin, streptomycin, Opti-MEM I reduced serum medium, and trypsin/EDTA were purchased from Life Technologies, Inc. (Gaithersburg, MD). TRI reagent was from Molecular Research Center (Cincinnati, OH). The Prime-IT II kit and QuikHyb solution were from Stratagene (Menasha, WI). Monofluor was purchased from National Diagnostics (Atlanta, GA), and myo-[3H]inositol (1000 μCi/ml) was from NEN Life Science Products Inc. (Wilmington, DE). Common buffer salts were obtained from Fisher (Pittsburgh, PA). All other chemicals and reagents, including Dulbecco’s modified Eagle’s Medium (DMEM) with 25 mM Hepes and 4.5 g/liter glucose and calf serum, were from Sigma.

**Cell Culture**—VSMCs were isolated from male Sprague-Dawley rat thoracic aortas by enzymatic digestion as described previously (20). Cells were grown in DMEM supplemented with 10% calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin and incubated in a humidified incubator with 5% CO_2/95% air at 37°C. After reaching confluence, cells were trypsinized and seeding into 75-cm² flasks. For experiments, cells between passages 6 and 15 were used at confluence.

**Stable Transfection of βARK1ct Expression Plasmid**—To transfect CHO cells with a plasmid encoding the Gβγ-binding region of the carboxyl terminus of β-adrenergic receptor kinase 1 (βARK1ct) DNA (19), a kind gift from Dr. Robert J. Lefkowitz, was digested with EcoRI and XbaI and cloned into the eukaryotic expression plasmid pcDNA3. Transcription of pcDNA3/βARK1ct DNA 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 μg 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 40–50% confluent VSMCs plated in 60-mm dishes in OptiMEM 1 reduced serum medium and incubated 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 (4). 10-μg RNA samples were separated by electrophoresis in 1.0% agarose gels containing 6.6% formamide. RNA was transferred onto a nylon membrane and immobilized by UV cross-linking (Stratalinker, Stratagene). The probe, βARK1ct cDNA derived from EcoRI/XbaI digestion of pRC-βARK1ct DNA (19), was labeled with [α-32P]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 a[32P]-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 20 min at 50°C, the relative density of each band was determined using laser densitometry. Staining of the 28 S RNA band by ethidium bromide, after transfer to the membrane, was used for normalization.

**Measurement of IP_3_ Production**—Assay of PLC activity in intact VSMCs was performed as described previously (4). Cells grown on 35-mm dishes were labeled for 24 h with myo-[3H]inositol (15 μCi/ml) in 2 ml of culture medium. After washing, cells were incubated at 37°C for 5 min in buffered salt solution of the following composition (in mM, 130 NaCl, 5 KCl, 1 MgCl_2, 1.5 CaCl_2, 20 HEPES (buffered to pH 7.4 with Tris base)). Incubation buffer was removed and replaced with 1 ml of buffer with or without 100 nM Ang II for indicated times. The reaction was terminated by rapid aspiration of the buffer and addition of 1 ml of chloroform/methanol/HCl (1:2:0.05). Organic and aqueous phases of the extract plus a 0.5-ml rinse were separated by addition of 500 μl of chloroform and 900 μl of distilled water, followed by centrifugation for 10 min at 500 × g. Chloroform phases were removed and aqueous phases were washed with chloroform. IP_3_, IP_2_, and IP_1_ fractions extracted into the aqueous phases were sequentially eluted from AG-1-X8 anion exchange columns using 180 mM NH_4 formate, 5 mM sodium tetraborate; 400 mM NH_4 formate, 100 mM formic acid; 1 mM NaHCO_3, 100 mM formic acid, respectively. All inositol phosphates were quantified by liquid scintillation spectroscopy.

**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 plated with gold. Electroporation was performed in Hank’s balanced salt solution (HBSS) (in mM, 5 NaCl, 0.86 KH_2PO_4, 134 NaCl, 4 NaHCO_3, 0.3 KH_2PO_4, 1.8 KCl, 0.44 CaCl_2, 1.26 CaCl_2, 0.82 MgSO_4), containing antibodies at a concentration of 5 μg/ml. Cells were exposed to 1 pulse at 90 V for 40 ms (square wave) using a BTX Model T820 ElectroSquarePorator, similar to conditions used for electroporation of VSMCs in 100-mm culture plates (21). The tissue culture dishes were then incubated for 30 min at 37°C (5% CO_2), and then washed once with DMEM and further incubated in this same medium for 30 min at 37°C. The effectiveness of the transfection was measured by electroporation.
electroporation procedure was verified by measuring the intracellular incorporation of \(^{125}\text{I}\)-labeled rabbit IgG after electroporation. We found that 58.5 ± 3.3% (n = 8) of extracellular \(^{125}\text{I}\)-labeled rabbit IgG was efficiently incorporated within the cells without adversely affecting their viability. In contrast, VSMCs exposed to a mock experiment without electroporation contained undetectable levels of \(^{125}\text{I}\)-labeled rabbit IgG.

Protein Purification and Immunoblot Analysis—Confluent VSMCs in 100-mm dishes were washed three times with ice-cold PBS. Cells were scraped in 500 µl of ice-cold lysis buffer, pH 7.4 (in mM, 50 HEPES, 5 EDTA, 50 NaCl), containing 1% Triton X-100, protease inhibitors (10 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and phosphatase inhibitors (in mM, 50 sodium fluoride, 1 sodium orthovanadate, 10 sodium pyrophosphate). Solubilized proteins were centrifuged at 14,000 × g for 30 min, and supernatants were stored at −80 °C. Extracted protein was quantified by the Bradford assay. Proteins were separated on 5 or 10% polyacrylamide gels using SDS-PAGE and transferred to PVDF membranes (0.45 µm) overnight. Membranes were blocked for 1 h with PBS containing 5% nonfat dry milk and 0.1% Tween 20, and were incubated with primary antibody for 1 h in PBS containing 1% nonfat dry milk and 0.1% Tween 20, washed three times with PBS containing 0.2% nonfat dry milk and 0.1% Tween 20, and then incubated with HRP-conjugated goat anti-rabbit secondary antibody for 1 h. The ECL Western blotting detection kit (Amersham Corp.) was used for detection.

Analysis of Tyrosine Phosphorylation of PLC-γ—VSMCs in 100-mm dishes at near confluence were growth arrested in 0.1% serum-containing DMEM for 24 h. After washing three times with 5 ml of balanced salt solution buffer, cells were incubated at 37 °C for 20 min in balanced salt solution buffer. Incubation buffer was removed and replaced with 5 ml of buffer with or without 100 nM Ang II for the indicated times. The reaction was terminated by rapid aspiration of the buffer and addition of ice-cold PBS. Each plate was then treated with 0.5 ml of ice-cold lysis buffer and placed on ice for 30 min with occasional shaking. The solubilized cells were immunoprecipitated with Protein A/G Plus-agarose and anti-PLC-γ antibody (2 µg/mg) overnight at 4 °C. The immunoprecipitates were then recovered by centrifugation and washed five times with the lysis buffer. The immunoprecipitated proteins were dissolved in 50 µl of Laemmli buffer, boiled for 5 min, and separated by SDS-PAGE on 9% polyacrylamide gels and transferred to PVDF membranes. Blots were then probed with anti-phosphotyrosine monoclonal antibody (1:1000 dilution) and detected with HRP-conjugated goat anti-mouse secondary antibody (1:2000 dilution). The ECL Western blotting detection kit (Amersham Corp.) was used for detection.

Ang II Receptor Binding—The Ang II receptor binding assay was performed as described previously (13). A and B (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 value of <0.05 was considered to be statistically significant.

RESULTS

Phospholipase C Isozymes Expressed in Rat VSMCs—To identify the PLC isozymes expressed in rat aortic SMCs, immunoblot analysis was performed using antibodies raised against PLC-β1, -γ1, and -δ1. Anti-PLC-γ1 antibody reacted with a 145-kDa protein, consistent with the reported molecular mass for PLC-γ1 (Fig. 1A), and anti-PLC-β1 reacted with an 85-kDa protein of the reported molecular mass for PLC-β1 (Fig. 1C). In contrast to previous reports (12, 17), but in agreement with the findings of Schelling et al. (18, 22), an anti-PLC-β1 antibody identified a full-length 150-kDa protein as well as a 100-kDa fragment (Fig. 1B). Both bands detected with the anti-PLC-β1 antibody disappeared in the presence of control peptide used for antibody generation, indicating that this antibody specifically detects PLC-β1 (data not shown). Both the full-length 150-kDa protein and the 100-kDa fragment were detected using rat pituitary or rat-1 fibroblast homogenates as positive controls (Fig. 1B and data not shown). These results suggest that PLC-γ1, -δ1, and -β1 are expressed in rat VSMCs.

Effect of Electroporation of Antibodies against PLC Isozymes on Ang II-stimulated IP\(_3\) Formation—To determine which PLC isozymes are involved in AT\(_1\) receptor-PLC coupling, we measured IP\(_3\) production stimulated by Ang II at 15 s (peak response) in VSMCs electroporated with specific antibodies against PLC-β1, -γ1, and -δ1 (Fig. 2). 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 (21). IP\(_3\) production stimulated by Ang II in cells electroporated in the absence of antibody (mock electroporation) was increased by 119 ± 7% (n = 6), while that in cells without electroporation was increased by 126 ± 8% (n = 3). Cells electroporated with nonimmune rabbit IgG showed a decrease in the Ang II response (20%) compared with the cells with mock electroporation; therefore, the effects of specific antibodies were always compared with the response in the presence of rabbit IgG. When VSMCs were electroporated with anti-PLC-β1 antibody, there was no inhibition of Ang II-induced IP\(_3\) formation (3 ± 0.1% inhibition, n = 3). Electrophoration of anti-PLC-γ antibody showed a small and insignificant inhibitory effect (10 ± 5% inhibition, n = 4) at 15 s. In contrast, electrophoration of an antibody against PLC-β1 markedly inhibited IP\(_3\) production in response to Ang II (83 ± 5% inhibition, n = 3, p < 0.05). These data indicate a critical role for PLC-β1 in the initial AT\(_1\) receptor-PLC coupling in VSMCs.

Effect of Tyrosine Kinase Inhibition on Ang II-stimulated IP\(_3\) Formation—Judging from the time course that has been reported for PLC-γ phosphorylation by Ang II (12), we hypothesized that tyrosine kinase-dependent PLC-γ activation might be involved in IP\(_3\) generation during the later phases of the AT\(_1\) receptor signaling events in VSMCs. Therefore, we examined the effect of genistein, a tyrosine kinase inhibitor, on the time course of Ang II-induced IP\(_3\) formation. As shown in Fig. 3, genistein (100 µM) inhibited IP\(_3\) production only after 30 s of Ang II stimulation without affecting the peak response at 15 s, consistent with the inability of anti-PLC-γ antibody to inhibit maximum IP\(_3\) production during the initial phase of signaling. This genistein-induced inhibition was temporally correlated with the PLC-γ tyrosine phosphorylation in response to Ang II (Fig. 3, inset). PLC-γ tyrosine phosphorylation peaked at 30 s and returned to control levels by 10 min. Furthermore, Ang II-induced IP\(_3\) formation measured at 30 s was markedly inhibited by electrophoresis of anti-PLC-γ antibody (40 ± 6% inhibition).

![Fig. 1. Immunoblot analysis of phospholipase C isozyme expression in rat VSMCs.](image-url)
Fig. 2. Effect of electroporation of antibodies against PLC isozymes on Ang II-stimulated IP formation. myo-[3H]Inositol-labeled VSMCs were electroporated with anti-PLC-6 (Santa Cruz), PLC-γ1, or PLC-81 antibodies (5 μg/ml), and then stimulated with 100 nM Ang II for 15 s. Rabbit IgG was used as a negative control. Bars indicate the increase in IP production by Ang II in the presence or absence of the indicated antibodies, expressed as the percent increase in IP production over that in unstimulated cells. Values are the mean ± S.E. for three to six independent experiments performed in triplicate. ** p < 0.05 for the increase in IP production by Ang II in cells electroporated with rabbit IgG versus anti-PLC antibody.

Fig. 3. Effect of genistein on the time course of Ang II-stimulated IP3 formation. myo-[3H]Inositol-labeled VSMCs were treated with (closed circles) or without (open circles) genistein (100 μM) for 30 min, and then stimulated with 100 nM Ang II for the indicated times. The values indicate the increase in IP3 production by Ang II, expressed as the percent increase in IP3 production over that in unstimulated cells at each time point, and are the mean ± S.E. for three independent experiments performed in triplicate. ** p < 0.05 for IP3 production by Ang II in untreated cells versus cells treated with genistein. Inset shows the time course of PLC-γ tyrosine phosphorylation in response to Ang II (100 nM).

Effect of Electroporation of Antibodies against G Protein Subunits on Ang II-stimulated IP3 Formation—Since PLC-6 has been shown to be activated by Gα and Gβγ subunits of heterotrimeric G proteins (11), we next investigated the primary subunits involved in the early AT1 receptor-PLC-6 coupling. For this purpose, we measured IP3 production by Ang II at 15 s (peak response) in cells electroporated with specific antibodies against Gαq, Gαq11, Gα12, Gα13, and Gβ. As shown in Fig. 4, electroporation of anti-Gαq and Gα13 antibodies, as well as rabbit IgG (negative control), had no effect on Ang II-induced IP3 production. In contrast, anti-Gαq11 and Gα12 antibodies significantly inhibited the Ang II response (56 ± 4% inhibition, n = 5, p < 0.05 and 62 ± 5% inhibition, n = 7, p < 0.05, respectively). 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-αq12 + anti-αq12, Fig. 4). Furthermore, when anti-Gαq11 and anti-Gα12 were combined, their inhibitory effects were additive and nearly complete (93% inhibition, n = 3), suggesting that both Ga subunits can couple to the AT1 receptor. The partial involvement of Gαq11 in PLC coupling is consistent with our previous finding that in Gαq11 down-regulated cells, Ang II-stimulated IP3 formation is inhibited by 30% (4). The effectiveness of anti-Gαq11 and Gα12 antibodies was abolished when they were boiled (100 °C for 30 min) prior to electroporation, confirming that active antibody was required for the observed effect. Additionally, when VSMCs were electroporated with anti-Gα antibody, the Ang II response was also significantly inhibited (75 ± 6% inhibition, n = 6, p < 0.05), and this inhibition was reversed by boiling the antibody. Thus, these results suggest that early AT1 receptor-PLC coupling is mediated by the Gαq11βγ and Gα12βγ complex of heterotrimeric G proteins, and that Gβγ, as well as the Ga subunits, may serve as an active molecule for transducing the AT1 receptor signal to PLC.

Effect of Overexpression of βARK1ct on Ang II-stimulated IP3 Formation—To further assess the role of Gβγ in the early phase of AT1 receptor-PLC coupling, we stably transfected the plasmid encoding the βARK1ct (effectively a Gβγ antagonist) (19) into cultured rat VSMCs. Control cells were transfected with vector only. The efficacy of βARK1ct cDNA transfection was evaluated and confirmed by Northern analysis (Fig. 5, left panel). As shown in Fig. 5, IP3 production by 100 nM Ang II at 15 s was significantly inhibited in cells stably overexpressing βARK1ct compared with that in 2 different cell lines transfected with vector alone (average inhibition 43 ± 4%, n = 3). We verified by measuring equilibrium binding of β3H-Ang II that AT1 receptor expression was not different in vector- and in βARK1ct-overexpressing cells (data not shown). Thus, these results strongly suggest that Gβγ acts as a signal transducer for PLC activation.

DISCUSSION

The mechanisms by which AT1 receptors activate PLC in rat VSMCs are controversial, because the expression of PLC-β1 protein has not been consistently detected (12, 17), and both direct coupling to G proteins (4, 13) and downstream tyrosine kinase-dependent activation mechanisms (12) have been reported. Here, we provide evidence that PLC-β1 and PLC-γ are both functionally expressed in rat VSMCs and demonstrate that they may be sequentially activated by Ang II. Their mechan-isms of activation are quite different: PLC-β1 coupling to the AT1 receptor appears to be mediated by Gαq11βγ and Gα12βγ heterotrimeric G proteins, while PLC-γ activation is dependent on a downstream tyrosine kinase. It appears that the active subunit transducing PLC-β1 stimulation may include Gβγ derived from a nonpertussis toxin-sensitive G protein α-subunit.

In the present study, we identified a full-length (150 kDa) PLC-β1 as well as a 100-kDa fragment in rat VSMCs using an antibody raised against the carboxyl terminus of rat PLC-β1 (Santa Cruz) (Fig. 1). These two major bands of PLC-β1 were also detected by a different mouse monoclonal antibody raised against the amino terminus of PLC-β1 of rat origin (Transducion Laboratories) (data not shown), and disappeared when the antibody was preincubated with the control peptide used for antibody generation, suggesting that both bands represent PLC-β1 isoforms or fragments. Schelling et al. (22) have previously detected the 100-kDa PLC-β1 fragment in rat aortic
FIG. 4. Effect of electroporation of antibodies against specific Go and Gβ proteins on Ang II-stimulated IP₃ formation. myo-[³H]inositol-labeled VSMCs were electroporated with anti-Gα, Gαq/11, or Gα₁₃ and Gβ antibodies (5 μg/ml), and then stimulated with 100 nM Ang II for 15 s. Ang II-induced IP₃ formation in mock electroporated cells was 94% of that in cells not exposed to electroporation. Bars indicate the increase in IP₃ production by Ang II in the presence or absence of the indicated antibodies, expressed as the percent increase in IP₃ production over that in unstimulated, electroporated cells. Values are the mean ± S.E. for three to 11 independent experiments performed in triplicate. *, p < 0.05; **, p < 0.01 for the increase in IP₃ production by Ang II in cells electroporated with rabbit IgG - versus anti-G protein antibody.

FIG. 5. Effect of overexpression of the carboxyl terminus of βARK1 on Ang II-stimulated IP₃ formation. A, Northern blot analysis of expression of βARK1 carboxyl terminus (βARK1ct) mRNA levels in VSMCs stably transfected with plasmid DNA encoding βARK1ct. 10 μg of total RNA was used in each lane. The βARK1ct cDNA derived from EcoRI/XbaI digestion with pRK-βARK1ct DNA (19) was used as a probe. The upper panel shows a representative autoradiogram of βARK1ct mRNA levels in two of the vector-transfected clones (clone 1 and clone 2) and one of the selected βARK1ct-transfected clones. The size of the βARK1ct mRNA band is 880 base pairs. The lower panel shows the 28 S ribosomal RNA band (detected by ethidium bromide fluorescence of the membrane). B, Ang II-induced IP₃ formation in vector-transfected cells (clone 1 and clone 2) and βARK1ct-overexpressing cells. myo-[³H]inositol-labeled VSMCs were stimulated with 100 nM Ang II for 15 s. Bars indicate the increase in IP₃ production by Ang II, expressed as the percent increase in IP₃ production over that in unstimulated cells. Values are the mean ± S.E. for three independent experiments performed in triplicate. *, p < 0.05 for IP₃ production by Ang II in βARK1ct-overexpressing cells versus vector-transfected cells.

VSMCs using the same rabbit polyclonal PLC-β1 antibody. Thus, the 100-kDa band may represent a PLC proteolytic fragment (22) or a truncated PLC-β1 isomeric reported in other species (23, 24). The discrepancies regarding PLC-β1 detection between our results and those of others (12, 17) may be due to variations in VSMC phenotype or differences in the antibodies or the cell extraction methods used for immuno detection.

We also provided direct evidence that PLC-β1 plays an important role in the early phase of AT₁ receptor-PLC coupling in rat VSMCs, because electroporation of specific anti-PLC-β1 antibody markedly inhibited the maximum IP₃ generation observed at 15 s after Ang II stimulation (Fig. 2). Consistent with our results, Schelling et al. (18) have recently demonstrated that introduction of PLC-β1 antibody inhibits Ang II-stimulated inositol phosphate production in β-escin-permeabilized human aortic VSMCs. We found that a tyrosine kinase is involved in the later phase of AT₁ receptor-PLC coupling, because the tyrosine kinase inhibitor genistein attenuated only the later phase (≥30 s) of Ang II-stimulated IP₃ formation (Fig. 3). This result is consistent with the reports by Marrero et al. (12) who showed inhibition of Ang II-induced IP₃ formation by genistein, but the earliest time point measured was 30 s. The genistein-induced inhibition of IP₃ production is temporally correlated with increased tyrosine phosphorylation of PLC-γ in response to Ang II (Fig. 3)(12). Furthermore, the later phase of IP₃ production (at 30 s) was markedly inhibited by electroporation of anti-PLC-γ antibody, indicating that AT₁ receptors couple to PLC-γ in the later phase of PLC activation. However, a partial involvement of PLC-β1 in the later phase cannot be ruled out, because the inhibition by genistein was incomplete and anti-PLC-β1 antibody also inhibited the Ang II-induced IP₃ production at 30 s by 38% (data not shown). In contrast, Schelling et al. (18) failed to detect inhibition of the Ang II-induced PLC activation by a tyrosine kinase inhibitor in human VSMCs. This discrepancy may be due to the fact that they measured total inositol phosphate accumulation during 10 min of Ang II stimulation as PLC activation, which may mostly reflect the peak PLC-β1 mediated response and mask the con-
of IP3 in intact VSMCs. Our present result extends the original concept that Gβγ transduces the signal for pertussis toxin-sensitive pathways (28). Gβγ has recently been shown to mediate PTX-insensitive activation of PLC in Xenopus oocytes (29), and to be involved in c-Jun kinase activation by Gαq-coupled m1 muscarinic receptors in COS-7 cells (30). We recently found that the Gβγ derived from G12/13 transduces AT1 receptor-mediated tonic phospholipase D activation in rat VSMCs. Thus, Gβγ appears to be a common signal transducer in both PTX-sensitive and -insensitive signaling pathways in certain cell types.

In summary, the present study demonstrates that the early phase of Ang II-induced PLC activation occurs through coupling to PLC-β via Gαq/11 and Gα12 as well as their associated Gβγ subunits, and the later phase involves a downstream tyrosine kinase, presumably via phosphorylation of PLC-γ. The sequential coupling of AT1 receptors to PLC-β and PLC-γ suggests a novel mechanism for a temporally controlled, highly organized and convergent Ang II-signaling network in VSMCs.

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REFERENCES

1. Griendling, K. K., and Alexander, R. W. (1994) in Textbook of Hypertension (Swales, J. D., ed) pp 244–253, Blackwell Scientific, Oxford

2. Sasaki, K., Yamano, Y., Bardhan, S., Iwai, N., Murray, J. J., Hasegawa, M., Matsuda, Y., and Inagami, T. (1991) Nature 351, 250–253

3. Murphy, T. J., Alexander, R. W., Griendling, K. K., Rengev, M. S., and Bernstein, K. E. (1991) Nature 351, 233–236

4. Kai, H., Fukui, T., Lassègue, B., Shah, A., Minieri, C. A., and Griendling, K. K. (1996) Mol. Pharmacol. 49, 96–104

5. Okuda, K., Kawahara, Y., and Yokoyama, M. (1996) Am. J. Physiol. 271, H595–H601

6. Macrez-Lепrêtre, N., Kalkbrenner, F., Morel, J. L., Schultz, G., and Mironneau, J. (1997) J. Biol. Chem. 272, 10905–10102

7. Macrez, N., Morel, J.-L., Kalkbrenner, F., Viard, P., Schultz, G., and Mironneau, J. (1997) J. Biol. Chem. 272, 21380–21385

8. Post, G. R., Collins, L. B., Kennedy, E. D., Moskowitz, S. A., Aragoy, A. M., Goldstein, D., and Brown, J. H. (1996) Mol. Cell. Biol. 17, 1679–90

9. Griendling, K. K., Tsuda, T., Berk, B. C., and Alexander, R. W. (1989) J. Cardiovasc. Pharmacol. 14, S27–S33

10. Alexander, R. W., Brock, T. A., Gimbrone, M. A., Jr., and Rittenhouse, S. E. (1985) Hypertension 7, 447–451

11. Rhee, S. G., and Bae, Y. S. (1997) J. Biol. Chem. 272, 15045–15048

12. Marrero, M. B., Paxton, W. J., Berk, B. C., and Bernstein, K. E. (1994) J. Biol. Chem. 269, 10935–10939

13. Soccoro, L., Alexander, R. W., and Griendling, K. K. (1990) Biochem. J. 265, 799–807

14. Timmermans, P. B. M. W., Wang, P. C., Chiu, A. T., Herin, B. W., Benfield, P., Carini, D. J., Lee, R. J., Weerse, R. E., Slaye, J. A. M., and Smith, R. D. (1993) Pharmacol. Rev. 45, 205–251

15. Koyama, Y., Sakamoto, H., Tsunoda, M., Aoki, M., Takenawa, T., and Ooyama, H. (1991) J. Biol. Chem. 266, 2895–2900

16. Griendling, K. K., Taubman, M. B., Akers, M., Mendlowitz, M., and Alexander, R. W. (1991) Am. J. Physiol. 260, C1558–C1566

17. Koch, W. J., Hawes, B. E., Allen, L. F., and Lefkowitz, R. J. (1994) Mol. Pharmacol. 46, 205–212

18. Schelling, J. R., Gentry, D. J., and Dubyak, G. R. (1994) J. Biol. Chem. 269, 23180–23185

19. Schelling, J. R., Nkemere, N., Konieczkowski, M., Martin, K. A., and Dubyak, G. R. (1997) Am. J. Physiol. 273, C1555–C1566

20. Koch, W. J., Hawes, B. E., Inagami, T., and Lefkowitz, R. J. (1994) J. Biol. Chem. 269, 23180–23185

21. Schelling, J. R., Gentry, D. J., and Dubyak, G. R. (1994) Am. J. Physiol. 267, F682–F690

22. Xu, X., Chen, F., and Hokin, L. E. (1994) J. Biol. Chem. 269, 12925–12931

23. Bahk, Y. Y., Lee, Y. H., Lee, T. G., Seo, J., Ryu, S. H., and Suh, P. G. (1994) J. Biol. Chem. 269, 23180–23185

24. Schelling, J. R., Nkemere, N., Konieczkowski, M., Martin, K. A., and Dubyak, G. R. (1997) Am. J. Physiol. 273, C1558–C1566

25. Koch, W. J., Hawes, B. E., Inagami, T., and Lefkowitz, R. J. (1994) J. Biol. Chem. 269, 6193–6197

26. Dhanasekaran, N., and Kalkbrenner, F. (1996) J. Biol. Chem. 271, 28418–28424

27. Zhang, B. X., and Muallem, S. (1992) J. Biol. Chem. 267, 6714–6720

28. Schelling, J. R., Nkemere, N., Konieczkowski, M., Martin, K. A., and Dubyak, G. R. (1997) Am. J. Physiol. 273, C1558–C1566

29. Koch, W. J., Hawes, B. E., Inagami, T., and Lefkowitz, R. J. (1994) J. Biol. Chem. 269, 6193–6197

30. Soccoro, L., Alexander, R. W., and Griendling, K. K. (1990) Biochem. J. 266, 20519–20524

31. Homma, Y., Sakamote, H., Tsumada, M., Aoki, M., Takenawa, T., and Ooyama, H. (1991) Biochem. J. 290, 649–653

32. Schelling, J. R., Nkemere, N., Konieczkowski, M., Martin, K. A., and Dubyak, G. R. (1997) Am. J. Physiol. 273, C1558–C1566

33. Koch, W. J., Hawes, B. E., Inagami, T., Luttrell, L. M., and Lefkowitz, R. J. (1994) J. Biol. Chem. 269, 6193–6197

34. Griendling, K. K., Taubman, M. B., Akers, M., Mendowitz, M., and Alexander, R. W. (1991) J. Biol. Chem. 266, 15498–15504

35. Marrero, M. B., Schiefber, B., Paxton, W. G., Schiefber, E., and Bernstein, K. E. (1995) J. Biol. Chem. 270, 15734–15738

36. Schelling, J. R., Gentry, D. J., and Dubyak, G. R. (1996) Am. J. Physiol. 270, F682–F690