0026-895X/96/010096-09$3.00/0

MOLECULAR PHARMACOLOGY, 49:96–104 (1996).

Prolonged Exposure to Agonist Results in a Reduction in the Levels of the G_q/G_{11} α Subunits in Cultured Vascular Smooth Muscle Cells

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Received February 22, 1995; Accepted September 25, 1995

SUMMARY

Recent studies have shown that G proteins are a potential regulatory site in the transmembrane signaling cascade. The aim of this study was to examine the effects of prolonged agonist exposure on expression of the G_q class of G protein α subunits ($G_{\alpha q}/G_{\alpha 11}$) in cultured rat vascular smooth muscle cells (VSMC). Treatment with 100 nM angiotensin II (Ang II) led to a substantial sustained down-regulation of cellular levels of immunologically detectable $G_{\alpha q}/G_{\alpha 11}$ by 50% within 6 hr. The effect of Ang II was dose dependent with an EC_{50} of 2 nM and was specifically blocked by the vascular type-1 Ang II receptor-specific antagonist losartan. The Ang II-induced reduction in cellular levels of G protein α subunits was specific for $G_{\alpha q}/G_{\alpha 11}$. The calcium ionophore ionomycin or activators of ubiquitous protein kinases (phorbol-12-myristate-13-acetate, forskolin, and 8-bromo-cGMP) did not mimic the effects of Ang II. However, [Arg^8]vasopressin also induced a significant loss in cellular $G_{\alpha q}/G_{\alpha 11}$ levels. Ang II-induced $G_{\alpha q}/G_{\alpha 11}$ down-regulation was reversed by prevention of cellular receptor processing with phenylarsine oxide or chronic potassium depletion. The effects of Ang II on $G_{\alpha q}/G_{\alpha 11}$ levels were inhibited when protein kinase C activity was abolished. $G_{\alpha q}$ mRNA levels were down-regulated by 30% after 4-hr incubation with Ang II, in part by transcriptional regulation. Although a short term vasopressin pretreatment had no effect on inositol-1,4,5-trisphosphate (IP_3) generation in response to subsequent Ang II stimulation, a partial heterologous desensitization of the IP_3 response was induced after a long term vasopressin pretreatment, which concurrently down-regulated cellular $G_{\alpha q}/G_{\alpha 11}$ levels. Homologous desensitization of IP_3 generation on a second Ang II stimulation was observed after both a short and long term Ang II pretreatment. In conclusion, prolonged exposure to Ang II induces down-regulation of cellular $G_{\alpha q}/G_{\alpha 11}$ levels in intact VSMC. The effect of Ang II appears to be mediated by the signaling pathway sensitive to inhibition of receptor processing. The present study raises the possibility that agonist-induced $G_{\alpha q}/G_{\alpha 11}$ down-regulation participates in the mechanism of long term desensitization of the $G_{\alpha q}/G_{\alpha 11}$-mediated signaling system in VSMC.

A large family of heterotrimeric G proteins mediates signal transduction between the transmembrane receptors and the effector systems involved in the generation of intracellular second messengers (1). Many hormones and neurotransmitters apparently regulate phosphatidylinositol-specific PLC via a G protein-mediated pathway, resulting in elevation of intracellular levels of DG and IP_3 with consequent activation of PKC and mobilization of intracellular calcium (2). Recent studies suggest that at least two separate mechanisms are involved in the agonist regulation of PLC activity (1). In some systems where the receptor interacts with a pertussis toxin-sensitive G protein, $\beta\gamma$ subunits liberated from the activated G protein activate PLC-β_2 and/or -β_3 (3). More commonly, the receptor interacts with the pertussis toxin-insensitive G_q class of G proteins, and it is the α subunits that activate PLC-β1 (4–6). Functional intervention with antibodies against the carboxyl termini of G_q and G_{11} α subunits ($G_{\alpha q}/G_{\alpha 11}$) have indicated that this latter mechanism is used by Ang II, vasopressin, bradykinin, histamine, and thromboxane A_2 (7, 8).

Cellular responses to agonists wane rapidly even when these agonists are continuously present. This phenomenon, termed desensitization, has been observed in a variety of G protein/effector systems (9, 10). The desensitization appears to be biphasic in several systems. Short term (rapid) desen-

This work was supported by National Institutes of Health Grants HL47557 and HL38206.

ABBREVIATIONS: VSMC, vascular smooth muscle cells; Ang II, angiotensin II; $AT_{1A}R$, vascular type-1A angiotensin II receptor; m1CR, muscarinic m_1 cholinergic receptor; PLC, phospholipase C; DG, 1,2-diacylglycerol; IP_3, inositol-1,4,5-trisphosphate; PK, protein kinase; PMA, phorbol-12-myristate-13-acetate; PDBu, phorbol-12,13-dibutyrate; PAO, phenylarsine oxide; PBS, phosphate-buffered saline solution; CHO, Chinese hamster ovary; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSS, HEPES-buffered saline solution; SSC, sodium chloride/sodium citrate buffer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

sitization involves functional uncoupling of the receptors from the G protein/effector systems, which diminishes the effectiveness with which stimulated receptors are able to activate G proteins and therefore their effectors. The change in the efficiency of receptor coupling to the G protein/effector seems to occur in seconds to minutes and does not correlate with a change in the expression of proteins involved in the signaling pathway. In contrast, long term desensitization involves agonist-induced down-regulation, a decrease in the levels of the signal transduction pathway proteins. Many studies have demonstrated that the receptor is a predominant site of regulation of desensitization of G protein-mediated signal transduction systems (9, 10).

Recently, however, there has been increasing evidence that such regulation may also exist at postreceptor sites, most notably at the level of G proteins. Agonist-induced reduction of cellular G protein levels has been observed in several G protein families. Prolonged exposure to agonists for prostanoid PGI_2 (IP) or adenosine A_2 receptors, which activate adenylate cyclase, induces a substantial reduction, by 50–70% in cellular levels of $G_{\alpha s}$ in NG108–15 cells (11–13). It has also been demonstrated that chronic exposure to agonists for prostanoid PGE_1 (EP) or adenosine A_1 receptors, which are negatively linked to adenylate cyclase via the pertussis toxin-sensitive G_i family of G proteins, results in down-regulation of $G_{\alpha i\text{-}1}$, $G_{\alpha i\text{-}2}$ and $G_{\alpha i\text{-}3}$ by 50–90% in cultured rat adipocytes (14, 15). Furthermore, evidence for agonist-induced down-regulation of $G_{\alpha q}/G_{\alpha 11}$ levels has been recently presented in CHO cells transfected to express human m1CR (16). These studies suggest that agonist-induced down-regulation of G protein levels is generally restricted to the G protein with which the receptor interacts and that down-regulation of the G protein occurs concurrent with down-regulation of the receptor to which the G protein is coupled (17). However, the functional implications of the agonist-induced down-regulation of these G proteins and the mechanisms responsible for the reduction in protein levels are not yet known in each G protein-mediated signal transduction system.

We have recently shown that in cultured rat VSMC, prolonged exposure of Ang II induces down-regulation of the $AT_{1A}R$ mRNA and protein (18). Because coordinate regulation of receptors and their coupled G proteins has been observed in other systems, we investigated the ability of Ang II to regulate G proteins in these cells. At present there are no available data focusing on the regulation of cellular levels of the G_q class of G proteins in VSMC by calcium-mobilizing agonists, including Ang II and vasopressin. The aims of the present study were thus, first, to examine whether prolonged exposure to Ang II alters cellular $G_{\alpha q}/G_{\alpha 11}$ levels in intact VSMC and, second, if so, to assess the underlying mechanisms and the functional implications of the Ang II-induced reduction in $G_{\alpha q}/G_{\alpha 11}$ levels. We found agonist-induced sustained down-regulation of immunologically detectable $G_{\alpha q}/G_{\alpha 11}$ levels, with an accompanying smaller decrease in $G_{\alpha q}$ mRNA levels. It appears that receptor processing initiated by agonist/receptor interaction is required for Ang II-induced $G_{\alpha q}/G_{\alpha 11}$ down-regulation. Activation of PKC may also be involved, since the effects of Ang II on $G_{\alpha q}/G_{\alpha 11}$ levels were reversed by down-regulation or inhibition of PKC activity. These observations suggest that regulation of the coupling G protein levels may provide an additional level of control of long term signal transduction in cultured VSMC.

Experimental Procedures

Materials. Ang II, ionomycin, PMA, forskolin, 8-bromo-cGMP, PDBu, PAO, sodium orthovanadate, and [Arg^8]vasopressin were purchased from Sigma Chemical Co. (St. Louis, MO). GF-109203X was obtained from LC Laboratories (Woburn, MA). Losartan was a kind gift from Dr. R. D. Smith (DuPont de Nemours Co., Wilmington, DE). Anti-$G_{\alpha q}/G_{\alpha 11}$ polyclonal antibody was purchased from New England Nuclear Research Products (Cambridge, MA). This antibody was developed against the carboxyl-terminal peptide common to $G_{\alpha q}$ and $G_{\alpha 11}$ and has no cross-reactivity for other G protein α subunits. Polyclonal antibody specific for $G_{\alpha q}$ was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-$G_{\alpha i\text{-}1/2}$, anti-$G_{\alpha o}/G_{\alpha i\text{-}3}$ and anti-$G_{\alpha s}$ polyclonal antibodies were purchased from Calbiochem Co. (La Jolla, CA). The full-length cDNA for mouse $G_{\alpha q}$ was generously provided by Dr. J. J. Murtagh, Atlanta Veterans Administration Medical Center, and the GAPDH cDNA was a gift from Dr. Gadiparthi N. Rao. Other sources for materials were: *myo*-2-[^{3}H]inositol and [α-^{32}P]UTP, DuPont-NEN (Boston, MA); [^{32}P]dCTP, Amersham (Arlington Heights, IL); Dulbecco's modified Eagle's medium, Sigma (St. Louis, MO); calf serum, GIBCO Laboratories (Chagrin Falls, OH); Immobilon-P, Millipore (Bedford, MA); ECL Western blot analysis reagent system, Amersham (Arlington Heights, IL); Magna NT nylon hybridization membranes, Micron Separations Inc. (Westboro, MA); AG-1-X8 anion exchange resin and Biospin P30, Bio-Rad Laboratories (New York, NY); TRI reagent, Molecular Research Center (Cincinnati, OH); Prime-It II kit, Stratagene (La Jolla, CA); Denhardt's solution, 5′3′, Inc. (West Chester, PA); human β-actin clone, American Type Culture Collection (Rockville, MD); and nucleotides, Boehringer Mannheim (Indianapolis, IN). All other chemicals were of molecular biological grade or the highest grade commercially available. Vanadyl hydroperoxide was freshly prepared just before each experiment according to the method of Bourgoin *et al.* (19). The composition of PBS was 100 mM NaCl, 80 mM Na_2HPO_3, and 20 m NaH_2PO_3, pH 7.4. BSS contained 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM glucose, and 20 mM HEPES, buffered to pH 7.4 with Tris base.

Culture of VSMC. Primary cultures of VSMC were obtained by enzymatic dissociation of aortic medial tissue from male Sprague-Dawley rats, as described previously (20). Cells were passaged in Dulbecco's modified Eagle's medium containing 10% calf serum and antibiotics, as described elsewhere (20). For experiments, VSMC from passages 5–16 were seeded onto 35- or 60-mm dishes, fed every other day, and used on reaching confluence. Chronic potassium depletion was achieved by exposing VSMC to standard medium with 10% calf serum but without additional potassium for 24 hr before the experiment (final K^+ concentration of medium, 0.3–0.84 mM) (21).

Cell preparation. Ang II or other agents were added to culture media (37°) for the final period of incubation as indicated. High cell viability (>95%) was maintained throughout all the experiments, as assessed by the Trypan blue exclusion test. The stimulation was terminated by aspirating the medium and by washing three times with ice-cold PBS. VSMC were dissolved in 400 μl of ice-cold lysis buffer (50 mM HEPES, pH 7.5, 1% Triton X-100, 50 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 10 μg/ml aprotinin) by several passages through a 27-gauge needle with syringe, and the whole-cell lysate was placed on ice for 30 min. The lysate was centrifuged at 6000 × g for 20 min at 4° to remove insoluble materials, and the supernatant was collected into prechilled tubes. Cellular proteins were precipitated by adding the same volume of ice-cold 10% trichloroacetic acid (final concentration, 5%) and incubating on ice for 1 hr. After washing three times with ice-cold PBS, proteins were dissolved in Laemmli sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol).

In some experiments, crude membrane preparations were obtained from VSMC as follows (22). The cells were harvested in ice-cold PBS and were centrifuged at 500 × g for 5 min (4°). The pellet was resuspended and then dounced in ice-cold hypotonic solu-

tion containing 5 mM Tris, pH 8.0, 1 mM $MgCl_2$, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride as well as 2 μg/ml leupeptin and 10 μg/ml aprotinin. The cell homogenate was centrifuged at 500 × g for 10 min at 4° to remove nuclei and unbroken cells. The membrane fraction was then collected by centrifugation at 20,000 × g for 30 min at 4° and resuspended in the hypotonic solution. The final supernatant was saved as the cytosolic fraction. Membrane and cytosolic proteins were precipitated with trichloroacetic acid and dissolved in Laemmli sample buffer, as described above.

Immunoblotting. Sample proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis consisting of 12% acrylamide. Separated proteins were electrophoretically transferred to Immobilon-P. Immunoblotting was performed according to the method of Molloy *et al.* (23). Anti-$G_{\alpha q}/G_{\alpha 11}$, anti-$G_{\alpha q}$, anti-$G_{\alpha i\text{-}1/2}$, anti-$G_{\alpha i\text{-}3}$, anti-$G_{\alpha o}/G_{\alpha i\text{-}3}$, or anti-$G_{\alpha s}$ rabbit polyclonal antibody was used for the primary antibody (1:1000 dilution). The immunoreactive bands were visualized with horseradish peroxidase-conjugated donkey anti-rabbit IgG and the ECL Western blot analysis reagent system. Bands obtained from the chemiluminescence films were quantified by laser densitometry. In control experiments, immunoblotting of increasing amounts of VSMC protein with anti-$G_{\alpha q}/G_{\alpha 11}$ antibody showed a linear increase in immunoreactive signals when cellular protein was between 0.5 and 15 μg/lane. Based on this result, 5 μg of cellular protein was applied routinely to each gel lane for all experiments with anti-$G_{\alpha q}/G_{\alpha 11}$ antibody. Similarly, linearity was ensured when the protein amount applied to each lane was 25 μg, 10 μg, 15 μg, or 20 μg for immunoblotting with anti-$G_{\alpha q}$, anti-$G_{\alpha i\text{-}3}$, anti-$G_{\alpha o}/G_{\alpha i\text{-}3}$, or anti-$G_{\alpha s}$ antibody, respectively. Anti-$G_{\alpha i\text{-}1/2}$ antibody did not react with cellular protein from VSMC (up to 100 μg/lane).

RNA purification and Northern blotting. RNA was isolated using the one-step TRI reagent method. After VSMC were lysed with TRI reagent, lysates were extracted with chloroform followed by isopropanol precipitation and then extracted once with buffered phenol chloroform and precipitated with ethanol. Final total RNA concentration and purity were assessed by measurement of the absorbances at 260 and 280 nm. Samples were stored at −80° until use. Northern blotting was performed as described elsewhere (18). Briefly, 10 μg total RNA samples were lyophilized and denatured in gel loading buffer containing 0.4 mg/ml ethidium bromide and separated by electrophoresis on 1% denaturing formaldehyde agarose minigels. RNA was transferred overnight to Magna NT nylon hybridization membranes. Consistency of RNA loading among samples was assessed by densitometric measurement of RNA fluorescence after transfer. After immobilization by UV cross-linking, blots were prehybridized at least for 4 hr at 42° in the following solution: 1 M NaCl, 50 mM Tris, pH 7.4, 5 × Denhardt's solution, 50% formamide, 0.5% SDS, and 100 μg/ml sheared and denatured salmon sperm DNA. Overnight hybridization was carried out with denatured ^{32}P-labeled probe under identical conditions, except for the omission of the Denhardt's solution. The blots were rinsed in 1× SSC and washed three times for 60 min at 52° in 0.5× SSC with 0.1% SDS. The membranes were then subjected to autoradiography for 24–48 hr at −80°. The results were quantified by laser densitometry. The cDNA probe for $G_{\alpha q}$ was prepared by random priming labeling of 12.5 ng cDNA/miniblot with 25 μCi [^{32}P]dCTP using the Prime-It II kit.

Nuclear run-on assay. This assay was a modification of the method of Groudine *et al.* (24). VSMC were harvested with trypsin-EDTA and washed with culture medium with serum and with the following buffer: 150 mM KCl, 4 mM Mg acetate, and mM 10 Tris·HCl, pH 7.4. Cells were pelleted at 4° and lysed for 10 min on ice in the same buffer with 0.5% Nonidet P-40. Lysates were layered over buffer containing 0.6 M sucrose, and nuclei were harvested by centrifugation at 1000 × g for 10 min at 4°. Nuclear pellets were resuspended in 40% glycerol, 50 mM Tris, 5 mM $MgCl_2$, and 0.1 mM EDTA and stored at −80°. Transcription reactions were carried out at 30° for 30 min with 5–10 × 10^6 nuclei in the following buffer: 151 mM KCl; 36 mM Tris; 6 mM $MgCl_2$; 4 mM dithiothreitol; 0.5 mM of ATP, CTP, and GTP; 100–200 μCi (0.2–0.3 μM) [α-^{32}P]UTP (>3,000 μCi/mmol); and 25% glycerol. The TRI reagent LS was used to terminate the reaction and purify the transcripts. Unincorporated label was removed by filtration on a Biospin P30 column. Labeled nuclear transcripts were partially hydrolyzed for 10 min on ice in the presence of 0.2 M NaOH, neutralized with 0.24 M HEPES, ethanol precipitated, and dissolved in hybridization buffer.

DNA slot blots were prepared with 2.5 μg/slot of each of the following cDNAs: mouse $G_{q\alpha}$, human β-actin, and rat GAPDH. The mouse $G_{q\alpha}$ cDNA was a 1.5-kb insert containing the entire coding region (GeneBank M55412), excised from its cloning vector by restriction enzyme digestion, and gel purified. The human β-actin cDNA was a 908-bp PCR product from the coding region of the HFBCC49 clone (American Type Culture Collection) amplified with the following sense and antisense primers: 5′-GGCGACGAGGCCCAGAGCAAGAGAG-3′ and 5′-GCCGGACTCGTCATACTCCTGCTTGCTGA-3′. The rat GAPDH cDNA was a 599-bp PCR product from the coding region (GeneBank X02231) amplified with the following primers: 5′-AATGGGGTGATGCTGGTGCTGAGTA-3′ and 5′-GGAAGAATGGGAGTTGCTGTTG AAG-3′. cDNAs were denatured, blotted on nylon membrane, and immobilized by UV cross-linking.

The blots were prehybridized at least 2 hr at 65° in 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, 0.2% SDS, 10 mM EDTA, 0.3 M NaCl, 250 μg/ml *Escherichia coli* tRNA, and 1× Denhardt's solution. Hybridization was carried out with 5 × 10^6 to 2 × 10^7 cpm labeled RNA transcripts for 36 hr at 65° in the same buffer without Denhardt's solution. The blots were washed in 2× SSC and 0.1% SDS, once for 10 min at room temperature and twice for 1 hr at 60°. After a 3-day autoradiography, results were quantified by laser densitometry.

Inositol phosphate assay. Cellular IP_3 content was measured as previously described (25). Briefly, VSMC on 35-mm dishes were labeled in culture medium with 15 μCi/ml *myo*-2-[^{3}H]inositol for 48 hr at 37°. In the short term pretreatment study, the labeled cells were washed three times with BSS and incubated in BSS (37°) for 20 min. Ang II (100 nM), vasopressin (100 nM), or vehicle (bovine serum albumin) was added to the cells for 10 min. After quickly washing five times with BSS, cells were then exposed to Ang II (100 nM) or vehicle in BSS for 15 sec. The reactions were terminated by aspirating the buffer and by immediately adding 1 ml of chloroform/methanol/HCl (1:2:0.05). In the long term pretreatment study, Ang II (100 nM), vasopressin (100 nM), or vehicle was added to the labeling medium 6 hr before the start of the experiment. After five BSS washes, VSMC were incubated in BSS for 20 min. Cells were then exposed to 100 nM Ang II or vehicle for 15 sec. The reactions were terminated as described above. The chloroform/methanol/HCl extract plus a 0.5-ml rinse were transferred to tubes, and organic and aqueous phases were separated as previously described (25). After two chloroform washes, the aqueous phase was analyzed by column chromatography on AG-1-X8 anion exchange resin. Inositol phosphates were eluted as described previously, and radioactivity in each fraction was counted using liquid scintillation spectroscopy. In control experiments, there was no significant difference in the cellular IP_3 contents after a 48- or 54-hr labeling period.

Statistical analysis. Data are expressed as mean ± standard error. Analysis of variance followed by multiple comparison tests was used for comparisons of initial data before expression as percent control. A value of $p < 0.05$ was considered statistically significant.

Results

In whole-cell preparations of VSMC subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, anti-$G_{\alpha q}/G_{\alpha 11}$ antibody identified a single band of molecular mass of 42 kDa (Fig. 1A). When VSMC were treated with 100 nM Ang II for 6 hr, a marked decrease in the levels of immu-

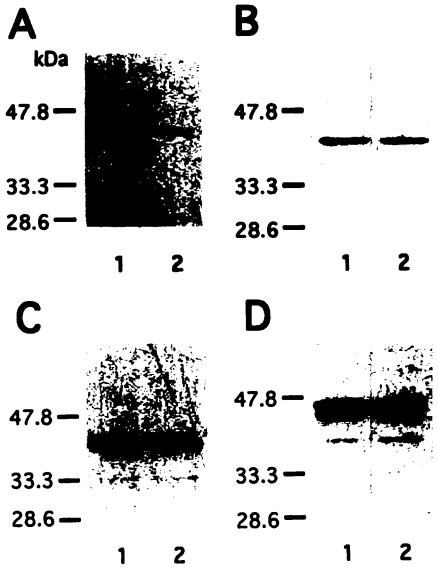

Fig. 1. Immunoblotting of G protein α subunits in VSMC. Representative immunoblots with anti-$G_{\alpha q}/G_{\alpha 11}$ (A), anti-$G_{\alpha i\text{-}3}$ (B), anti-$G_{\alpha o}/G_{\alpha i\text{-}3}$ (C), and anti-$G_{\alpha s}$ (D) antibodies using whole-cell lysates from untreated (*lane 1*) and Ang II (100 nM for 6 hr)-treated (*lane 2*) VSMC.

noreactive $G_{\alpha q}/G_{\alpha 11}$ was observed compared with untreated control cells (49.6 ± 11.2% control, four experiments; $p < 0.01$). Immunoblotting using a membrane preparation demonstrated a similar decrease in $G_{\alpha q}/G_{\alpha 11}$ levels on Ang II treatment (40.9 ± 9.8% control, three experiments; $p < 0.01$). No significant signals were obtained by immunoblotting the cytosolic protein sample from either Ang II-treated or untreated VSMC (up to 50 μg protein/lane). Therefore, it is unlikely that the Ang II-induced decrease in membrane $G_{\alpha q}/G_{\alpha 11}$ is due to the transfer of the G proteins to cytoplasm. Based on these findings, whole-cell lysates were used for immunoblotting in all subsequent experiments. Pretreatment with the $AT_{1A}R$-specific antagonist losartan (1 μM for 15 min) completely inhibited the Ang II-induced decrease in cellular $G_{\alpha q}/G_{\alpha 11}$ without altering the base-line level (losartan, 104.6 ± 11.3%; losartan plus Ang II, 93.4 ± 7.2% control; four experiments), indicating that the effects of Ang II are a consequence of Ang II/$AT_{1A}R$ interaction.

Immunoblotting of Ang II-treated and untreated VSMC was also carried out with antibodies for other G protein α subunits (Fig. 1, B–D). Immunoreactive bands for anti-$G_{\alpha i\text{-}3}$, anti-$G_{\alpha o}/G_{\alpha i\text{-}3}$, and anti-$G_{\alpha s}$ antibodies were not changed by a 6-hr treatment with Ang II (Table 1). Thus, it appears that the Ang II-induced reduction in cellular G protein α subunits is specific for $G_{\alpha q}/G_{\alpha 11}$.

Fig. 2 shows the time course of the Ang II-induced decrease in immunoreactive $G_{\alpha q}/G_{\alpha 11}$ in VSMC. Cellular $G_{\alpha q}/G_{\alpha 11}$ levels were gradually reduced to a lower steady state within 6 hr of incubation with 100 nM Ang II. Immunoblotting with antibody specific for $G_{\alpha q}$ also showed Ang II-induced sustained decrease of cellular $G_{\alpha q}$ levels with a similar time course to $G_{\alpha q}/G_{\alpha 11}$ (Ang II 6 hr, 50.1 ± 7.2% control; $p < 0.01$ versus untreated control; four experiments; see Fig. 3B). The decreased levels remained unchanged at least for 8 hr. As shown in Fig. 4, the effect of Ang II on $G_{\alpha q}/G_{\alpha 11}$ levels is dose dependent. When VSMC were exposed to Ang II for 6 hr, the half-maximal decrease was observed at 2 nM, and the minimal concentration required to induce the maximal effect was 100 nM Ang II.

Because Ang II causes PKC activation and calcium mobilization, we investigated the effects of exogenous activators of these pathways on cellular $G_{\alpha q}/G_{\alpha 11}$ levels. As shown in Fig. 5, a 6-hr treatment with either the PKC activator PMA (100 nM) or the calcium ionophore ionomycin (2 μM) did not mimic the effects of Ang II on the cellular $G_{\alpha q}/G_{\alpha 11}$ levels. Simultaneous application of PMA and ionomycin also had no effect on G protein levels. Although PKA and PKG are not normally activated on Ang II stimulation in VSMC (26), we tested whether these protein kinases might act as heterologous modulators of $G_{\alpha q}/G_{\alpha 11}$ levels because these protein kinases have been shown to inhibit agonist-induced PLC activation (27). However, a 6-hr treatment with either the adenylate cyclase activator forskolin (10 μM) or the membrane-permeable cGMP analogue 8-bromo-cGMP (100 μM) did not significantly change $G_{\alpha q}/G_{\alpha 11}$ levels in VSMC. In contrast to these kinase activators, [Arg^8]vasopressin (100 nM for 6 hr), which activates the similar signaling pathways to Ang II, induced a

TABLE 1

Effects of Ang II on G protein α subunits

VSMC were treated with 100 nM Ang II for 6 hr. Whole-cell lysates were subjected to immunoblotting with anti-$G_{\alpha q}$, anti-$G_{\alpha i\text{-}3}$, anti-$G_{\alpha i\text{-}3}/G_{\alpha o}$, and anti-$G_{\alpha s}$ antibodies.

G protein	100 nM Ang II for 6 hr
	% Control
$G_{\alpha q}/G_{\alpha 11}$	49.6 ± 11.2[a]
$G_{\alpha i\text{-}3}$	87.3 ± 7.4
$G_{\alpha i\text{-}3}/G_{\alpha o}$	91.0 ± 14.5
$G_{\alpha s}$ (heavy band)	106.6 ± 5.3

Data are expressed as mean ± standard error of four independent experiments.

[a] $p < 0.01$ versus untreated cells.

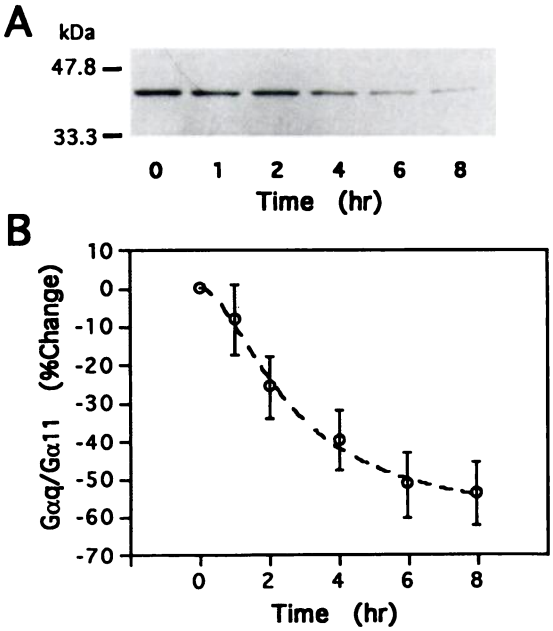

Fig. 2. Time course of decrease in cellular $G_{\alpha q}/G_{\alpha 11}$ levels during Ang II treatment. A, Representative immunoblot showing the time-dependent decrease in $G_{\alpha q}/G_{\alpha 11}$ levels induced by 100 nM Ang II. B, Immunoreactive bands were quantified by densitometric scanning. Data points represent the mean ± standard error (four experiments) expressed as percent change from untreated control.

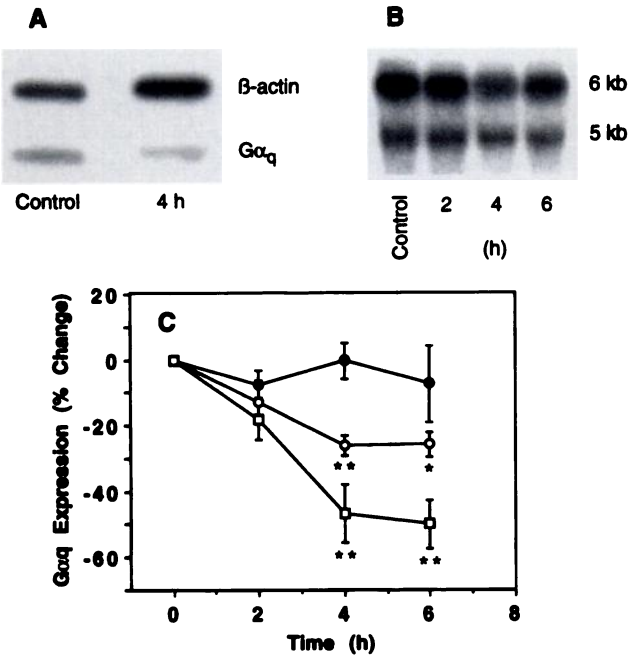

Fig. 3. Effects of Ang II on $G_{\alpha q}$ mRNA and protein. A, Representative nuclear run-on of $G_{\alpha q}$ in VSMC. Transcription was allowed to proceed for 30 min in the presence of [α-^{32}P]UTP in nuclei isolated from cells treated with or without Ang II for 4 hr. Purified transcripts were hybridized with cDNAs for β-actin and $G_{\alpha q}$ immobilized on nylon membranes. Autoradiograph was subjected to laser densitometry. B, Representative Northern blot of $G_{\alpha q}$ in VSMC. We probed with full length $G_{\alpha q}$ cDNA 10 μg total RNA from VSMC that were incubated with 100 nM Ang II for 2, 4, or 6 hr. Autoradiograph was subjected to laser densitometry. C, Time course of Ang II-induced changes of $G_{\alpha q}$ mRNA (●, 5-kb band; ○, 6-kb band; □, protein). $G_{\alpha q}$ protein levels were determined using immunoblotting with $G_{\alpha q}$-specific antibody. Data are mean ± standard error (three experiments). *, $p < 0.05$ versus untreated cells. **, $p < 0.001$ versus untreated cells.

significant loss of the $G_{\alpha q}/G_{\alpha 11}$ levels in VSMC (46.3 ± 7.4% control, $p < 0.01$).

In contrast to the effect of the exogenous PKC activator, endogenous activation of PKC was apparently involved in the Ang II-induced decrease in cellular $G_{\alpha q}/G_{\alpha 11}$levels. In the present study, we examined the effects of abolishing PKC activity on the Ang II-induced loss of cellular $G_{\alpha q}/G_{\alpha 11}$ (Fig. 6). Down-regulation of PKC by exposure to 200 nM PDBu for 24 hr slightly decreased the basal levels of cellular $G_{\alpha q}/G_{\alpha 11}$ (84.6 ± 8.9% control) and abolished the Ang II-induced decrease in $G_{\alpha q}/G_{\alpha 11}$. When VSMC were treated with the PKC inhibitor GF-109203X (2 μM), a staurosporine analogue, for 20 min before Ang II, basal $G_{\alpha q}/G_{\alpha 11}$ levels decreased slightly yet not significantly to 80.7 ± 9.1% control, and no further loss of protein was observed on Ang II stimulation.

The response of VSMC to Ang II is biphasic (28–30). The sustained phase of signal generation is attenuated by prevention of receptor sequestration with PAO or chronic potassium depletion (29, 30). Thus, we examined whether receptor processing is involved in the mechanism underlying the agonist-induced loss of $G_{\alpha q}/G_{\alpha 11}$. As shown in Fig. 7, pretreatment of VSMC with 2 μM PAO for 20 min before Ang II addition completely inhibited the Ang II-induced decrease in $G_{\alpha q}/G_{\alpha 11}$ while having no effect on base-line levels (PAO, 101.7 ± 15.9%; PAO plus Ang II, 94.0 ± 16.4% control). Similar effects of PAO were observed on the vasopressin-induced reduction in $G_{\alpha q}/G_{\alpha 11}$ levels (PAO plus vasopressin,

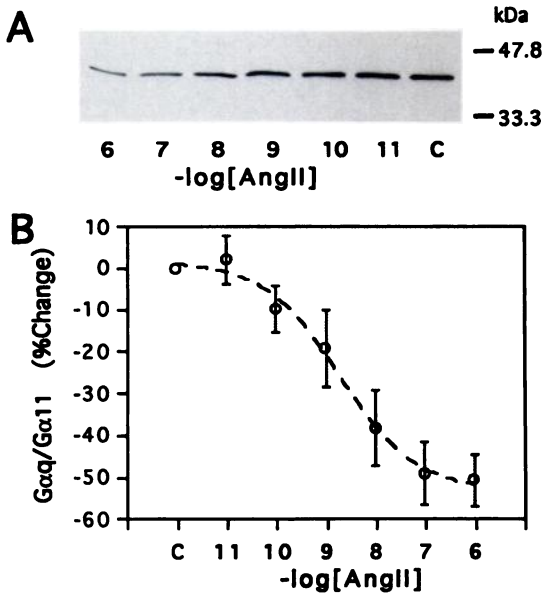

Fig. 4. Dose-dependent decrease in cellular $G_{\alpha q}/G_{\alpha 11}$ levels during Ang II treatment. A, Representative immunoblot showing the dose-dependent decrease in cellular $G_{\alpha q}/G_{\alpha 11}$ levels during a 6-hr treatment with Ang II. B, Concentration-effect relationship. Data points represent the mean ± standard error of five independent experiments expressed as percent change from untreated control. EC_{50} = 2 nM.

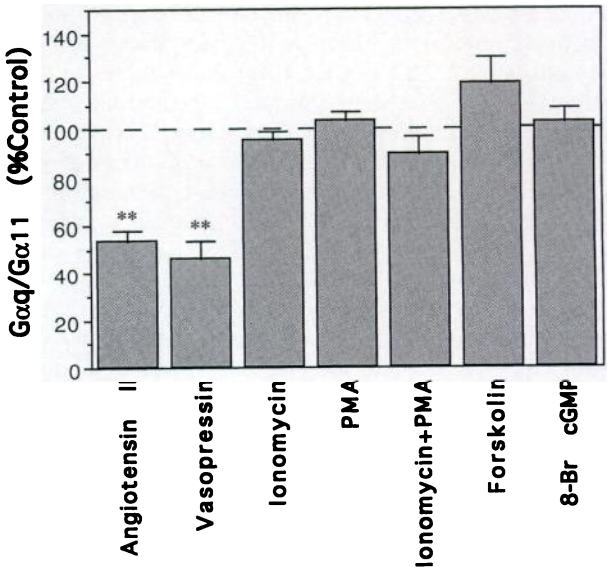

Fig. 5. Comparison of the effects of receptor-mediated and non-receptor-mediated stimulation on the cellular $G_{\alpha q}/G_{\alpha 11}$ levels. *Bars*, effects of a 6-hr treatment with 100 nM Ang II, 100 nM vasopressin, 2 μM ionomycin, 100 nM PMA, simultaneous administration of ionomycin and PMA, 10 μM forskolin, or 100 μM 8-bromo-cGMP on cellular $G_{\alpha q}/G_{\alpha 11}$ levels in VSMC. Data are mean ± standard error of four experiments. **, $p < 0.01$ versus untreated control.

102.3 ± 6.8% control; five experiments). Furthermore, chronic potassium depletion also reversed the Ang II-induced $G_{\alpha q}/G_{\alpha 11}$ reduction (95.8 ± 12.9% control) without altering the base-line level (104.9 ± 6.0%). To rule out the possibility that PAO is exerting its effects via tyrosine phosphatase inhibition (31), we examined the effects of other tyrosine phosphatase inhibitors, vanadyl hydroperoxide and sodium orthovanadate, on the Ang II-induced reduction in $G_{\alpha q}/G_{\alpha 11}$ levels. Treatment with 100 μM vanadyl hydroperoxide for 20

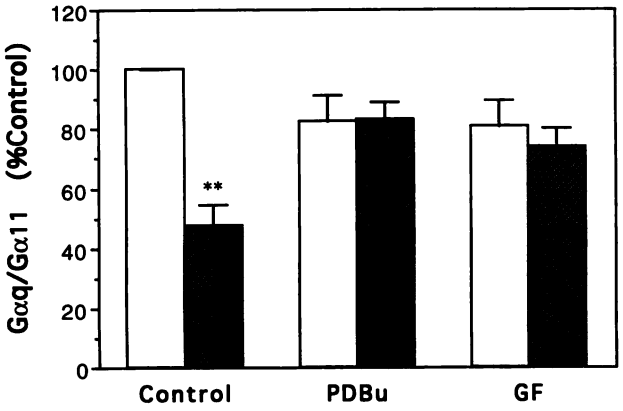

Fig. 6. Effects of down-regulation or inhibition of protein kinase C on the Ang II-induced reduction in $G_{\alpha q}/G_{\alpha 11}$ levels. *Left*, Untreated control experiment. *Middle*, PKC was down-regulated by exposing VSMC to 200 nM PDBu for 24 hr before the experiment. *Right*, PKC was inhibited by pretreatment with 2 μM GF-109203X (*GF*) for 20 min. After pretreatment, 100 nM Ang II (*striped bars*) or vehicle (base-line, *open bars*) was added to the medium, and cells were incubated for 6 hr. Data represent the mean ± standard error of six independent experiments. **, $p < 0.01$ versus untreated control.

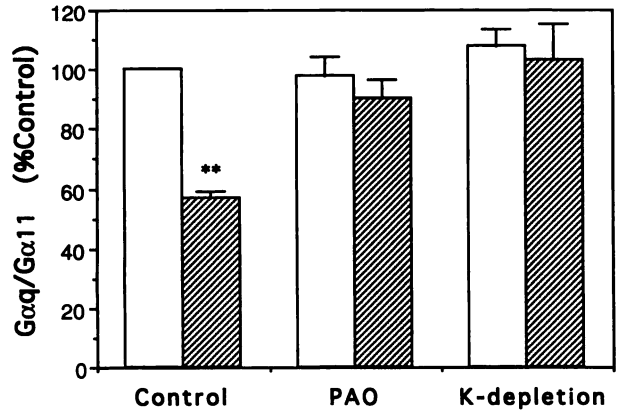

Fig. 7. Effects of inhibition of receptor internalization on the Ang II-induced change in cellular $G_{\alpha q}/G_{\alpha 11}$ levels. *Bars*, effects of pretreatment with 2 μM PAO for 20 min (*middle*) or chronic potassium (K^+) depletion with low-potassium medium for 24 hr (*right*). After pretreatment, 100 nM Ang II (*striped bars*) or vehicle (base-line, *open bars*) was added to the medium, and cells were incubated for 6 hr. Data are mean ± standard error (six experiments). **, $p < 0.01$ versus untreated control.

min before Ang II stimulation did not significantly alter base-line $G_{\alpha q}/G_{\alpha 11}$ levels (86.8 ± 6.5% control; five experiments) and did not affect the Ang II-induced reduction in cellular $G_{\alpha q}/G_{\alpha 11}$ levels (63.1 ± 4.3%, not significant versus Ang II, $p < 0.05$ versus control or vanadyl hydroperoxide alone; five experiments). Pretreatment with 100 μM sodium orthovanadate for 1 hr showed the similar results (orthovanadate, 84.0 ± 5.4%; orthovanadate plus Ang II, 69.7 ± 4.9% control; five experiments). Therefore, it appears that the ability of PAO to prevent the agonist-induced $G_{\alpha q}/G_{\alpha 11}$ down-regulation is independent of its inhibitory action on tyrosine phosphatases.

To determine whether alterations in $G_{\alpha q}$ mRNA levels could contribute to the agonist-mediated regulation of the G proteins, Northern blotting with the cDNA for $G_{\alpha q}$ was performed in 100 nM Ang II-treated VSMC (Fig. 3). Two bands of 5 and 6 kb were detected in control VSMC, in accord with previous observations (32). Ang II induced a delayed reduction in $G_{\alpha q}$ mRNA levels in the major band (6 kb) at 4 hr (73.9 ± 3.1% control, $p < 0.001$ versus untreated control; seven experiments), which was maintained for up to 6 hr. In the accessory band (5 kb), mRNA levels did not change significantly. Nuclear run-on analysis showed that Ang II decreased $G_{\alpha q}$ mRNA expression by 54% at the transcriptional level at 4 hr (Fig. 3A).

To assess the functional consequence of the reduction in $G_{\alpha q}/G_{\alpha 11}$ levels, we compared the effects of short or long term pretreatment with Ang II or vasopressin on subsequent Ang II-stimulated IP_3 formation. We reasoned that a short term exposure of VSMC to agonist would measure desensitization that was not regulated by a change in $G_{\alpha q}/G_{\alpha 11}$ levels, whereas long term agonist exposure would correlate with desensitization due to decreases in receptor and/or G protein levels. By using two different agonists, we attempted to separate the contribution of receptor and G protein down-regulation.

In VSMC without pretreatment, Ang II induced a transient IP_3 increase with a peak at 15 sec (212.3 ± 9.1% control). IP_3 levels returned to near base-line within 10 min (126.8 ± 13.5%). IP_3 generation in response to vasopressin showed a similar pattern, with a peak at 20 sec (191.8 ± 12.9%) and a return to the base-line level within 10 min (116.6 ± 5.1%). In the short term pretreatment study (Fig. 8A), VSMC were stimulated by 100 nM Ang II or 100 nM vasopressin for 10 min, and then a second Ang II stimulation (100 nM) was applied to the cells. After a 10-min pretreatment with vasopressin, subsequent stimulation with Ang II evoked a peak increase in cellular IP_3 content (198.6 ± 8.7%) to an extent similar to that of Ang II in untreated cells. In contrast, no significant IP_3 response to a second Ang II stimulation was observed when cells were pretreated with Ang II for 10 min (117.4 ± 6.8%, $p < 0.01$ versus Ang II response in untreated cells), implying that short term desensitization of Ang II-stimulated IP_3 formation was apparent after a 10-min pretreatment with Ang II but that vasopressin did not alter Ang II/AT_{1A}R/PLC coupling.

In the long term pretreatment study (Fig. 8B), VSMC were exposed to Ang II or vasopressin for 6 hr. After the treatment, IP_3 levels were indistinguishable from those in untreated cells. Pretreatment with Ang II dramatically inhibited the IP_3 increase in response to a second Ang II treatment (121.9 ± 10.2%, $p < 0.01$ versus Ang II response in untreated cells [228.2 ± 13.7% control]). A 6-hr pretreatment with vasopressin partially, yet significantly, reduced subsequent Ang II-stimulated IP_3 formation to 176.3 ± 10.8% ($p < 0.05$ versus Ang II response in untreated cells).

As shown in Fig. 8C, when $G_{\alpha q}/G_{\alpha 11}$ levels were down-regulated by a 6-hr pretreatment with Ang II, the ability of vasopressin to stimulate IP_3 formation was nearly abolished. The more complete desensitization to Ang II after long term pretreatment with Ang II compared with vasopressin is most likely indicative of the fact that Ang II regulates both AT_{1A}R and $G_{\alpha q}/G_{\alpha 11}$ levels (present study and Ref. 18).

Discussion

The results of this study demonstrate that in intact VSMC, prolonged exposure to Ang II induces a substantial sustained reduction in cellular $G_{\alpha q}/G_{\alpha 11}$ levels in a dose-dependent

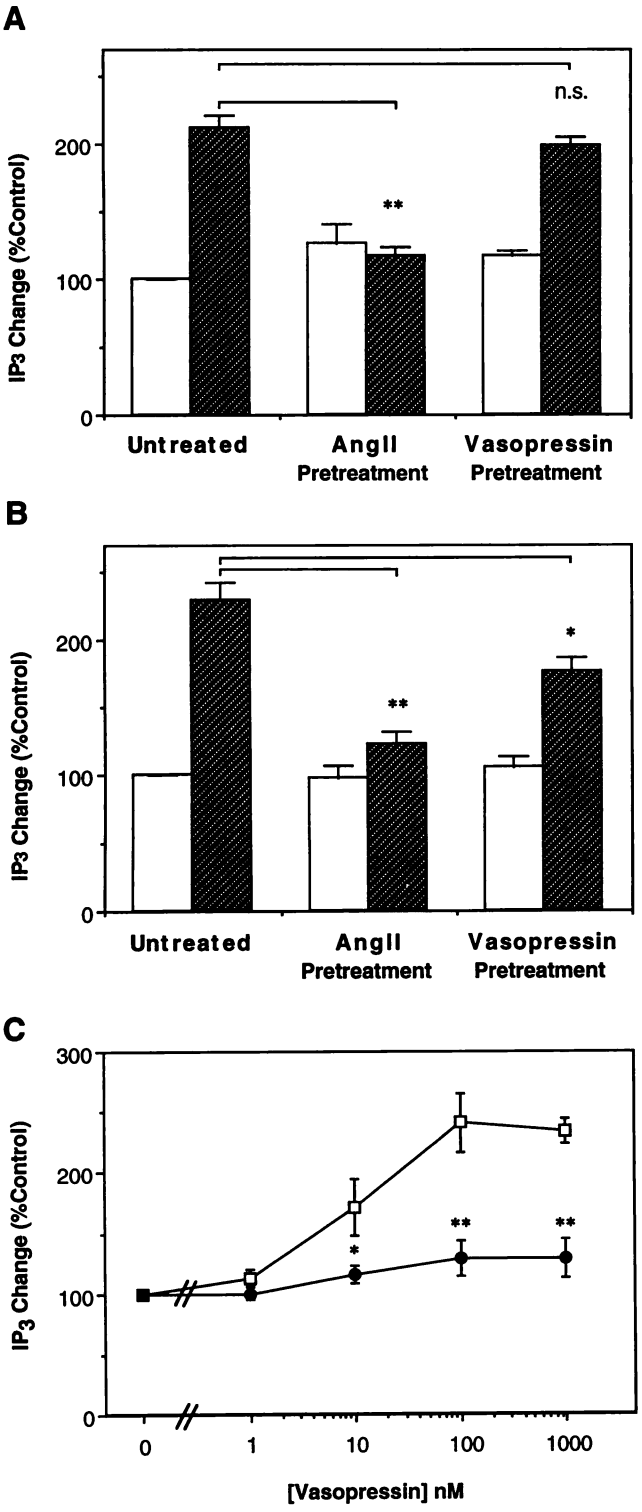

Fig. 8. Effects of short and long term pretreatment with an agonist on IP_3 generation in response to subsequent Ang II stimulation. A, VSMC labeled with *myo*-[^{3}H]inositol were at first stimulated with 100 nM Ang II (*middle*) or 100 nM vasopressin (*right*) for 10 min (*left*, vehicle alone) and then received a subsequent 15-sec 100 nM Ang II (*striped bars*) stimulation. *Open bars*, base-line (vehicle). B, After a 6-hr pretreatment with 100 nM Ang II (*middle*), 100 nM vasopressin (*right*), or vehicle (*left*), a subsequent 15-sec Ang II stimulation (100 nM, *striped bars*) was applied to the cells. *Open bars*, base-line. Data are expressed as percentage change from base-line value in untreated cells (mean ± standard error, four experiments). C, After pretreatment with 100 nM Ang II (●) or vehicle (□), a subsequent 20-sec stimulation with the indicated concentration of vasopressin was applied to the cells (mean ± standard error, three experiments). *n.s.*, no significant difference; *, $p < 0.05$ versus response in untreated cells; **, $p < 0.01$ versus response in untreated cells.

manner, with an accompanying smaller and transient decrease in $G_{\alpha q}$ mRNA levels. The Ang II-induced reduction in G protein α subunit expression is specific for $G_{\alpha q}/G_{\alpha 11}$. The ability of Ang II to decrease cellular $G_{\alpha q}/G_{\alpha 11}$ is reversed by inhibition of receptor sequestration initiated by agonist/receptor interaction, as well as by inhibition of PKC activity. The decrease in $G_{\alpha q}/G_{\alpha 11}$ levels is accompanied by a decrease in functional coupling to PLC, as assessed by measurement of IP_3 formation. Thus, regulation of $G_{\alpha q}/G_{\alpha 11}$ expression may play a role in the long term signaling response.

Desensitization of transmembrane signal transduction is a complex of events involving alterations in receptor function, intracellular localization and expression, as well as alterations in expression of the G proteins. Short term desensitization is functional uncoupling of a receptor from the G protein and effector, as observed by the failure of an agonist to activate signaling when it is given to cells immediately after a short previous exposure. Studies have shown that rapid phosphorylation of G protein-coupled receptors induces receptor uncoupling by reducing the binding affinity for agonist ligands and/or reducing the ability to interact with the G protein/effector (33–35). Prolonged exposure of the cells to an agonist leads to degradation of the receptors, called down-regulation, which is responsible for long term desensitization (9, 36). Recently, it has been shown that G proteins represent an additional site of agonist-induced down-regulation (17). Agonist-induced down-regulation has been observed for members of the G_{sec} family (11–13), the G_i family (14, 15), and, more recently, G proteins coupled to PLC. In CHO cells transfected to express human m1CR, carbachol caused down-regulation of $G_{\alpha q}/G_{\alpha 11}$ levels by 40–50% during 8–16 hr treatments, whereas $G_{\alpha sec}$ and $G_{\alpha i}$ levels were not altered (16). $G_{\alpha q}$ and $G_{\alpha 11}$ were down-regulated nonselectively (37). Although much phenomenological evidence exists that down-regulation of G protein α subunits occurs on agonist stimulation, the mechanisms responsible for and the physiological significance of down-regulation of the G proteins remain unclarified.

In many systems, down-regulation of the receptor and the G protein occurs simultaneously. In CHO cells expressing the m1CR, carbachol has been shown to down-regulate concurrently the m1CR and $G_{\alpha q}/G_{\alpha 11}$ (16). A similar concurrent regulation of the receptor and the related G protein was observed in the prostanoid IP receptor/$G_{\alpha sec}$ system (13). Recently, we demonstrated that prolonged exposure to Ang II down-regulates the $AT_{1A}R$ in VSMC (18). Treatment with Ang II for 4–8 hr induced the maximal reduction in the $AT_{1A}R$ protein and mRNA levels to 60% and 30% of control, respectively. Thus, Ang II appears to induce coordinate regulation of the $AT_{1A}R$ and $G_{\alpha q}/G_{\alpha 11}$ in VSMC. These data are consistent with the idea that a functional interaction between a receptor and a G protein is required to produce agonist-induced down-regulation of each. It is also possible that down-regulation of these two proteins is mechanistically distinct but is controlled by a common regulation mechanism generated early in the signaling pathways. Although the loss of two proteins seems to be closely linked, the target for the

down-regulation is unlikely to be the receptor/G protein complex per se because most models of receptor/G protein interaction suggest that a physical dissociation of the components occurs after agonist-induced exchange of GDP to GTP on the α subunit of the G protein (36, 38).

The present study showed that Ang II regulated $G_{\alpha q}$ mRNA at the level of transcription. Ang II-induced down-regulation of $G_{\alpha q}$ mRNA was smaller than that of the protein. This suggests that an additional mechanism may be involved in the regulation of cellular $G_{\alpha q}/G_{\alpha 11}$. It has been shown that the turnover rate of the $G_{\alpha q}/G_{\alpha 11}$ protein was enhanced by carbachol treatment, especially during the early period (within 3 hr) in ^{35}S-labeled CHO cells (39). Thus, in VSMC, both transcriptional control and enhanced proteolytic degradation are likely to contribute to agonist-induced $G_{\alpha q}/G_{\alpha 11}$ down-regulation.

In an effort to provide insights into the signaling mechanisms by which Ang II stimulation induces $G_{\alpha q}/G_{\alpha 11}$ down-regulation, we studied the effects of the calcium ionophore ionomycin and activators of ubiquitous protein kinases. The initial signals resulting from the rapid activation of PLC, IP_3, and DG act as a trigger for initiation of calcium- and/or PKC-mediated events. However, because neither ionomycin, PMA, nor a combination of the two could mimic the effects of Ang II on $G_{\alpha q}/G_{\alpha 11}$ down-regulation, it is unlikely that the mechanism responsible for the down-regulation is directly regulated by these early-phase intracellular signals. On the other hand, Ang II-induced $G_{\alpha q}/G_{\alpha 11}$ down-regulation was blocked under conditions where the sustained phase of signaling was impaired by PAO, potassium depletion, or abolishment of PKC activity. It thus appears that a certain level of PKC activity may be necessary for agonist-induced down-regulation of the G protein, although activation of PKC alone is not sufficient to induce the down-regulation. It is possible that this PKC-dependent component is not in the mainstream of the signal transduction system but rather functions as an auxiliary regulatory cofactor of the system.

Ang II-induced $G_{\alpha q}/G_{\alpha 11}$ down-regulation was also dependent on those elements of receptor processing that are sensitive to PAO or chronic potassium depletion (21, 29). It is noteworthy that the $AT_{1A}R$ down-regulation on Ang II is also sensitive to PAO (18). Although the precise mechanism of action of PAO remains unclear, PAO has been shown to prevent the appearance of the receptors in a light vesicular fraction on agonist stimulation (40), suggesting that PAO exerts its effect early in the internalization pathway, probably at the level of the plasma membrane. Chronic potassium depletion decreases coated-pit formation and endocytosis of receptor-bound low density lipoprotein in human fibroblasts (41), and assembly of the clathrin lattice of the coated pit is potassium dependent (42). Taken together, these data suggest that movement of the receptor within the plane of the membrane is a necessary requisite for Ang II-induced $AT_{1A}R$ and $G_{\alpha q}/G_{\alpha 11}$ down-regulation. Because prolonged DG production is also sensitive to receptor processing (21, 29, 30), it is possible that signals from this pathway represent the mechanism responsible for down-regulation. The ability of a PKC inhibitor to interfere with $G_{\alpha q}/G_{\alpha 11}$ down-regulation is consistent with this possibility. Alternatively, an unknown factor(s) at the level of coated pits or some other membrane region may function as a common mediator for divergent signals that link the sustained cellular signals and down-regulation mechanisms.

Our measurements of IP_3 were designed to examine the possible functional implications of agonist-induced $G_{\alpha q}/G_{\alpha 11}$ down-regulation. A 10-min pretreatment with Ang II showed short term homologous desensitization of the IP_3 response to subsequent Ang II stimulation, whereas vasopressin did not induce a rapid heterologous desensitization. Because down-regulation of neither $AT_{1A}R$ (18) nor $G_{\alpha q}/G_{\alpha 11}$ was observed in these conditions, the homologous short term desensitization may be attributed to receptor uncoupling. The lack of effect of vasopressin also shows that it has no effect on Ang II binding to the $AT_{1A}R$. In contrast, prolonged treatment (6 hr) with either Ang II or vasopressin caused desensitization of IP_3 generation in response to subsequent Ang II or vasopressin stimulation. This decreased IP_3 generation is not due to depletion of phosphatidylinositol-4,5-bisphosphate by prolonged exposure to agonists as the agonist-induced hydrolysis of polyphosphoinositides is transient and phosphatidylinositol-4,5-bisphosphate is rapidly replenished within 10 min after stimulation despite the continued presence of Ang II (29). Because prolonged Ang II, but not vasopressin, treatment decreases the $AT_{1A}R$ levels (18), the ability of vasopressin to decrease subsequent Ang II-stimulated IP_3 generation may be related to its ability to down-regulate $G_{\alpha q}/G_{\alpha 11}$. However, the inhibition of vasopressin-stimulated IP_3 generation after heterologous down-regulation appears to be greater than that of the Ang II response after heterologous down-regulation (Fig. 8, B and C), raising the possibility that additional mechanisms may be involved. The more pronounced inhibition of Ang II-stimulated IP_3 production in Ang II-pretreated VSMC compared with vasopressin-pretreated cells (Fig. 8B) could be explained by concurrent $AT_{1A}R$ and $G_{\alpha q}/G_{\alpha 11}$ down-regulation after Ang II pretreatment. It is interesting that some signal generation still occurs with Ang II, even with substantially reduced levels of G proteins. Taken together, these data suggest that agonist-induced $G_{\alpha q}/G_{\alpha 11}$ down-regulation may be a mechanism underlying long term desensitization of the calcium-mobilizing signaling systems in VSMC.

In conclusion, we demonstrated that prolonged exposure to Ang II down-regulates specifically $G_{\alpha q}/G_{\alpha 11}$ levels in intact VSMC. Our data suggest that the agonist-induced $G_{\alpha q}/G_{\alpha 11}$ down-regulation is involved in part in mechanisms of long term desensitization of the $G_{\alpha q}/G_{\alpha 11}$-mediated signaling system in VSMC. The Ang II-induced down-regulation appears to be mediated by the sustained signaling pathways that are sensitive to inhibition of receptor processing. These observations raise the possibility that modulation of $G_{\alpha q}/G_{\alpha 11}$ expression in VSMC is an important mechanism regulating cellular responses to vasoactive agonists.

Acknowledgments

We are very thankful to Dr. R. Wayne Alexander for his helpful suggestions and critical reading of the manuscript. We are also grateful to Dr. Ronald D. Smith for kindly supplying us with losartan, to Dr. James J. Murtagh for generously providing the cDNA probe for $G_{\alpha q}$, and to Dr. Gadiparthi Rao for providing the cDNA for GAPDH. We thank Stephen Baker for technical assistance and Barbara Merchant for editorial assistance.

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