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Desensitization of angiotensin-stimulated inositol phosphate accumulation in human vascular smooth muscle cells

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Abstract

The effect of angiotensin II treatment on desensitization of phospholipase C (PLC)-mediated inositol phosphate accumulation has not been quantitated in human aortic vascular smooth muscle (HVSM) cells. We determined the angiotensin II pretreatment dose dependency and time course for desensitization of PLC activation in HVSM cells and the effect of protein kinase C (PKC) activators on angiotensin II-mediated inositol phosphate accumulation. Our results with PKC activators and direct G protein stimulators suggest that PKC activation may play a negative feedback role in desensitization of angiotensin II-activated signaling in HVSM cells by modifying the Gq transducer, PLC-β effector, or related proteins in the signaling pathway. However, neither angiotensin II nor PKC activator affected basal phosphorylation levels of PLC-β1 or PLC-β3 in HVSM cells; PLC-β isoenzymes were shown to be phosphorylated in unstimulated cells independent of PKC inhibition. We suggest that desensitization of G protein-stimulated inositol phosphate accumulation in HVSM differs from other cell types in which phosphorylation of PLC-β isoenzymes accompanies desensitization.

Keywords: Vascular smooth muscle; Phospholipase C-beta; Phosphatidylinositol; Tachyphylaxis; Phosphorylation

1. Introduction

Angiotensin II, a peptide hormone, acts on vascular smooth muscle tissue to increase tone in the short term, and migration and growth regulation in the long term (reviewed by Huckle and Earp, 1994). The primary effects of angiotensin II in aortic vascular smooth muscle are mediated by angiotensin AT_1 receptors, heptahelical transmembrane receptors which activate G_q-type guanine nucleotide binding proteins (G proteins). Activation of G_q-type G proteins stimulates phosphatidylinositol phospholipid hydrolysis by phospholipase C (PLC)-β effector activation in the inositol phospholipid signaling pathway. Activation of PLC-β by angiotensin II treatment results in accumulation of inositol 1,4,5 triphosphate (IP_3) and diacylglycerol, second messengers which increase cytoplasmic calcium levels and activate protein kinase C (PKC) isoenzymes (Bkaily et al., 2003; Griendling et al., 1989; Murphy et al., 1992; Smith et al., 1984; Takata et al., 1990).

Brief, repeated applications of angiotensin II to vascular smooth muscle induces a long-term tachyphylaxis (Peach, 1977), or desensitization, as a putative means of negative feedback regulation of the inositol phospholipid hydrolysis pathway. Receptor-based mechanisms for desensitization of G protein-coupled receptor-stimulated phosphatidlyinositol phospholipid hydrolysis include receptor phosphorylation, internalization, and long-term degradation (Fisher, 1995). Membrane levels of angiotensin AT_1 receptors downregulate in cultured rat aortic vascular smooth muscle (RVSM) cells following exposure to angiotensin II. However, receptor downregulation alone is insufficient to fully account for desensitization of angiotensin II-stimulated inositol lipid signaling (Bkaily et al., 2003; Lassegue et al., 1995; Ullian and Linas, 1990). Modifications of PLC-β and/or G_q levels or activity are another means by which inositol phospholipid hydrolysis may be desensitized by

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repeated or chronic agonist treatment. $G\alpha_{q/11}$ protein levels decreased in RVSM cells following 6 h of angiotensin II treatment (Kai et al., 1996). Protein levels of PLC-β1 decreased in SH-SY5Y neuroblastoma cells following agonist activation of G_q-coupled muscarinic receptors (Sorensen et al., 1998).

Activation of PKC has been proposed to modify G protein-stimulated inositol phospholipid hydrolysis. Application of 4β-phorbol 12β-myristate 13α acetate (PMA), a direct PKC activator, inhibits G protein-coupled receptor stimulation of inositol phospholipid hydrolysis in many systems by mechanisms downstream of various G_q-linked G protein-coupled receptors, e.g. muscarinic (Orellana et al., 1985; Willars et al., 1996), purine P2Y (Chen and Chen, 1997; Galas and Harden, 1995), α_1 adrenoceptor (Lynch et al., 1985), bombesin (Brown et al., 1987), and oxytocin (Yue et al., 2000). Inhibition of angiotensin II receptor-stimulated inositol lipid signaling by PMA pretreatment in RVSM cells also is not accompanied by a change in agonist binding (Brock et al., 1985), angiotensin AT_1 receptor number, or agonist affinity (Pfeilschifter et al., 1989). Additionally, PMA had no effect on phospholipid pools or basal Ca^{2+} levels in RVSM cells (Brock et al., 1985), suggesting that PKC activation affected non-receptor proteins (e.g., $G\alpha_q$, PLC-β) in the signal cascade.

Covalent modification of PLC-β effectors may be a mechanism for PKC-dependent inhibition of inositol lipid signaling. Evidence exists in vitro for PKC-promoted phosphorylation of PLC-β isoenzymes, including avian PLC-βT (Filtz et al., 1999), PLC-β1 (Litosch, 1996; 1997; Ryu et al., 1990), and PLC-β3 (Yue et al., 2000). Evidence in avian erythrocytes (Filtz et al., 1999; Galas and Harden, 1995), NG108-15 cells (Strassheim et al., 1998), and several transfected cell lines (Ali et al., 1997; Yue et al., 2000) has suggested PKC-dependent PLC-β phosphorylation in vivo coincident with desensitization.

Human aortic vascular smooth muscle (HVSM) cells, similarly to RVSM cells, respond to angiotensin II treatment with IP_3 accumulation, increased cytoplasmic Ca^{2+}, and activation of PKC-α in a G_q- and PLC-β-dependent manner (Assender et al., 1997; Gutowski et al., 1991; Schelling et al., 1997). Although studied in RVSM cells, a time course for angiotensin II-induced desensitization and the effect of PKC activation on G protein-stimulated inositol phospholipid signaling in HVSM cells has not been previously quantitated.

2. Materials and methods

2.1. Materials

myo-[2-^{3}H]Inositol (17.0 Ci/mmol) and protein A Sepharose were purchased from Amersham Bioscience (Amersham, Piscataway, NJ). Human recombinant fibroblast growth factor and human recombinant epidermal growth factor were from BD Biosciences (Bedford, MA). Goat anti-rabbit-IgG alkaline phosphatase conjugate, nitrocellulose membrane (0.45-μm pore size), Immune-Star® chemiluminescent substrate and AG1-X8 resin were from Bio-RAD Laboratories (Hercules, CA). Penicillin/streptomycin antibiotic mix and Dulbecco's modification of Eagle's medium (DMEM) containing 4.5 g/l glucose, L-glutamine, and sodium pyruvate were from Cellgro (Herndon, VA). Phosphate-free DMEM and inositol-free DMEM were purchased from GIBCO BRL Life Technologies (Rockville, MD). Standard fetal bovine serum was purchased from HyClone (Logan, UT). Insulin and inorganic [^{32}P]orthophosphate were obtained from ICN Pharmaceuticals (Costa Mesa, CA). Polyclonal rabbit anti-PLC-β1 (C-terminal, G-12),-PLC-β2 (C-terminal, Q-15),-PLC-β3 (C-terminal, C-20), and-PLC-β4 (C-terminal, C-18) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Angiotensin II, PMA, 4α-phorbol 12, 13-didecanoate (4αPDD), and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO). GF109203X (2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide) was purchased from TOCRIS (Ellisville, MO). PLC-β2 was purified from PLC-β2 baculovirus-infected Sf9 cell lysates as previously described (Paterson et al., 1995).

2.2. Culture of human aortic vascular smooth muscle cells and 1321N1 astrocytoma cells

HVSM cells, >90% homogeneous smooth muscle phenotype by flow cytometry, were purchased from Cascade Biologics (Portland, OR) at passage 4. HVSM cells from passages 4 to 20 were routinely seeded at a density of 5×10^4 cells/cm^2 and maintained in bicarbonate-buffered DMEM supplemented with 5% fetal bovine serum, 4.5 mg/l glutamine, 10 μg/l human recombinant epidermal growth factor (EGF), 2 μg/l human recombinant fibroblast growth factor (FGF), 5 mg/l insulin, 5000 U/l penicillin, and 5 mg/l streptomycin in a 5% CO_2 humidified atmosphere at 37 °C. 1321N1 human astrocytoma cells were cultured as previously described (Filtz et al., 1994).

2.3. Detection of PLC-β isoenzymes

Flasks (150 cm^2) of HVSM cell monolayers (80% confluent), 1321N1 cell monolayers (100% confluent), or fresh-thawed rat cerebellum (Zivic Laboratories, Pittsburgh, PA) were washed in ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3) and incubated for 10 min in lysis buffer containing 10 mM Tris–HCl (pH 7.4), 2 mM EDTA, 5 mM $MgCl_2$ and protease inhibitors, (200 nM benzamidine, 10 μM leupeptin, 1 μM pepstatin A, and 200 nM phenylmethylsulfonyl fluoride) at 0.5 ml/flask cells or 1 ml/100 mg cerebellum wet weight. Lysis was completed by 15 strokes of Dounce homogenization. Cell lysates were

extracted with buffer containing 50 mM HEPES (pH 7.4), 2.5 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, and 1% Triton X-100 with protease inhibitors for 1 h followed by centrifugation at 16,000 rpm, 4 °C for 30 min. The soluble extract (2 ml/sample) was incubated with 1 μg/ml of isoenzyme-selective anti-PLC-β antibodies overnight at 4 °C for immunoprecipitation, followed by incubation with 50 μl of protein A-Sepharose beads for 1 h at 4 °C. (Protein A-Sepharose beads were pre-equilibrated in extraction buffer in accordance with manufacturer's specifications.) Protein A-immunoprecipitated complexes were washed three times with extraction buffer, resuspended in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, and subjected to 7.5% polyacrylamide (w/v) SDS-PAGE. SDS-PAGE size-fractionated proteins were transferred to nitrocellulose membranes and Western blots were performed by incubation with 1:3000 dilution of anti-PLC-β antibody as previously described (Filtz et al., 1999). Immunoreactive bands were visualized by enhanced chemiluminescence alkaline phosphatase substrate (Immune-Star®) and exposure to X-ray film for 10–300 s.

2.4. Measurement of phospholipase C-β activity

HVSM cells were seeded in 24-well plates overnight and radiolabeled by incubation in 0.5 ml of bicarbonate-buffered, serum-free, inositol-free DMEM containing 1 μCi *myo*-[^{3}H]inositol for 24 h. Following radiolabeling, cells were exchanged into serum-free DMEM buffered with 10 mM HEPES (pH 7.4), removed to room air, and incubated at 37 °C. Cells were pretreated as indicated. Angiotensin II and aluminum magnesium fluoride (AlF_4^-; 10 mM NaF, 20 μM $AlCl_3$) were prepared as 50×concentrated stocks in 10 mM HEPES (pH 7.0). PMA, 4αPDD, and GF109203X were prepared as 1000× concentrated stocks in DMSO. Stimulators (angiotensin II or AlF_4^-) were added in the presence of 10 mM LiCl to allow for accumulation of inositol phosphates. Incubations were terminated by aspiration of the drug-containing medium and addition of 0.5 ml ice-cold 5% (w/v) trichloroacetic acid. Acid-soluble cell fractions were ether extracted and [^{3}H]inositol phosphates collected by anion exchange chromatography as described previously (Filtz et al., 1994). Total accumulated [^{3}H]inositol phosphates (i.e. inositol trisphosphate, inositol bisphosphate, and inositol monophosphate) were quantitated by liquid scintillation spectrometry. Acid precipitates containing cell-incorporated [^{3}H]inositol phospholipids were collected and quantitated by liquid scintillation spectrometry. Experimental response was calculated as response to drug treatment minus basal inositol phosphate accumulation in the presence of vehicle only. Percent maximal conversion reflecting stimulated inositol phosphate accumulation was calculated as the fraction of inositol phospholipids converted to inositol phosphates, relative to maximal conversion obtained with 10 μM angiotensin II treatment.

2.5. Detection of PLC-β1 and PLC-β3 phosphorylation in HVSM cells

HVSM cells (5×10^6cells/sample) were collected, washed with phosphate-free DMEM, and radiolabeled by incubation with 400 μCi/ml [^{32}P]orthophosphate in serum-free, phosphate-free DMEM for 90 min. Cells were then treated with drugs for indicated times, washed with ice-cold PBS (pH 7.4), and extracted for 1 h with inversion at 4 °C in buffer containing 50 mM HEPES (pH 7.4), 2.5 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 1% Triton X-100, 50 mM NaF, 10 mM β-glycerophosphate, 10 nM microcystin-LR, and protease inhibitors. Extracted samples were centrifuged at 16,000 rpm, 4 °C, for 30 min to pellet insolubles. Following centrifugation, supernatants were treated with isoenzyme selective anti-PLC-β antibodies for immunoprecipitation as described above. Immunoprecipitates were separated by 7.5% (w/v) SDS-PAGE and anti-PLC-β isoenzyme-selective antibodies were used to visualize PLC-β proteins by Western blot analysis as described above. Densitometric analysis of radioactive and immunoreactive digitized band intensities from autoradiograph and Western blot films was performed by NIH Image v1.62 software for Macintosh.

2.6. Data analysis

Statistical analyses were performed using a two-tailed Student's *t*-test. Differences among treatment groups were considered significant at $p<0.05$. Data from time course and dose–response curves for stimulation of inositol phospholipid hydrolysis were analyzed by nonlinear regression using Prism 4 GraphPad® software (GraphPad, San Diego, CA).

3. Results

3.1. Detection of PLC-β isoenzymes in HVSM cells

PLC-β1 was previously shown to be expressed in HVSM cells (Schelling et al., 1997), but no other PLC-β isoenzymes were assessed. To identify the complete complement of PLC-β isoenzymes expressed in HVSM cells, Western blot analysis was performed on nuclear-free cell extracts with each of four human-reactive PLC-β isoenzyme-selective antibodies. The major PLC-β isoenzymes expressed by HVSM cells as detected immunologically are PLC-β1 and PLC-β3 migrating at 150 kDa; PLC-β2 and PLC-β4 were not expressed at detectable levels (Fig. 1). Positive controls for immunologic detection of each isoenzyme included human astrocytoma 1321N1 cell homogenates which previously were shown to express PLC-β1 and PLC-β3 isoenzymes (McCullar et al., 2003), purified recombinant PLC-β2 protein, and cerebellum which expresses PLC-β4 (Min et al., 1993). PLC-β1 is

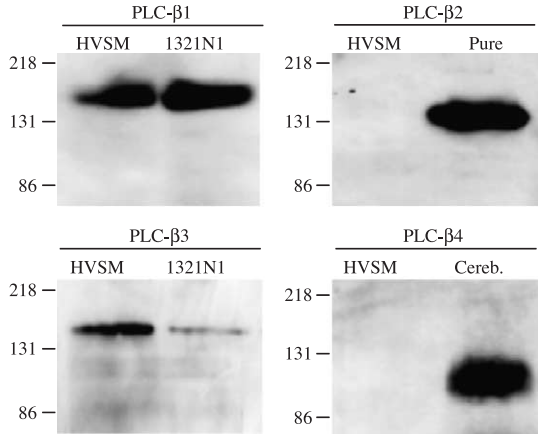

Fig. 1. Immunoblot detection of PLC-β1 and PLC-β3 isoenzymes in HVSM cells. HVSM cell (HVSM), 1321N1 cell (1321N1), or bovine cerebellum (Cereb.) lysates were assessed by Western blotting protocols for PLC-β isoenzyme expression as indicated. Migration of molecular mass markers is indicated (kDa). As a positive control for PLC-β2 immunoprecipitation, 1 μg of purified recombinant PLC-β2 was included where indicated (Pure). Shown are representative experiments for each isoenzyme repeated two to four times.

known to be expressed in human tissue as two splice variants, PLC-β1a (150 kDa) and PLC-β1b (140 kDa) with reported cytosolic and nuclear expression, respectively (Bahk et al., 1998). We have detected a single immunologically reactive band for PLC-β1 on our blots, consistent with migration of PLC-β1a and the exclusion of nuclear fractions from these samples.

3.2. Angiotensin II stimulation and desensitization of inositol phosphate accumulation in HVSM cells

The dose–response characteristics and time-course for angiotensin II stimulation of inositol phospholipid hydrolysis in HVSM cells were determined (Fig. 2). Angiotensin II stimulated inositol phosphate accumulation in a dose-dependent manner with an EC_{50} value of 4.8±1.5 μM (Fig. 2A). Angiotensin II (1 μM) stimulated inositol phosphate accumulation in a time-dependent manner with a $t_{1/2}$ of 32±5 min, and reaching a plateau at 75–90 min (Fig. 2B). Inositol phosphate accumulation in the presence of LiCl alone was minor but significant. Therefore, the contribution of LiCl alone was quantitated and subtracted in all subsequent experiments.

To investigate the time course of desensitization to angiotensin II treatment, HVSM cells were treated with 10 μM angiotensin II for 0–180 min in the absence of LiCl to avoid inositol phosphate accumulation. To investigate the dose–response relationship of angiotensin II treatment to desensitization, HVSM cells were treated for 60 min with indicated concentrations of angiotensin II in the absence of LiCl. Following pretreatment with angiotensin II, HVSM cells were then challenged with 1 μM angiotensin II for 60 min in the presence of LiCl to allow for inositol phosphate accumulation. Pretreatment with angiotensin II reduced the effect of a subsequent stimulation with angiotensin II to increase inositol phosphate accumulation as a function of dose (Fig. 3A) or pretreatment time (Fig. 3B). The ED_{50} value for desensitization induced by a 60-min pretreatment of angiotensin II is 1.3±1.0 μM. Desensitization followed a two-phase time course, the first phase occurring with fairly rapid kinetics ($t_{1/2}$=6±3 min) declining to 60% of full response within 30 min of pretreatment. A second slower phase of desensitization then occurred reaching 30% of full response at 180-min pretreatment without reaching a plateau over this time period.

3.3. Effects of protein kinase C activation on inositol phosphate accumulation

To assess the effect of PKC activation on angiotensin II-stimulated inositol phosphate accumulation, HVSM cells were treated with the following: PMA, a PKC activator; 4αPDD, an inactive analogue of PMA; or GF109203X, a specific PKC inhibitor (Toullec et al., 1991). Following 5-min pretreatment with 1 μM PMA or 1 μM 4αPDD, HVSM

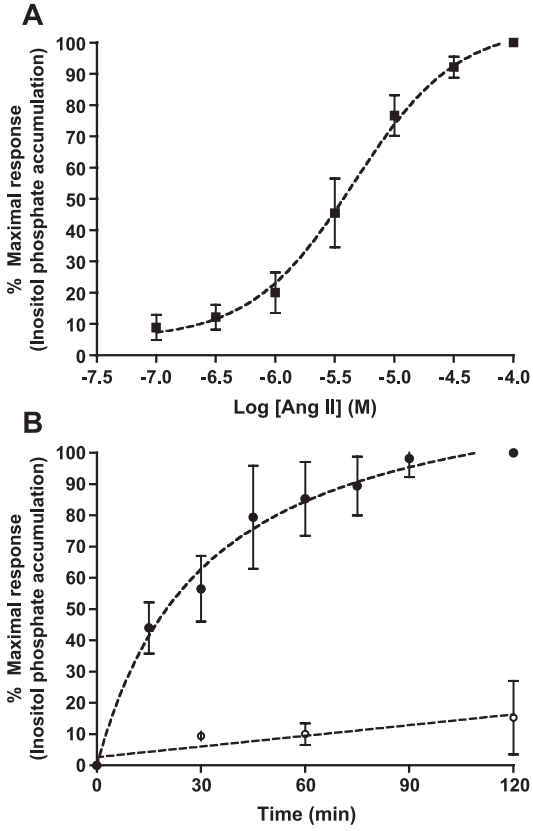

Fig. 2. Time course and dose–response curve for angiotensin II stimulation of inositol phosphate accumulation in HVSM cells. (A) HVSM cells were treated with the indicated concentrations of angiotensin II for 60 min in the presence of 10 mM LiCl. (B) HVSM cells were treated with 1 μM angiotensin II (●) or vehicle (○) for indicated times in the presence of 10 mM LiCl. Inositol phosphate accumulation is expressed as percent maximal response to 100 μM angiotensin II at 120 min. Shown is cumulative average data from three to five experiments conducted in triplicate±S.E.

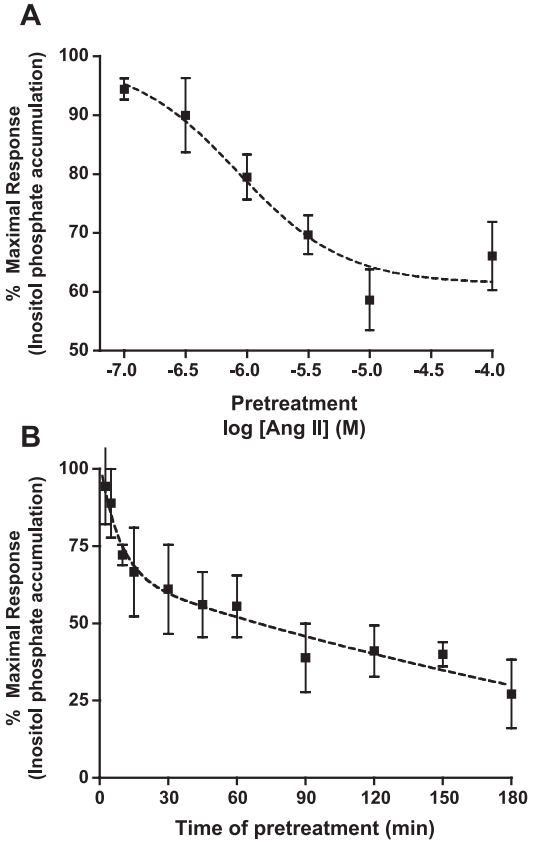

Fig. 3. Dose–response and time course of angiotensin II-induced desensitization in HVSM cells. (A) HVSM cells were pretreated with indicated concentrations of angiotensin II in the absence of LiCl for 60 min, washed twice, and then challenged with angiotensin II for 60 min in the presence of 10 mM LiCl. (B) HVSM cells were pretreated with 10 μM angiotensin II in the absence of LiCl for indicated times and washed twice, then challenged with 1 μM angiotensin II for 60 min in the presence of 10 mM LiCl. Inositol phosphate accumulation is expressed as percent maximal response to 1 μM angiotensin II at 60 min in the absence of pretreatment. Data shown are cumulative mean±S.E. of three experiments conducted in triplicate.

cells were stimulated by 1 μM angiotensin II in the presence of 10 mM LiCl for 60 min. Pretreatment with PMA, but not with 4αPDD, reduced angiotensin II-induced inositol phosphate accumulation to 56±4% of control. PMA-mediated inhibition of angiotensin II-induced inositol phosphate accumulation was completely attenuated by 30-min pretreatment with 10 μM GF109203X (Fig. 4A). Pretreatment with GF109203X alone increased angiotensin II-stimulated inositol phosphate accumulation two-fold. The stimulating effect of GF109203X reveals that endogenous PKC activation inhibits angiotensin II-induced signaling in HVSM cells.

To evaluate the contribution of angiotensin II receptor desensitization to decreased inositol phosphate accumulation following PKC activation, inositol phospholipid hydrolysis was stimulated by AlF_4^-, a direct activator of G proteins, bypassing angiotensin AT_1 receptor stimulation. AlF_4^- activation of inositol phosphate accumulation is dependent only upon G_q stimulation of PLC-β activity in the signaling pathway, eliminating receptor modification as a means for desensitization. HVSM cells were pretreated with 1 μM PMA or 1 μM 4αPDD for 5 min, or 10 μM GF109203X for 30 min, followed by stimulation with AlF_4^- in the presence of LiCl for 60 min. Pretreatment with PMA, but not 4αPDD, completely inhibited AlF_4^--stimulated inositol phosphate accumulation (1±12%). Pretreatment with GF109203X completely attenuated PMA-mediated inhibition (Fig. 4B). These data suggest that PKC activation affects inositol phosphate accumulation downstream of the angiotensin AT_1 receptor in HVSM cells in the inositol phospholipid signaling pathway, affecting, for example, the G_q protein transducer or PLC-β effector enzyme. Pretreatment with GF109203X alone did not enhance AlF_4^--stimulated inositol phosphate accumulation, unlike angiotensin II-stimulated inositol phosphate accumulation.

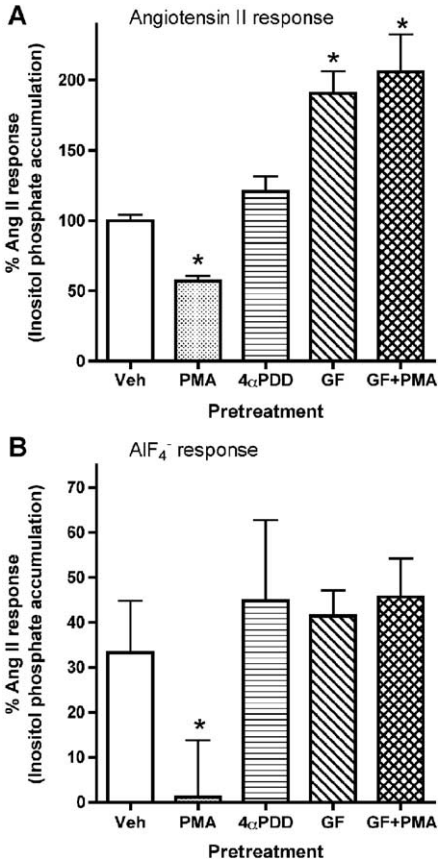

Fig. 4. Effects of PMA, 4αPDD and GF109203X pretreatment on inositol phosphate accumulation. HVSM cells were pretreated with 0.1% DMSO vehicle for 30 min (Veh), with 0.1% DMSO for 25 min followed by 1 μM PMA for 5 min (PMA), with 0.1% DMSO for 25 min followed by 1 μM 4αPDD for 5 min (4αPDD), with 10 μM GF109203X for 30 min (GF), or with 10 μM GF109203X for 25 min followed by 1 μM PMA for 5 min (GF+PMA). (A) Pretreatment was followed by 1 μM angiotensin II stimulation for 60 min in the presence of 10 mM LiCl. (B) Pretreatment was followed by 1× AlF_4^- stimulation for 60 min in the presence of 10 mM LiCl. Inositol phosphate accumulation is expressed as percent maximal response to 1 μM angiotensin II for 60 min in the absence of pretreatment for both data sets. Data shown are cumulative mean±S.E. of three to five experiments performed in triplicate.

3.4. Effects of angiotensin II, PMA, and GF109203X on phosphorylation of PLC-β isoenzymes

Since PMA inhibited AlF_4^--induced inositol phosphate accumulation, we investigated the possibility that angiotensin II or PMA induces phosphorylation of PLC-β isoenzymes in HVSM cells. [^{32}P]Orthophosphate-radiolabelled HVSM cells were treated with 1 μM angiotensin II, 1 μM PMA, and/or 10 μM GF109203X as indicated, followed by cell lysis and immunoprecipitation of PLC-β1 or PLC-β3 (Fig. 5A). [^{32}P]Phosphate-radiolabelled immunoreactive bands at 150 kDa were detected for each treatment condition including vehicle. [^{32}P]Phosphate incorporation was paralleled by intensity of immunoreactive bands across several experiments, and no consistent differences among treatments for [^{32}P]phosphate incorporation into PLC-β immunoreactive bands were observed over several experiments. Additionally, no consistent treatment-dependent differences were observed for PLC-β1 or PLC-β3 protein levels assessed by Western blot. Autoradiography as a measure of [^{32}P]phosphate incorporation, and Western blot intensity as a measure of immunoprecipitated protein levels, were quantitated by densitometry. The ratio of phosphate incorporation to protein level was calculated for each treatment and normalized to vehicle control for each experiment. No statistically significant differences were observed among treatments (Fig. 5B).

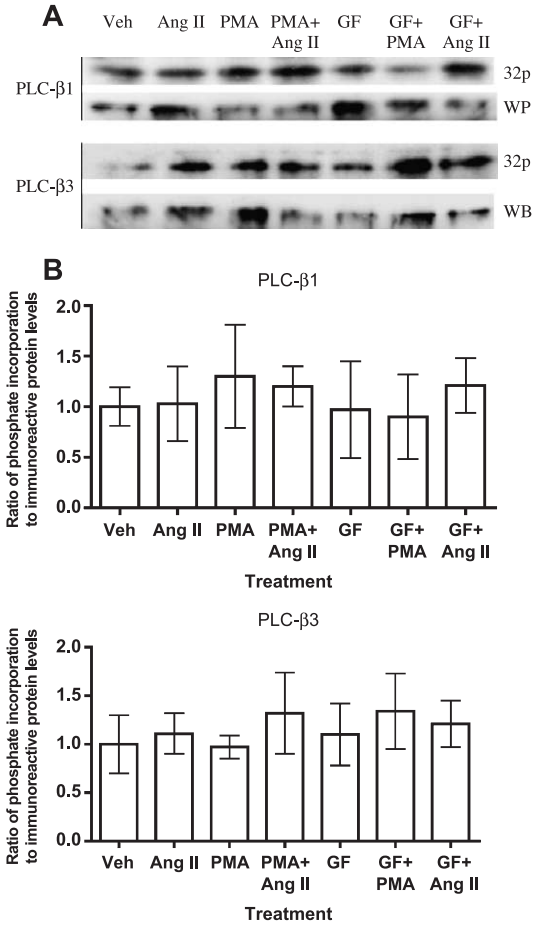

Fig. 5. Phosphorylation of PLC-β1 and PLC-β3 in HVSM cells. HVSM cells were radiolabeled with [^{32}P]orthophosphate and initially treated with 0.1% DMSO for 30 min (Veh, Ang II), with 0.1% DMSO for 25 min followed by 1 μM PMA for 5 min (PMA, PMA+Ang II), with 10 μM GF109203X for 30 min (GF, GF+Ang II), or with 10 μM GF109203X for 25 min followed by 1 μM PMA for 5 min (GF+PMA). The cells were then further treated for 60 min with DMEM vehicle (Veh, PMA, GF, GF+PMA) or 10 μM angiotensin II (Ang II, PMA+Ang II, GF+Ang II). Following treatment, PLC-β1 or PLC-β3 were immunoprecipitated separately as indicated from whole cell detergent extracts, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were exposed to X-ray film for detection of [^{32}P]phosphate incorporation by autoradiography followed by Western blotting protocols with anti-PLC-β selective antibodies for protein detection. (A) Shown are autoradiography bands (^{32}P) that migrate coincident with PLC-β1 or PLC-β3 immunoreactive bands (WB). Data shown are representative of three experiments. (B) Quantitative ratio of [^{32}P]phosphate labeling to immunoprecipitated protein levels obtained following PLC-β1 or PLC-β3 immunoprecipitation by densitometric analysis of autoradiographic film and Western blots. Shown are average ratios of densities for each treatment group obtained from three separate experiments ± S.D. for PLC-β1 or PLC-β3, normalized to a ratio of 1 for the vehicle control group. No statistically significant differences among treatment groups were obtained.

However, both PLC-β1 and PLC-β3 incorporated [^{32}P]phosphate under non-stimulated (vehicle treatment) conditions, suggesting that basal PLC-β phosphorylation by kinases other than PKC may regulate PLC-β in HVSM cells. The addition of KN-93, a Ca^{2+}/calmodulin kinase inhibitor, had no effect on angiotensin II-stimulated inositol phospholipid turnover (data not shown), suggesting that Ca^{2+}/calmodulin kinase is not involved in feedback regulation of angiotensin II-stimulated inositol phospholipid hydrolysis.

4. Discussion

The complement of G proteins and PLC-β isoenzymes involved in G protein-mediated inositol phospholipid signaling in cardiovascular tissues has been investigated in a piecemeal fashion. In RVSM cells, angiotensin II stimulation of the inositol phospholipid hydrolysis pathway required at least activation of $G\alpha_q$, Gβγ, and PLC-β1 (Ushio-Fukai et al., 1998). In HVSM cells, stimulation of the same pathway required at least activation of $G\alpha_q$ and PLC-β, but not PLC-γ or PLC-δ (Schelling et al., 1997). With the exception of the central nervous system, PLC-β1 and PLC-β3 are much more widely expressed throughout the body than PLC-β2 and PLC-β4 (Fukami, 2002; Smrcka and Sternweis, 1993), and may be present alone or together depending on the tissue. In cardiovascular tissues, PLC-β3 but not PLC-β1 was observed in rat aorta (Hansen et al., 1995); and PLC-β1 and PLC-β3 isoforms were detected in rat and human renal artery (Blayney et al., 1998) and rat neonatal cardiomyocytes (Arthur et al., 2001). In HVSM cells, we detected expression of PLC-β1 and PLC-β3, but not PLC-β2 or PLC-β4 (Fig. 1).

Although often co-expressed, as in HVSM cells, PLC-β1 and PLC-β3 isoenzymes may be differentially regulated. In vivo, PLC-β1 is most likely exclusively activated by $G\alpha_q$, whereas PLC-β3 is sensitive to both $G\alpha_q$ and $G\beta\gamma$ in vitro and exclusively regulated by $G\beta\gamma$ in some systems (Rebecchi and Pentyala, 2000). Arthur et al. (2001) demonstrated in rat neonatal cardiomyocytes that PLC-β1 and PLC-β3 isoenzymes are activated differentially and specifically by α_1-adrenoceptors and purine P2Y receptors, respectively, although both receptor subtypes couple to G_q-type G proteins. Since both PLC-β1 and PLC-β3 isoenzymes are expressed in HVSM cells, which are known to express multiple G protein-coupled receptors linked to inositol phospholipid signaling, e.g., purine P2Y receptors (Erlinge, 1998), endothelin receptors (Resink et al., 1990), angiotensin AT_1 receptors (Bkaily et al., 2003), PLC-β1 and PLC-β3 differential activation may underlie different vascular smooth muscle responses to various hormones (e.g. ATP, endothelin, angiotensin). We are pursuing means for studying differential PLC-β isoenzyme activation in HVSM cells.

As previously noted, tachyphylaxis to successive angiotensin II applications is seen in vascular smooth muscle, and desensitization of inositol phosphate accumulation to repeated angiotensin II treatments was noted in RVSM cells. However, angiotensin II desensitization had not been previously quantitated in HVSM cells. We have found that the ED_{50} values for activation and desensitization of angiotensin II-mediated inositol phosphate accumulation in HVSM cells, 1.3 ± 1.0 and 4.8 ± 1.5 μM, respectively, are sufficiently similar to suggest that activation of angiotensin AT_1 receptors is closely coupled to desensitization of inositol phosphate accumulation. The slightly lower ED_{50} value for desensitization of signaling may indicate more spare receptors for desensitization than activation of signaling by angiotensin II.

The time course of desensitization of angiotensin II-activated inositol phospholipid hydrolysis varies widely among cell lines (Richard et al., 1997; Tang et al., 1995). Our quantitation of the time course of desensitization in HVSM cells shows a rapid desensitization over several minutes, followed by a slower rate of desensitization over minutes to hours (Fig. 4). This time course is similar to that seen for desensitization of G protein-coupled inositol phospholipid signaling in other systems, potentially reflecting rapid phosphorylation of signal transduction proteins followed by redistribution or degradation over a longer time period (Fisher, 1995).

Activation of PKC appears to inhibit angiotensin II-stimulated inositol phosphate accumulation in HVSM cells (Fig. 4A). To discount the contribution of receptor modification to desensitization, we stimulated inositol phosphate accumulation independently of angiotensin AT_1 receptor activation by using the G protein activator, AlF_4^-. This approach also discounts the desensitizing effect of angiotensin receptor phosphorylation by G protein-coupled receptor kinases (GRK), which most likely contributes to rapid desensitization of the signaling pathway. As we are interested in the effects of PKC activation on desensitization which are more likely to develop over a longer time course than GRK activation (reviewed by Fisher, 1995), we have quantitiated desensitization and phosphorylation of PLC-β isoenzymes at 60 min following angiotensin II treatment, after the rapid desensitization phase is complete. The effect of PMA on AlF_4^--stimulated inositol phosphate accumulation suggests that PKC activation affects PLC-β, G_q, or associated RGS (regulators of G protein signaling) proteins, inhibiting inositol phosphate accumulation and leading to signal desensitization (Fig. 4B). Our phosphorylation data suggest that neither PLC-β1 nor PLC-β3 phosphorylation is regulated by angiotensin II or PKC activation (Fig. 5). Phosphorylation of $G\alpha_q$ seems unlikely, given repeated evidence that $G\alpha_q$ is not a direct PKC substrate (Cunningham et al., 1999; Kozasa and Gilman, 1996). However, the possibility cannot be ruled out that PKC may mediate activation of downstream kinases for which $G\alpha_q$ is a substrate.

$G\beta\gamma$ dimers are another potential substrate for PKC. The PLC-β3 isoenzyme is potentially regulated by $G\beta\gamma$, but the contribution of each isoenzyme of PLC-β (1 or 3) to angiotensin II stimulation of inositol phosphate accumulation in vascular smooth muscle is not yet quantified.

Another protein candidate affected by PKC leading to inhibition of G protein-stimulated inositol phospholipid signaling is a $G\alpha_q$-associated GTPase stimulatory protein, RGS2. RGS2 is a substrate for PKC, but PKC-dependent phosphorylation of RGS2 attenuates stimulation of $G\alpha_q$'s intrinsic, self-limiting GTPase activity, which would be expected to amplify, not inhibit, inositol phospholipid hydrolysis (Cunningham et al., 2001). However, Grant et al. (2000) demonstrate that PKC activation will increase levels of RGS2 mRNA in RVSM cells. Whether this transient elevation in mRNA levels over a few hours results in elevation of RGS2 protein levels over a similar time period is as yet unknown and should not be assumed, but it could result inhibition of PLC-β isoenzyme activation and PKC-dependent desensitization of inositol phospholipid signaling.

An alternative explanation for PKC-dependent desensitization of angiotensin II-stimulated inositol phospholipid hydrolysis in the absence of PLC-β phosphorylation is that PKC affects a portion of the angiotensin II-stimulated inositol phospholipid signaling pathway that is not dependent on G protein transduction. Of interest, we demonstrate that GF109203X (a direct PKC inhibitor) potentiates angiotensin II-but not $AlF4^-$-stimulated inositol phosphate accumulation; this data suggests that in the non-stimulated state, PKC is involved in phosphorylation of a protein upstream of the G protein in the signaling pathway. Basal phosphorylation of angiotensin receptor or G protein-coupled receptor-associated proteins such as G protein-coupled receptor kinases, arrestins, or scaffolding proteins

may also play a regulatory role in receptor activation of G protein-stimulated inositol phospholipid hydrolysis. PKC may affect different proteins in the signaling pathway differently resulting in various affects under stimulated and non-stimulated conditions.

Our finding that PLC-β isoenzymes are not phosphorylated in HVSM cells following angiotensin II treatment or PKC activation was unexpected, given evidence for PLC-β phosphorylation following activation in other cell lines. However, the extensive basal phosphorylation of both PLC-β1 and PLC-β3 in HVSM cells may suggest alternative mechanisms of regulation of these isoenzymes in smooth muscle. Given that PLC-β activation is associated with smooth muscle hypertrophy as well as contraction, under normal conditions PLC-β may not be fully active in smooth muscle as a protective mechanism. Perhaps dephosphorylation of the PLC-β isoenzymes results in greater activity under yet to be determined conditions. We intend to identify sites of basal phosphorylation on PLC-β isoenzymes in HVSM cells as one means to understand the consequences of phosphorylation.

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