AN ABSTRACT OF THE THESIS OF

Yoshiko Niibori for the degree of Master of Science in Pharmacy presented on March 6, 2003.

Title: Regulation of Inositol Phospholipid Hydrolysis by Extended Treatment with Angiotensin II in Human Aortic Smooth Muscle Cells.

Abstract approved: Theresa M. Filtz

Long-term stimuli of many systems leads to decreased cellular responsiveness, or desensitization. We characterized the desensitization of angiotensin II (Ang II)-mediated inositol phospholipid (IP) hydrolysis in cultured human aortic smooth muscle cells (HASMC). Although it has been suggested that the desensitization induced by long-term Ang II exposure may result partially from down-regulation of Ang II receptor, this is not sufficient to explain fully desensitization in many systems. Post-receptor desensitization of IP hydrolysis may also result from phosphorylation or changes in protein levels of the effector enzyme, PLC-β. We identified the major PLC-β isoenzymes expressed by HASMC as PLC-β1 and PLC-β3. Ang II pretreatment reduced IP accumulation induced by Ang II (1μM) in a time-dependent manner. Phorbol ester-12-myristrate-13-acetate (PMA), a protein kinase C (PKC) activator, also reduced Ang II-stimulated IP accumulation. These results suggest that PKC activation may negatively regulate Ang II-stimulated IP signaling in HASMC, similar to rat cells. In addition, PKC also reduced IP accumulation stimulated by AlF_4^-, directly activating the G protein. It suggests that the majority of PKC-induced desensitization of Ang II-stimulated IP signaling occurs downstream of the Ang II receptor in HASMC. However, both PLC-β1 and PLC-β3, expected candidates for PKC phosphorylation, were phosphorylated independently of PKC activation or inhibition, indicating that PKC might not be involved in direct phosphorylation of PLC-β1 and PLC-β3. Furthermore, PLC-β1, but not PLC-β3, was highly phosphorylated under basal conditions, suggesting that PLC-β1 and PLC-β3 may play different roles in IP signaling in HASMC.

Regulation of Inositol Phospholipid Hydrolysis by Extended Treatment with Angiotensin II in Human Aortic Smooth Muscle Cells

by

Yoshiko Niibori

A THESIS

Submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented March 6 2003

Commencement June 2003

Master of Science thesis of Yoshiko Niibori presented on March 6, 2003.

APPROVED:

Redacted for Privacy

Major Professor, representing Pharmacy

Redacted for Privacy

Dean of the College of Pharmacy

Redacted for Privacy

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State university libraries. My signature below authorizes release of my thesis to my reader upon request.

Redacted for Privacy

Yoshiko Niibori, Author

ACKNOWLEDGMENTS

Truly, I would not have completed this study without the help and support from many people, friends, and my family.

First, I would like to thank Dr. Theresa Filtz as my major professor for her guidance and financial support throughout the course of this study.

Second, thanks and appreciations are extended to all of my friends in Filtz Lab, College of Pharmacy, and Oregon State University for their kindness. I would like to thank Ms. Setsuko Nakajima, Ms. Marcey Bamba, and Ms. Satoko Kimpara for cordially understanding, encouragement, and helping me out of disappointments. The best thing that I obtained through this study is definitely to have my great friends who are from all over the world (including Japan) and shared in anguish and joy. They always encouraged and supported me. I would like to thank them. I will not forget their hearts and empathy.

Finally, to my family, Michio, Masako, and Masaaki Niibori, I would not have completed this study in the United States without their uninterrupted encouragement and moral support. Cordial thanks and appreciations are extended to them.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

REGULATION OF INOSITOL PHOSPHOLIPID HYDROLYSIS BY EXTENDED TREATMENT WITH ANGIOTENSIN II IN HUMAN AORTIC SMOOTH MUSCLE CELLS

1. INTRODUCTION

1.1 Angiotensin II

Angiotensin II (Ang II), an octapeptide hormone, is a direct vasoconstrictor of arteries in the short term and a stimulus for vascular smooth muscle cell hyperplasia and hypertrophy in the long term (reviewed by Griendling *et al.*, 1996; Helin *et al.*, 1997; Kim and Iwao, 2000). Clinically, the stimulation of vascular smooth muscle to hypertrophy is thought to underlie the development of arteriosclerosis and the pathology of hypertension (Ushio-Fukai *et al.*, 1998a, Geisterfer *et al.*, 1988; Berk *et al.*, 1989). Physiologically, Ang II is derived from angiotensinogen, a glycoprotein derived mainly from the liver and synthesized by the liver as well as the kidney, heart, brain, vasculature and fat by the sequential actions of proteolytic enzymes (Figure 1.1). Angiotensinogen is first catabolized to Ang I, the biologically inactive decapeptide, by renin synthesized in the kidney juxtaglomerular cells. Ang I is then catabolized by angiotensin converting enzyme (ACE), which occurs in plasma, kidneys, brain, adrenal glands, ovaries, and possibly other tissues (Johnston, 1992).

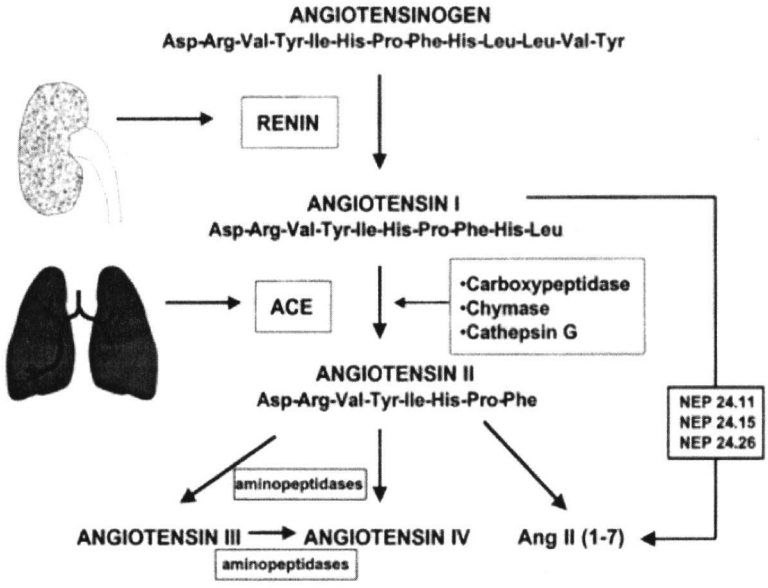

Figure adapted from Touyz and Schiffrin (2000) Figure 1

Figure 1.1 Scheme of the classic pathway of renin-angiotensin system. Renal-derived renin catalyzes hepatic-derived angiotensinogen to Ang I. Sequent catalytic enzyme, ACE, secreted from juxtaglomerular cells in the kidneys, converts Ang I to Ang II. Further proteolytic cleavage generates Ang III and Ang IV.

1.2 The signal transduction pathway of Angiotensin II

The primary effects of Ang II in vascular smooth muscle are mediated through the Ang II type-1 (AT1) receptor, which is coupled to Gq-type heterotrimeric guanine nucleotide-binding regulatory proteins (G protein) (Sasaki *et al.*, 1991, Griendling *et al.,* 1997) (Figure 1.2). Although there are two major classes of Ang II receptors, AT1 and AT2 (Timmermans *et al.*, 1993), the AT1 receptor mediates virtually all the known Ang II-mediated physiological actions in cardiovascular, renal, neuronal, hepatic, and other cells. Thus, the AT1 receptor is a major target for drug design in the treatment of hypertension, congestive heart failure, and cardiac hypertrophy (Timmermans *et al.*, 1993). Structure-function studies of the AT1 receptor reveal that the carboxyl terminus (C-terminus) of Ang II binds within the transmembrane domain and that Ang II and other peptides may also be influenced by the extracellular domain of the receptor (Bihoreau *et al.*, 1993; Ji *et al.*, 1994; Noda *et al.*, 1995; Schambye *et al.*, 1995).

After AT1 receptor stimulation, the inositol phospholipid-signaling pathway is stimulated by Gαq/11 activation of phospholipase C (PLC) (Ushio-Fukai *et al.*, 1998b). An activated PLC hydrolyzes phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2, or PIP2] to two second messengers, inositol (1,4,5)-trisphosphate [Ins(1,4,5)P_3, or IP3] and diacylglycerol, (DAG) (Ohyama *et al.*, 1992). IP3 releases calcium from calcium stores (endoplasmic reticulum) to increase levels of intracellular calcium ([Ca^{2+}]i). Ang II also increases the entry of calcium into the

cell through channels in the cell membrane (Spat *et al.*, 1991). DAG promotes the activation of protein kinase C (PKC) and calcium activates calcium calmodulin (CaM) kinase through CaM activation. Both PKC and CaM kinase catalyze the phosphorylation of proteins that ultimately regulate the Ang II-induced cell functions (Tsuda *et al.*, 1993). Therefore, PKC and CaM kinases may also catalyze the phosphorylation of proteins that regulate cell functions mediated by Ang II.

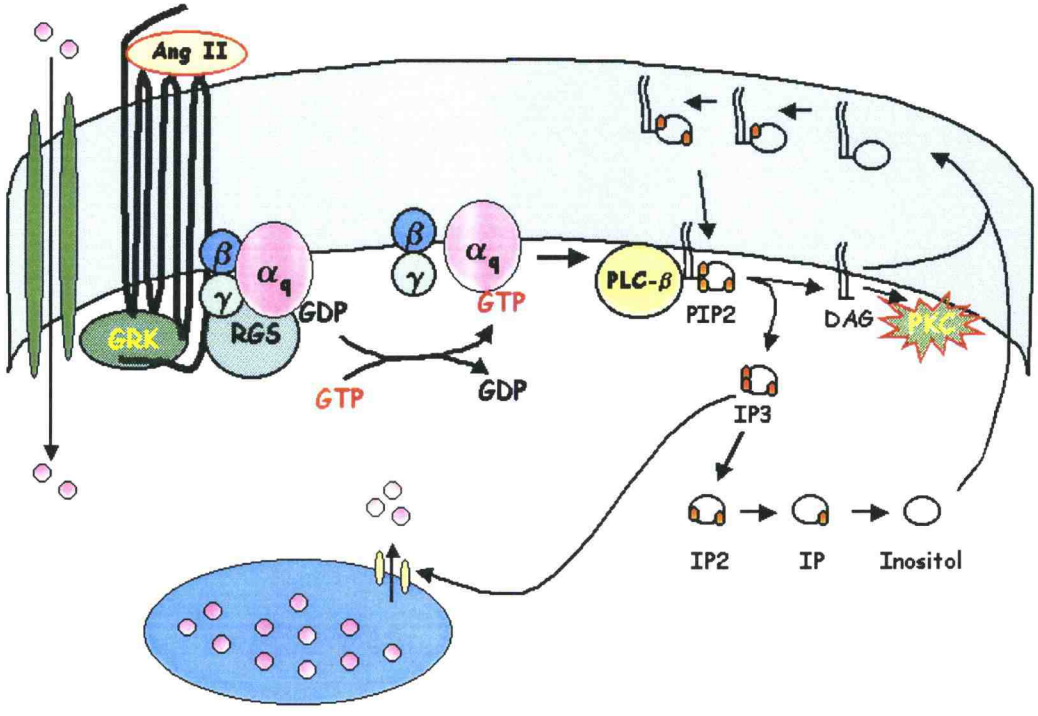

Figure 1.2 Schematic diagram of Ang II-induced intracellular signaling transduction pathway. The binding of Ang II to the AT1 receptor leads to the dissociation of subunits of the G protein (Gq/11), which activates PLC to generate two second messengers, DAG and IP3 (Ohyama *et al.*, 1992). IP3 then releases calcium from intracellular stores. Ang II also increases the entry of calcium into the cell through channels in the cell membrane (Spat *et al.*, 1991). Increased $[Ca^{2+}]i$ and membranous DAG activate enzymes, including PKC and CaM kinases that catalyze the phosphorylation of proteins, which regulate cell functions affected by Ang II (Tsuda *et al.*, 1993).

1.3 Angiotensin II-induced desensitization

In signaling transduction pathways, the rapid attenuation of responses, including inositol phospholipid turnover, by prolonged or repeated application of agonist (Lajat *et al.*, 1998) is termed desensitization. Ang II-induced desensitization of inositol phospholipid signaling has been reported (Abdellatif *et al.*, 1991; Oppermann *et al.*, 1996). Moreover, down-regulation of intracellular components in receptor/G protein-coupled inositol phospholipid signaling, such as heterotrimeric G-proteins (α, β and γ subunits) and effectors (*i.e.* PLC-β isoenzymes), has been studied in many tissues including rat aortic smooth muscle cells (Berridge and Irvine, 1984; Brock *et al.*, 1985; Fisher, 1995; Ulan and Lianas, 1990; Lassegue *et al.*, 1995). From these reports, it is expected that Ang II-induced inositol phospholipid signaling in human aortic smooth muscle cells (HASMC) may also be desensitized by down-regulation of component(s) in the signaling pathway.

1.4 Possible mechanisms for Angiotensin II-induced desensitization

Among the possible mechanisms for Ang II-induced desensitization in inositol phospholipid signaling are; 1) decreases in the membrane levels of receptors, such as AT1 receptors, 2) deactivation of G protein transducers including

activation of guanosine triphosphatase (GTPase)-activating proteins (GAP), 3) deactivation of effectors, such as PLC-β enzymes, and/or 4) activation of membrane signaling proteins which interfere in the agonist-induced signaling, *i.e.* regulators of G-protein signaling (RGS) proteins and G protein-coupled receptor kinase (GRK) (Berridge and Irvine, 1984; Fisher, 1995).

Because enzymatic activation or deactivation of proteins, such as the above-listed proteins, is often a consequence of protein modifications (*e.g.* phosphorylation), we are interested in the regulation of Ang II-induced inositol phospholipid signaling by PKC. PKC, one of the first activated enzymes following inositol phospholipid hydrolysis, is a serine/threonine protein kinase that initiates a phosphorylation cascade resulting in modification of activities for a variety of cell signaling proteins. In fact, PKC has been implicated in a negative feedback loop in inositol phospholipid signaling. Meszaros *et al.* (2000) verified that 10 min-pretreatment with a PKC activator, phorbol-12-myristate-13-acetate (PMA) attenuated Ang II-induced PLC-β activity by approximately 25%, and depletion of PKC activity by incubation with PMA for 18-20 hours amplified Ang II-induced PLC-β activity in rat cardiac fibroblasts. Also, Huwiler *et al.* (1997) showed that 30 min-pretreatment with a potent PKC activator, 12-O-tetradecanoylphorbol 13-acetate (TPA) decreases ATP-stimulated inositol phospholipid signaling in glomerular endothelial cells, and this inhibition was not observed in the cells incubated with TPA for four hours. Longer-term pretreatment (10 to 48 hr) with TPA to deplete PKC activity potentiated ATP-stimulated inositol phospholipid

signaling. Therefore, it is assumed that PKC phosphorylates these components, thereby altering their enzymatic activity, and induces desensitization of Ang II-induced inositol phospholipid signaling.

1.5 Factors as a potential site for PKC-induced desensitization

1.5.1 AT1 receptors and G protein coupled receptor kinase (GRK)

One PKC substrate candidate is the AT1 receptor. There are many reports suggesting that down-regulation of AT1 receptors in membranes may be involved in rapid agonist-induced desensitization (Oppermann *et al.*, 1996; Olivares-Reyes *et al.*, 2001; Smith *et al.*, 1998; Balmforth *et al.*, 1997; Thomas *et al.*, 1998, Qian *et al.*, 1999). Down-regulation mechanisms of receptors have been studied extensively for β2-adrenergic receptors (β2AR). Down-regulation of the receptor results from 1) the uncoupling of the receptor from heterotrimeric G proteins in response to receptor phosphorylation (Bouvier *et al.*, 1988; Hausdorff *et al.*, 1989; Lohse *et al.*, 1990 a and b), 2) the internalization of cell surface receptors to intracellular membranous compartments (Hermans *et al.*, 1997; Trejo and Coughlin, 1999; Oakley *et al.*, 1999; Anborgh *et al.*, 2000), and 3) the down-regulation of the total cellular complement of receptors due to degradation of receptors, reduced receptor mRNA, and protein synthesis (Doss *et al.*, 1981; Hadcock and Malbon

1988; Valiquette *et al.*, 1990, 1995; Jockers *et al.*, 1999; Pak *et al.*, 1999). The direct phosphorylation of the AT1 receptor, which may be involved in desensitization, is likely mediated by two kinases, GRKs (Ishizaka *et al.*, 1997; Oppermann *et al.*, 1996; Olivares-Reyes *et al.*, 2001; Smith *et al.*, 1998) and PKCs (Balmforth *et al.*, 1997; Smith *et al.*, 1998; Thomas *et al.*, 1998, Qian et al, 1999). However, GRKs and PKCs phosphorylate different positions within the serine/threonine-rich region of the C-terminus of AT1 receptors. The phosphorylation sites are at Ser^{335} and/or Thr^{336} for GRKs (Smith *et al.*, 1998) and at Ser^{331} (Smith *et al.*, 1998; Qian *et al.*, 1999), the Thr^{332}-Ser^{338} region (Thomas *et al.*, 1998), Ser^{338}, and Ser^{348} (Qian et al, 1999) for PKCs. Interestingly, GRK2 and GRK5, which are expressed in cultured vascular smooth muscle cells (Ishizaka *et al.*, 1997) are phosphorylated *in vitro* and within intact cells by PKC (Oppermann *et al.*, 1996, Chuang *et al.*, 1995; Winstel *et al.*, 1996; Pronin and Benovic, 1997) suggesting that GRKs are phosphorylated and activated by PKC. According to Winstel *et al.* (1996), PKC-mediated GRK2 phosphorylation may accelerate its translocation to the membrane rather than its catalytic activity. However, PKC-mediated GRK5 phosphorylation may attenuate its catalytic activity (Pronin and Benovic, 1997). Phosphorylated AT1 receptors recruit arrestins to prevent further interaction with the G proteins and lead to internalization and clathrin-coated pit-dependent endocytosis (Ferguson, 2001; Goodman *et al.*, 1996). From these reports, it is postulated that PKC may phosphorylate AT1 receptors in a direct and/or indirect manner to attenuate phosphorylation and degradation of AT1

receptors. Furthermore, the desensitization of Ang II-induced inositol phospholipid signaling may partially result from the down-regulation of membrane levels of AT1 receptors.

1.5.2 G proteins

Phosphorylation of the G protein as the second substrate candidate for PKC phosphorylation also has been reported. G proteins, members of a superfamily of GTPases, are essentially conserved in a variety of species and play many roles in miscellaneous aspects of cell regulation (Gilman, 1995). The family of receptor-coupled G proteins has a unique heterotrimeric composition, and there is structural and functional diversity among each of the three components (α-, β-, and γ-subunits) (Hepler and Gilman, 1992; Strathmann *et al.*, 1989). Although it has been reported that rat AT1 receptor binds various α subunits of heterotrimeric G proteins (Gq, Gq/11, Gi/o, and G12/13) under differing conditions (Kai *et al.*, 1996, Macrez *et al.*, 1997, Macrez-Lepretre *et al.*, 1997, Ushio-Fukai *et al.*, 1998b), the precise subunit to which a specific receptor couples may play different roles with respect to the specific complement of signaling pathways through activated effectors. In rat vascular smooth muscle cells, coupling of the AT1 receptor to PLC is mediated by Gαq/11 and Gα12 (Timmermans *et al.*, 1993, Kai *et al.*, 1996, Ushio-Fukai *et al.*, 1998b). The phosphorylation of Gαq/11 leads to an increase in binding to AT1 receptors and stimulates PLC-β signaling in vascular smooth

muscle (Ushio-Fukai *et al.*, 1998b; Kai *et al.*, 1996). Gαq/11 phosphorylation by PKC leads to an increase in affinity for AT1 receptors (Liu S *et al.*, 2002; Liu WW *et al.*, 1996; Umemori *et al.*, 1997; Umemori *et al.*, 1999) to support the enzymatic activity of Gαq/11. However, several other reports have demonstrated that PKC does not directly phosphorylate Gα11 (Cunningham, 1999; Kozasa and Gilman, 1996; Lounsbury *et al.*, 1993); these findings support that PKC induced reduction of Gαq mRNA levels (Kai *et al.*, 1996), but not Gαq deactivation. These reports suggest that phosphorylation of Gαq subunits is not a strong candidate for a desensitization mechanism. Although Gα12 is coupled to the AT1 receptor, Gα12 subunits mediate AT1 receptor coupling to tonic phospholipase D (PLD) activation via pp60(c-src)-dependent mechanisms rather than to PLC (Ushio-Fukai *et al.*, 1999).

A few reports have shown that some βγ subunits of heterotrimeric G-proteins, which stimulate PLC-β3 enzymes but not PLC-β1 (Camps *et al.*, 1992; Smrcka and Sternweis, 1993; Lee *et al.*, 1994; Park *et al.*, 1993), may also be phosphorylated (Wieland *et al.*, 1993). The Gβ subunit is phosphorylated transiently by GTP and dephosphorylated readily by GDP (Wieland *et al.*, 1993). Furthermore, the phosphorylated residue may be histidine (Wieland *et al.*, 1993), which is not a PKC phosphorylated residue.

1.5.3 Regulators of regulators of G-protein signaling (RGS) proteins

RGS proteins, guanosine triphosphatase (GTP)-activating proteins (GAP) and modulators of the AT1 receptors/Gαq/PLC-β interaction (Grant *et al.*, 2000; Cunningham *et al.*, 2001; Dowal et al, 2001), are also possible phosphorylation substrates for PKC. RGS proteins possessing a conserved domain (RGS domain) form a large family of highly diverse, multifunctional signaling proteins which bind directly to activated Gα subunits to modulate G protein signaling. RGS proteins differ widely in their size and amino acid identity, and many family members possess a variety of structural domains and motifs that regulate their actions and/or allow for interaction with protein binding partners with diverse cellular tasks (Hepler, 1999; Siderovski *et al.*, 1999).

It has been shown that Ang II-induced PLC-β activity in vascular smooth muscle is mediated by Gq/11 (Ushio-Fukai *et al.*, 1998b) and that the Gαq subunit interacts with RGS2 and RGS4 (Cunningham *et al.*, 2001; Grant *et al.*, 2000; Xu X *et al.*, 1999). RGS2 has been demonstrated to have specificity for Gq (Heximer *et al.*, 1997) and to be phosphorylated by PKC (Cunningham *et al.*, 2001) to increase GAP activity, which should inhibit Gαq. Additionally, mRNA levels of RGS2 in rat vascular smooth muscle cells are increased by PKC activation (Grant *et al.*, 2000). While direct and indirect RGS activation by PKC may reduce PLC-β activity, it has also been reported that RGS4 binds not only activated Gαq, but also Gβγ and PLC-β1 (Dowal *et al.*, 2001) and increases Gβγ activity in HEK293 cells

(Bunemann and Hosey, 1998; Camps *et al.*, 1992; Katz *et al.*, 1992), which may stimulate PLC-β3 isoenzymes. These reports suggest that RGS may be one of the PKC substrates required for PKC-related desensitization of agonist-induced inositol phospholipid hydrolysis; but functional consequences of RGS phosphorylation on PLC-β activity in whole cells remains to be investigated.

1.5.4 Phospholipase C-β

PLC-β as a component of the receptor/G protein coupled signaling pathway has been reported to be involved in the desensitization of agonist-mediated inositol phospholipid signaling. PLC-β, one of four PLC subfamilies (PLC-β, -δ, -γ, and -ε) (Rhee and Choi, 1992; Cockcroft and Thomas, 1992; Berridge, 1993; Noh *et al.*, 1995; Lee and Rhee, 1995, Lopez *et al.*, 2001; Song *et al.*, 2001), is subdivided into four isoenzymes (PLC-β1, PLC-β2, PLC-β3 and PLC-β4) in mammals. PLC-β1 is the most widely expressed of the PLC-β isoenzymes, and the highest concentration is found in specific regions of the brain (Gerfen *et al.*, 1988; Homma *et al.*, 1989; Mizuguchi *et al.*, 1991). The highest expression levels of PLC-β2 are observed in hematopoietic cells (Lee *et al.*, 1996). PLC-β3 protein is also widely expressed, similar to PLC-β1. The highest concentration of PLC-β3 is found in brain, liver, and parotid gland (Jhon *et al.*, 1993; Tanaka and Kondo, 1994). PLC-β4 is observed in the cerebellum (Min *et al.*, 1993 a and b; Tanaka and Kondo, 1994) and the retina (Jiang *et al.*, 1994; Lee *et al.*, 1994).

Among desensitization reports involving PLC-β isoenzymes, Sorensen *et al.* (1998) demonstrated that protein levels of PLC-β1, but not PLC-β3 or PLC-γ1 isoforms, were attenuated in SH-SY5Y neuroblastoma cells, following 24 hour-stimulation of muscarinic receptors by oxotremorine-M, the muscarinic receptor agonist (activating PLC-β via a seven transmembrane domain G protein-coupled receptor, GPCR). In addition, Filtz *et al.* (1999) reported that stimulation by P2Y1 receptor (one of GPCRs, stimulating inositol lipid signalling) or by PMA augments phosphorylation of the G protein-activated PLC-βT (a PLC-β subtype in turkey erythrocytes, similar to mammalian PLC-β2) and decreases PLC-βT activity *in vitro*. Filtz *et al.* (1999) also revealed that PLC-βT and PLC-β2, but not PLC-β1, were phosphorylated by PKC *in vitro*, and concluded that phosphorylation by PKC was isoenzyme-specific. Additionally, they demonstrated that PKC-induced or physiologically induced phosphorylation of PLC-βT may be involved in loss of enzyme activity, reflected as a decrease in overall catalytic activity, rather than as a specific modification of G protein-regulated activity. Furthermore, Ali *et al.* (1997) demonstrated that platelet-activating factor (PAF, activating PLC-β via GPCR) or PMA pretreatment inhibited PAF-mediated PLC-β activity by approximately 90% in RBL-2H3 cells. Of the 90% inhibition, 50% may be unrelated to receptor phosphorylation. Ali *et al.* (1997) concluded that PAF-mediated desensitization results from PKC-mediated phosphorylation at both the receptor and PLC-β3. From these reports, it is assumed that phosphorylation of

PLC-β isoenzymes are tissue and receptor specific. We examined which PLC-β isoenzymes in HASMC are phosphorylated.

1.6 Hypothesis

We hypothesized that PLC-β enzyme activity is decreased or desensitized by extended Ang II stimulation in HASMC. First, we identified which of the four known mammalian subtypes of PLC-β isoenzymes, PLC-β1, PLC-β2, PLC-β3, and PLC-β4, (Rhee and Bae, 1997; Rebecchi and Pentyala, 2000; Rhee, 2001) were expressed in HASMC. We then characterized the mechanism of inositol phospholipid signaling desensitization by Ang II in these cells.

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 corp. (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^{TM} chemiluminescent substrate, and AG1-X8 Resin were from Bio-RAD Laboratories (Hercules, CA). Dulbecco's Modification of Eagles Medium (DMEM) (containing 4.5g/l glucose, L-glutamine, and sodium pyruvate) and Penicillin/Streptomycin were from Cellgro (Herndon, VA). Standard fetal bovine serum was from HyClone (Logan, Utah). Insulin and Phosphorus-32 [^{32}P] were from ICN Pharmaceuticals, Inc (Costa Mesa, CA). Polyclonal rabbit anti-PLC-β_1, PLC-β_2, PLC-β_3, and PLC-β_4 antibodies: PLC-β1 (anti-PLC-β1, C-terminal, G-12, 1204-1216), PLC-β2 (anti-PLC-β2, C-terminal, Q-15, 1170-1181), PLC-β3 (anti-PLC-β3, C-terminal, C-20, 1198-1217) and PLC-β4 (anti-PLC-β4, C-terminal, C-18), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Ang II, phorbol-12-myristate-13-acetate (PMA), and 4α-phorbol 12, 13-didecanoate (4αPDD), Dimethyl sulfoxide (DMSO) were obtained from Sigma

Chemical Co. (St. Louis, MO), and Bisindolylmaleimide or GF109203X (GF), 8-(4-chlorophenylthio)-adenosine 3-cyclic monophosphate 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 Human Aortic Smooth Muscle Cells

Human aortic smooth muscle cells (HASMC;>90% homogeneous smooth muscle phenotype, by flow cytometry) were purchased from Cascade Biologics™ (Portland, Oregon). HASMC from *passages 4-12* were routinely seeded at a density of $5x10^4$ cells/cm^2. HASMC were maintained in bicarbonate-buffered Dulbecco's Modification of Eagles Medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 4.5mg/L glutamine, 10^{-6} % (w/v) human recombinant epidermal growth factor (EGF), $2x10^{-7}$ % (w/v) human recombinant fibroblast growth factor (FGF), $5x10^{-4}$ % (w/v) insulin, 50 units/ml penicillin, 50 mg/ml streptomycin in a 5% CO_2 humidified atmosphere at 37°C.

2.3 Detection of PLC-β isoenzymes in HASMC by Immunoprecipitation and Western blotting

HASMC were washed in ice-cold phosphate buffered saline (PBS: 137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.4mM KH_2PO_4, pH 7.3) and incubated for 10 minutes (min) in lysis buffer, containing 10mM Tris-HCl (pH 7.4), 2mM EDTA, 5mM $MgCl_2$ and protease inhibitors, (200nM benzamidine, 10μM leupeptin, 1μM pepstatine A, and 200nM phenylmethylsulfonyl fluoride), followed by 15 strokes of Dounce homogenization. HASMC lysates were extracted with buffer containing 50mM Hepes (pH 7.4), 2.5mM EDTA, 150mM NaCl, 1mM dithiothreitol (DTT), and 1% triton X-100 with protease inhibitors for 1 hour and centrifuged at 16,000 rpm at 4°C for 30 min. The soluble extract was incubated with 1μg/ml of isoenzyme selective anti-PLC-β antibodies overnight at 4°C for immunoprecipitation, followed by incubation with 50μl of suspended protein A-Sepharose beads (100mg/ml) for 1 hour at 4°C (Protein A-Sepharose beads were pre-equilibrated in extraction buffer in accordance with manufacturers specifications). Immune complexes were washed three times with extraction buffer and were resuspended in 25μl of extraction buffer plus 25μl of 2x-SDS-PAGE loading buffer containing 50mM Tris-HCl (pH 8.0), 30% Urea, 5% Sodium dodecyl Sulfate (SDS), 6% dithiothreitol (DTT), and 0.01% Bromophenol blue. Finally, samples were boiled for 5 min to separate from Protein A-Sepharose beads and centrifuged for 15 sec to pellet insoluble particulates. The immunoprecipitate

supernatants were separated by 7.5% (w/v) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 200V for 1 hour and size-fractionated proteins were transferred to nitrocellulose membranes at 100V for 1.5 hour for Western blotting. Western blots were performed by incubation with 1:3000 dilution of anti-PLC-β antibody in immunoblot buffer (20mM Tris-HCl, pH 7.4, 500mM NaCl) as described by Filtz *et al.* (1999). The blot was incubated with secondary anti-rabbit IgG antibody conjugated to alkaline phosphatase at 1:5000 dilution in immunoblot buffer containing 1% BSA and 0.02% sodium azide. Immunoreactive bands were visualized by enhanced chemiluminescence alkaline phosphatase substrate (Immune-Star^{TM}) and exposure to X-ray film for 10 second (sec) to 5 min.

2.4 Measurement of Phospholipase C–β Activity

HASMC were seeded in 24-well plates overnight and radiolabeled by incubation in 0.5ml of serum-free, inositol-free DMEM containing 1μCi of *myo*-[^{3}H]inositol for 24 hours. Drugs were added according to the following schedules: the cells were supplemented with serum-free DMEM containing 10mM HEPES, pH 7.4, and 10mM LiCl (final concentration) for 10 min followed by stimulators (Ang II or AlF_4^-) to allow for accumulation of inositol phosphate hydrolysis products. Incubations were terminated by aspiration of the drug-containing medium and addition of 0.5ml of ice-cold 5% (w/v) trichloroacetic acid (TCA).

TCA-soluble cell fractions were extracted 3 times with 2ml diethyl ether, and loaded onto a 1ml-column of anion exchange resin (AG1- X8 Resin, formate form; 200-400 mesh) to isolate [^{3}H] inositol phosphates. The columns were washed with water and a 50mM ammonium formate/0.1M formic acid solution, and eluted with 1.2M ammonium formate/0.1M formic acid solution, as described previously by Filtz *et al.* (1994). The eluted samples, containing accumulated total ^{3}H-inositol phosphates [*i.e.* inositol trisphosphate ($InsP_3$), inositol bisphosphate ($InsP_2$), and inositol monophosphate ($InsP_1$)], were quantitated by liquid scintillation spectrometry.

TCA precipitates containing accumulated ^{3}H-inositol phospholipids were solubilized with 0.5ml of 1N NaOH, and neutralized with 0.5ml 1N HCl. The neutralized samples were quantitated by liquid scintillation spectrometry. PLC activity was expressed as percentage of total inositol phosphates accumulated under varying conditions, relative to maximal response obtained with 1μM Ang II treatment, as described in data analysis.

2.5 Phosphorylation assay for PLC-β1 and PLC-β3

HASMC (>5x10^{6}cells/sample) were collected and washed with phosphate-free DMEM. The cells were radiolabeled by incubation in serum-free, phosphate-free DMEM with 200μCi/sample of [^{32}P] orthophosphate for 90 min. Cells were

then treated with drugs for indicated times. Following drug treatment, cells were washed with ice cold phosphate buffer saline (PBS) and incubated for 10 min in lysis buffer containing 10mM Tris-HCl (pH 7.4), 2mM EDTA, 5mM $MgCl_2$, protease inhibitors (see 2.3), 50mM NaF and 10mM β-glycerophosphate followed by 15 strokes of Dounce homogenization. HASMC lysates were extracted with buffer containing 50mM Hepes (pH 7.4), 2.5mM EDTA, 150mM NaCl, 1mM dithiothreitol (DTT), 1% triton X-100, 50mM NaF and 10mM β-glycerophosphate with protease inhibitors for 1 hour with inversion, and centrifuged at 16,000 rpm at 4°C for 30 min. After centrifugation, the supernatants were incubated with 6μg/sample of isoenzyme selective anti-PLC-β antibodies overnight at 4°C for immunoprecipitation, as described above. The immunoprecipitates were separated by 7.5% (w/v) SDS-PAGE and fractionated proteins were transferred to nitrocellulose membranes. The membranes were dried and exposed to X-ray film to detect [^{32}P]-radiolabeled, immunoprecipitated proteins, followed by anti-PLC-β Western blotting to detect proteins.

2.6 Data Analysis

Data from the inositol phosphates (InsP) accumulation assay were expressed as % of maximal response, using the equation below. <1> and <2> were raw data quantitated by liquid scintillation as the total accumulated ^{3}H-inositol

phosphates (InsP) and the recovered ^{3}H-inositol phospholipid (PtlIns), respectively. <3> % InsP accumulation is a calculation of the % of ^{3}H-inositol converted from ^{3}H-inositol phospholipid to ^{3}H-inositol phosphate. "Experimental response" is always expressed as response to drug treatment minus basal InsP accumulation in the presence of vehicle <4>. "Maximal response" in <5> refers to the "Experimental response" under conditions of treatment with 10μM Ang II.

Labeled Ins (dpm) = InsP..<1>

Labeled Ptdlns (dpm) = Ptdlns...<2>

$$\text{\% InsP accumulation (\% InsP acc)} = \frac{<1>}{<1> + <2>} \quad\ldots\ldots\ldots\ldots <3>$$

Experimental response = (Experimental % InsP acc)-(Control % InsP acc)........<4>

$$\text{\% of maximal response} = \frac{\text{Experimental response}}{\text{Maximal response}} \quad\ldots\ldots\ldots\ldots <5>$$

Data from dose-response curves for Ang II stimulation of inositol phospholipid hydrolysis in HASMC (Figure 3.3) were analyzed by a curve-fitting program using nonlinear regression to find the EC_{50} value (Prism™ by GraphPad Software).

2.7 Statistical Analysis

Statistical analysis was performed using the two-tailed Student's t test

3. RESULTS

3.1 Detection of PLC-β isoenzymes in HASMC

To identify which PLC-β isoenzymes are expressed in HASMC, Western blot analysis was performed with PLC-β isoenzyme selective antibodies shown to be cross reactive to human, rat, and bovine isotypes. The major PLC-β isoenzymes expressed by HASMC are PLC-β1 (150kDal) and PLC-β3 (133kDal) at predicted molecular weights: PLC-β1 and PLC-β3 are extensively expressed throughout the body, including heart tissue (Hansen *et al.*, 1995; Jhon *et al.*, 1993; Ju *et al.*, 1998; Rhee and Bae, 1997). PLC-β2 (120kDal) and PLC-β4 (90kDal and/or 130kDal) were not expressed at detectable levels (Figure 3.1) as expected. 1321N1 cells, which were recently shown to express PLC-β1 and PLC-β3 (Filtz, unpublished data), purified PLC-β2 protein, and bovine cerebellum which expresses PLC-β4 (Min *et al.*, 1993 a and b), were used as positive controls.

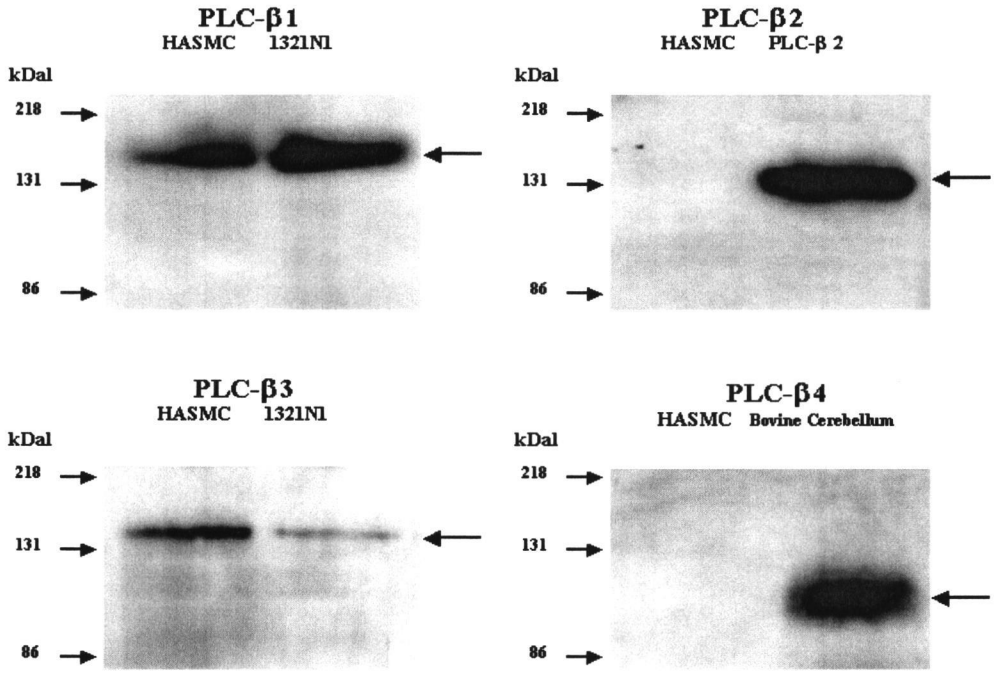

Figure 3.1: Immunoblot detection of PLC-* β1 *and PLC-* β3 *isoenzymes in HASMC. HASMC, 1321N1 cells, or bovine cerebellum lysates were extracted with 1% triton X-100 and the soluble fraction incubated with isoenzyme selective anti-PLC-β antibodies for immunoprecipitation and Western blotting with anti-PLC-β antibodies as indicated. Migration of molecular mass markers is indicated (kDal). As a positive control for PLC-β2 immunoprecipitation, 1μg of purified recombinant PLC-β2 was included where indicated. Each experiment for PLC-β isoenzymes was repeated 2-4 times.

3.2 Inositol Phosphates Accumulation Assay

3.2.1 Characteristics of Ang II on inositol phospholipid hydrolysis in HASMC

To characterize Ang II-induced inositol phospholipid signaling, the dose-response and time-course for Ang II treatment were determined (Figure 3.2 and Figure 3.3). HASMC were treated with 1μM Ang II for 0-120 min. Ang II stimulated inositol phosphate accumulation in a time-dependent manner and the time course for Ang II reached a plateau at 75-90 min. However, inositol phosphates also accumulated in the presence of LiCl and vehicle alone (open circle), time-dependently. When the effect of the vehicle was subtracted from the Ang II effect, the time-course for Ang II reached a plateau at 45-60 min (data not shown) (Figure 3.2). HASMC were also treated with 10^{-7}-10^{-4} M Ang II for 60 min. Ang II stimulated inositol phosphate accumulation in a dose-dependent manner with an EC_{50} value of 4.81 ± 0.79 μM (Figure 3.3).

To investigate the time course of desensitization to Ang II treatment, HASMC were treated with Ang II 10^{-5} M, a submaximal dose of Ang II, for 0-180 min in the absence of LiCl to avoid accumulation of inositol-1,4,5-trisphosphate (IP_3) product. HASMC were then challenged with a second dose of Ang II (10^{-6} M) in the presence of LiCl to allow for accumulation of IP products. In comparison to the control response (the effect of 10^{-6} M Ang II without Ang II pretreatment), the response pretreated with 10^{-5} M Ang II and challenged with

10^{-6} M Ang II was reduced as a function of pretreatment time with Ang II (Figure 3.4). The intensity of the desensitization induced by Ang II pretreatment depended on pretreatment time of Ang II. Desensitization of inositol phosphate accumulation to Ang II pretreatment reached a maximum 65% reduction at 60-90 min.

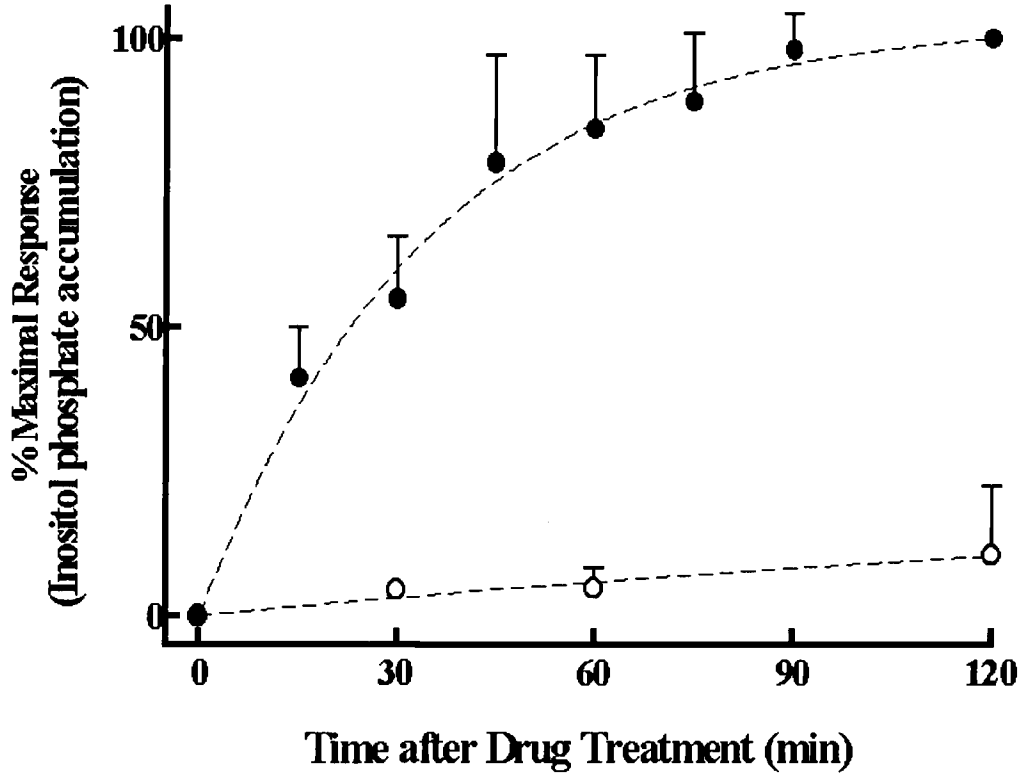

Figure 3.2: Time course of Ang II stimulation of inositol phospholipid hydrolysis in HASMC. HASMC were treated with Ang II (1μM) for indicated times in the presence of 10mM LiCl. PLC-β activity is expressed as percent maximal response to response of Ang II (1μM) for 120 min treatment minus basal levels of accumulation in the absence of stimulation. Each data point represents the cumulative mean ± S.E. of five (•: Ang II treatment), or three (o: vehicle treatment) experiments performed in triplicate. The time course reached a maximum plateau at approximately 60 min.

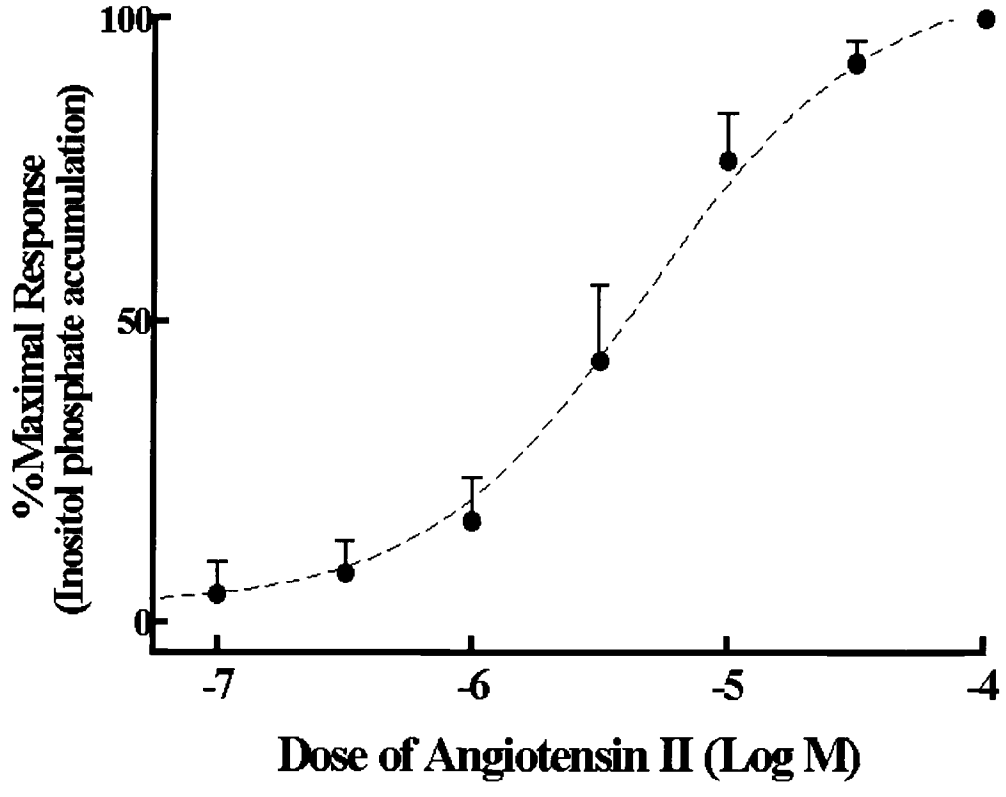

Figure 3.3: Dose-response curve for Ang II stimulation of inositol phospholipid hydrolysis in HASMC. HASMC were treated with the indicated concentrations of Ang II for 60 min in the presence of 10mM LiCl. PLC-β activity was expressed as percent maximal response to Ang II minus basal levels of accumulation in the absence of stimulation. Shown is cumulative average data from four experiments conducted in triplicate ± S.E. EC_{50}= 4.81± 0.79 μM.

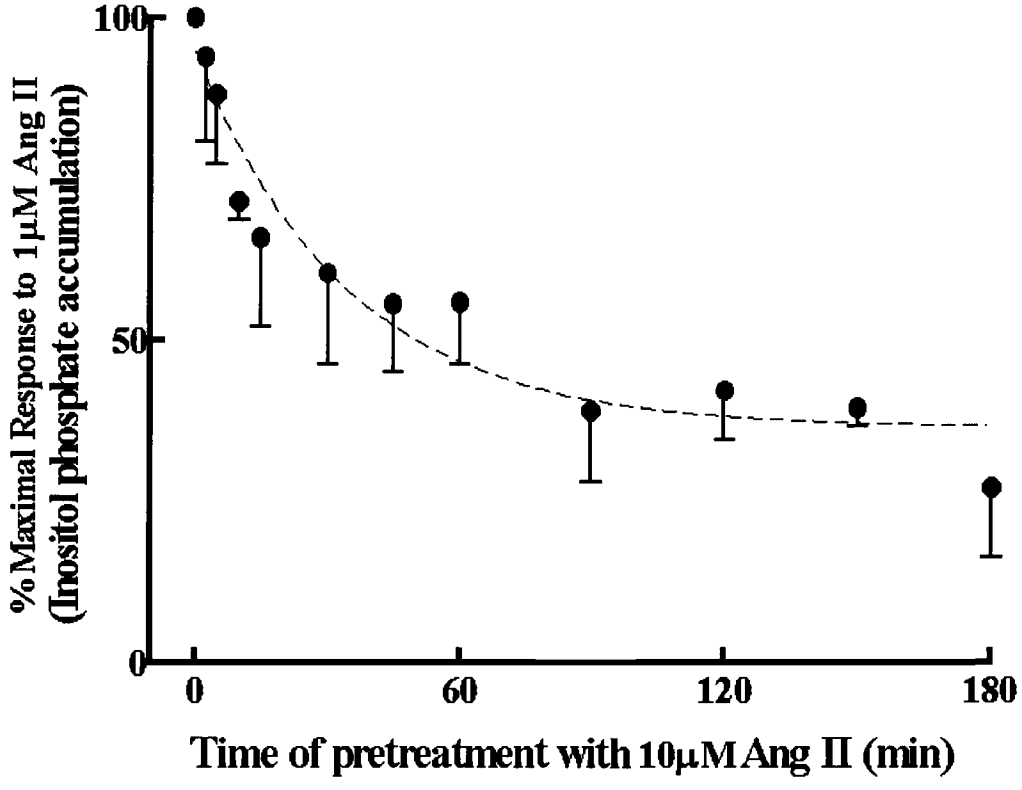

Figure 3.4: The time course of Ang II-induced desensitization in HASMC. HASMC were pretreated with 10μM Ang II in the absence of LiCl for indicated times and washed twice, then challenged with 1μM Ang II for 60 min in the presence of 10mM LiCl to allow for inositol phospholipid accumulation. PLC-β activity is expressed as percent maximal response to 1μM Ang II in the absence of pretreatment minus basal accumulation in the absence of stimulation. Data shown are cumulative mean ± S.E. of three experiments conducted in triplicate.

3.2.2 Investigation of regulation on inositol phospholipid hydrolysis by extended treatment with angiotensin II in HASMC

Having established the time course for desensitization of inositol phospholipid signaling to Ang II stimulation in HASMC, the possible mechanisms for desensitization were examined. Since PKC, one of the first activated enzymes following inositol phospholipid hydrolysis, has been implicated in a negative feedback loop in other systems (Meszaros *et al.*, 2000; Ozawa *et al.*, 1993; Xu A *et al.*, 2001), HASMC were treated with PMA (a PKC activator), 4αPDD (an inactive analogue of PMA), and GF109203X (a PKC specific inhibitor) to assess the role of PKC in Ang II-induced desensitization. Following 5 min pretreatment with 1μM PMA or 4αPDD, HASMC were stimulated by 1μM Ang II, and inositol phosphates were allowed to accumulate in the presence of 10mM LiCl for 60 min. Pretreatment with PMA, but not with 4αPDD, reduced Ang II-induced inositol phosphate accumulation by 50% (Figure 3.5). This reduction of Ang II-induced inositol phosphate accumulation by PMA was inhibited by 30 min pretreatment with 10μM GF109203X (Figure 3.6). Additionally, pretreatment with GF109203X alone increased inositol phosphate accumulation two-fold from basal levels, suggesting that basal PKC activating may affect activation of PLC.

To evaluate the contribution of Ang II receptor downregulation to inositol phospholipid desensitization, inositol phospholipid hydrolysis was stimulated by AlF_4^-, a direct activator of G proteins. HASMC were pretreated with PMA for 5 min, 4αPDD for 5 min, or GF109203X for 30 min. Pretreatment with PMA

inhibited AlF_4^--induced inositol phosphate accumulation by 50-100%. However, this inhibition is not statistically significant. Inhibition only occurred with PMA, but not with 4αPDD or GF109203X (Figures 3.7 and 3.8). As above, pretreatment with GF109203X enhanced AlF_4^--induced inositol phosphate accumulation by 50-100%. These data suggest that PKC affects Ang II-induced inositol phosphate accumulation downstream of the receptor at the G protein or at PLC-β.

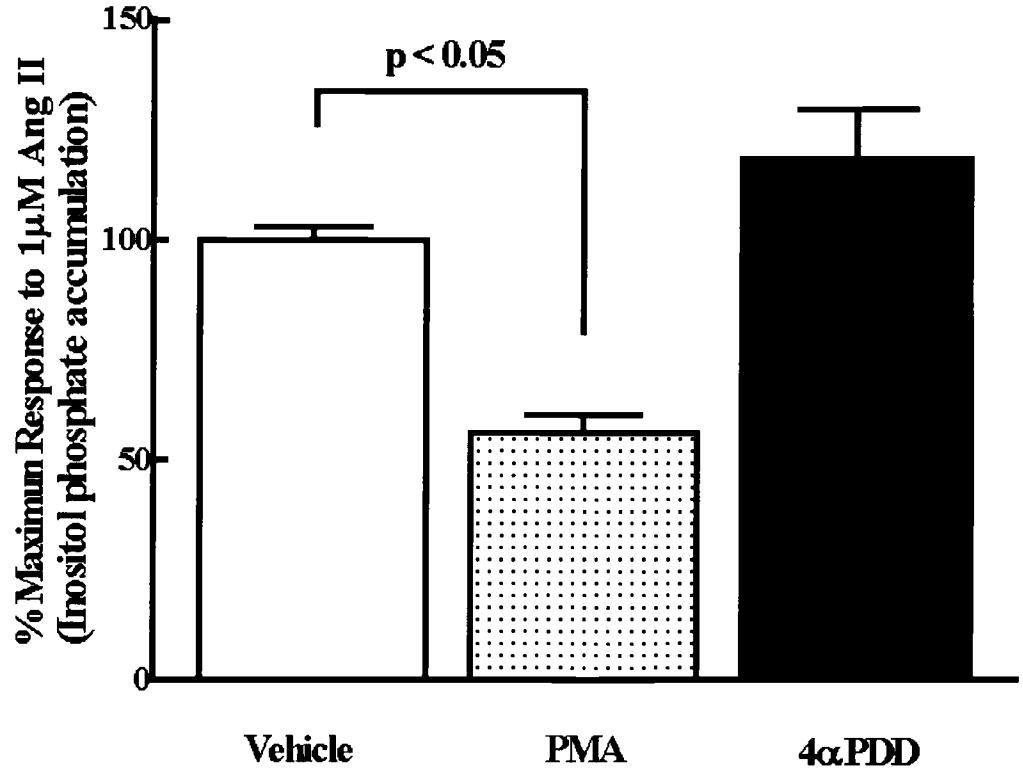

Figure 3.5: Effects of PMA, and 4αPDD pretreatment on Ang II-induced inositol phosphate accumulation. HASMC were pretreated with PMA (1μM), 4αPDD (1μM), or vehicle (DMSO, 0.1%) for 5 min, followed by Ang II (1μM) stimulation for 60 min in the presence of 10mM LiCl. PLC-β activity is expressed as percent maximal response to 1μM Ang II for 60 min in the absence of pretreatment minus basal accumulation in the absence of stimulation. Data shown are cumulative mean ± S.E. of five experiments preformed in triplicate.

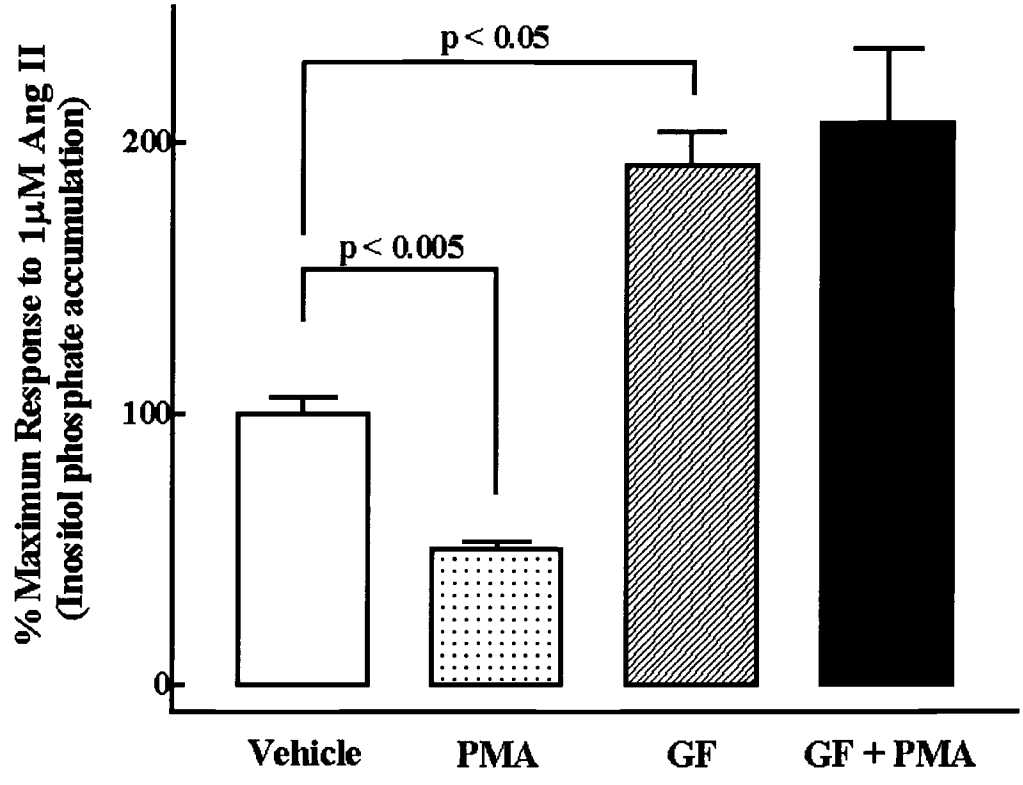

Figure 3.6: GF109203X inhibits the effect of PMA to reduced Ang II-induced inositol phosphate accumulation in HASMC. HASMC were pretreated with vehicle (0.1% DMSO, 30 min), PMA (1μM, 5 min), GF109203X (GF; 10μM, 30 min), or GF109203X + PMA (GF+PMA). Following pretreatment, 10mM LiCl was added with 1μM Ang II to stimulate inositol phosphate accumulation for 60 min. PLC-β activity is expressed as percent maximal response to 1μM Ang II in the presence of 0.1% DMSO pretreatment minus basal accumulation in the absence of stimulation following pretreatment. Data shown are cumulative mean ± S.E. from three experiments conducted in triplicate.

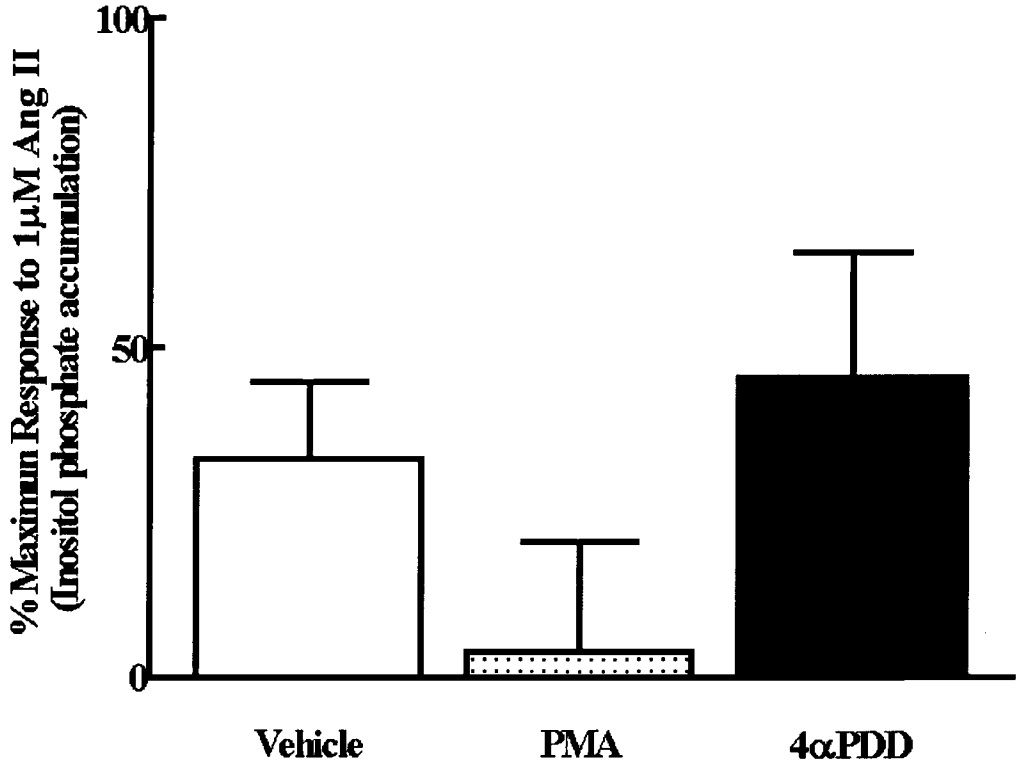

Figure 3.7: Effects of PMA, and 4αPDD pretreatment on basal and AlF_4^--induced inositol phosphate accumulation. HASMC were pretreated with PMA (1μM), 4αPDD (1μM), or vehicle (0.1% DMSO) for 5 min followed by AlF_4^--stimulation for 60 min in the presence of 10mM LiCl. PLC-β activity is expressed as a percentage of the maximal response to 1μM Ang II for 60 min in the absence of pretreatment. Basal accumulation in the absence of stimulation has been subtracted. Data shown are cumulative mean ± S.E. of seven experiments preformed in triplicate.

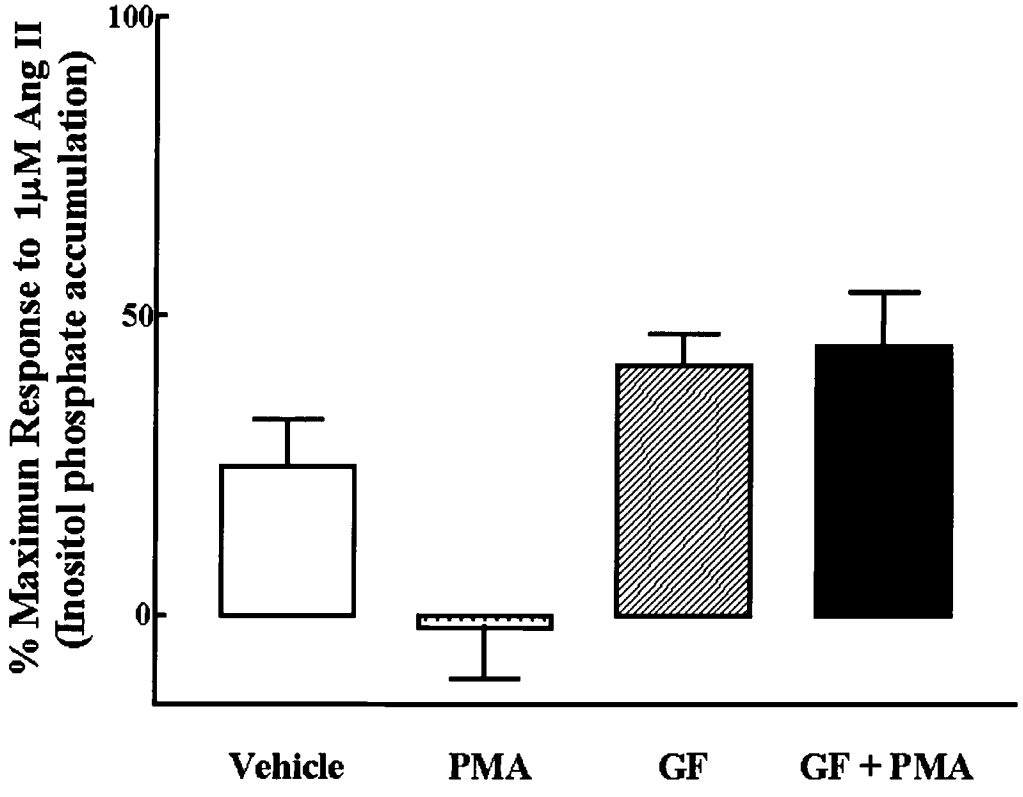

Figure 3.8: GF109203X inhibits the effect of PMA to reduce AlF_4^--induced inositol phosphate accumulation in HASMC. HASMC were pretreated with vehicle (0.1% DMSO, 30 min), PMA (1μM, 5 min), GF109203X (GF; 10μM, 30 min), or GF109203X + PMA (GF+PMA). Following pretreatment, AlF_4^- was added in the presence of LiCl to stimulate inositol phosphate accumulation for 60 min. PLC-β activity is expressed as a percentage of the maximal response to Ang II 1μM in the presence of vehicle (0.1% DMSO) pretreatment. Basal accumulation in the absence of AlF_4^--stimulation was subtracted. Data shown are cumulative mean ± S.E. from three experiments conducted in triplicate.

3.3 Phosphorylation of PLC-β isoenzymes in HASMC

Since PMA inhibited and GF109203X enhanced Ang II- and AlF_4^--induced inositol phosphate accumulation, we investigated the possibility that Ang II induces phosphorylation of PLC-β isoenzymes, PLC-β1 and PLC-β3, in HASMC through activation of PKC. HASMC were treated with 1μM Ang II, 1μM PMA, and 10μM GF109203X alone or in combination as indicated (Figure 3.9). PLC-β1 (Figure 3.9a) and PLC-β3 (Figure 3.9b) were immunoprecipitated from drug treated and [^{32}P]-radiolabelled HASMC. [^{32}P]-radiolabelled immunoactive bands at 133kDal (PLC-β3) and 150kDal (PLC-β1) were detected for each treatment. Differences among treatments for [^{32}P] incorporation were paralleled by differences in intensity of immunoreactive bands of the same size and were not consistent between experiments, suggesting that differences in [^{32}P] autoradiography were solely a result of sample-to-sample variability in loading and not a result of drug treatments. However, both PLC-β1 and PLC-β3 appear to incorporate [^{32}P] under non-stimulated conditions, suggesting that basal PLC-β phosphorylation by kinases other than PKC may regulate PLC-β in HASMC.

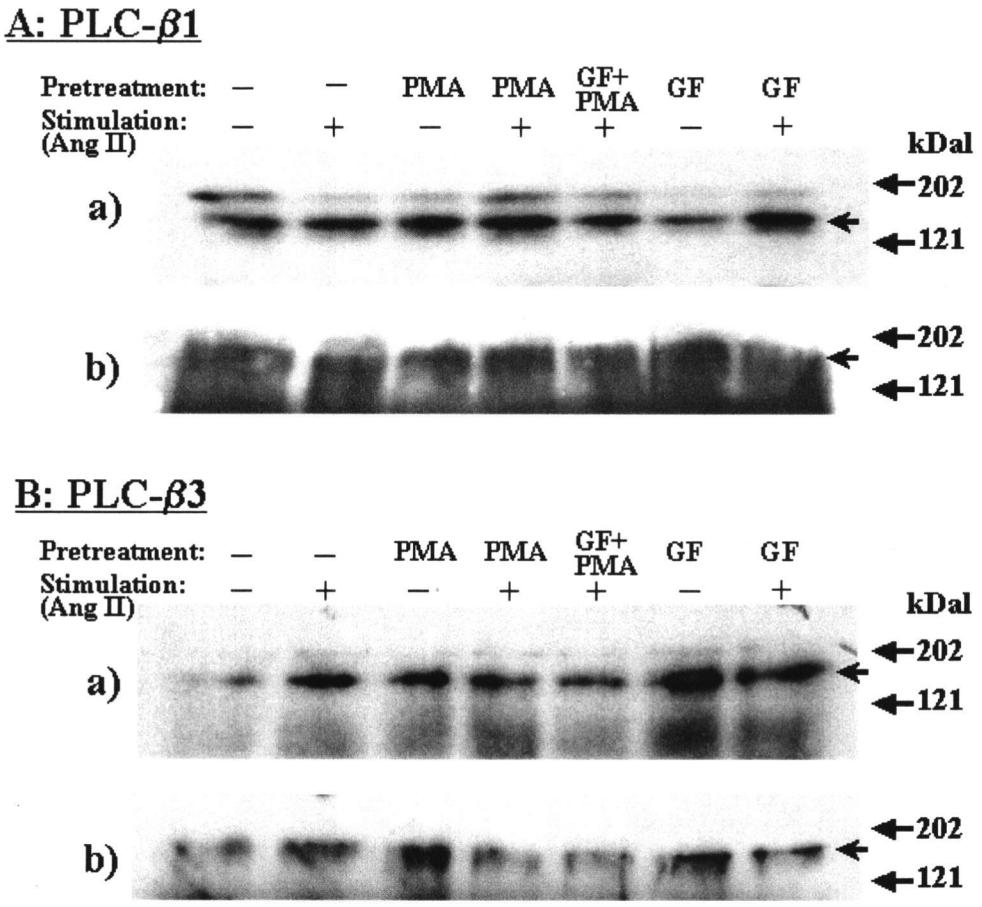

Figure 3.9: Immunoprecipitation with anti PLC-β1 antibodies (A) or anti PLC-β3 antibodies (B) in HASMC. Whole cells radiolabeled with [^{32}P] orthophosphate were extracted with detergent as indicated in methods. Following immunoprecipitation, SDS-PAGE separation, and transfer to nitrocellulose membranes, the membranes were exposed to X-ray film for 24-48 hrs for detection of [^{32}P] incorporation by autoradiography (a) or incubated with anti-PLC-β selective antibodies for Western blotting immunodetection (b). Treatments or pretreatments were as follows: 0.1% DMSO for 5 min and/or 30 min, 1μM PMA for 5 min, 10μM GF109203X for 30 min, 10μM Ang II for 60 min. Data shown are representation of three experiments.

4 DISCUSSION

4.1 Angiotensin II in vascular smooth muscle

Ang II in vascular smooth muscle is a clinically important hormone for the development of arteriosclerosis and the pathology of hypertension (Ushio-Fukai *et al.*, 1998a, Geisterfer *et al.*, 1988; Berk *et al.*, 1989). Therefore, we think that it is important to study the regulation, especially downregulation, of Ang II-induced signaling pathways in human vascular smooth muscle. Despite the fact that many studies have reported Ang II-induced desensitization of inositol phospholipid hydrolysis, none have utilized human vascular smooth muscle cells, with rodent smooth muscle cells being the closet cell type examined (Abdellatif *et al.*, 1991; Iglesias *et al.*, 2001; Lassegue *et al.*, 1995; Meszaros *et al.*, 2000; Ullian and Linas, 1990). In this study, we report the first detailed characterization of Ang II-induced desensitization in cultured, primary human aortic smooth muscle cells (HASMC).

4.2 Angiotensin II-induced inositol phospholipid signaling in HASMC

Both PLC-β1 and PLC-β3 were expressed in HASMCs (Figure 3.1), similar to reports using rat vascular smooth muscle cells (Ushio-Fukai *et al.*, 1998b; Blayney *et al.*, 1998). Ang II stimulated inositol phospholipid turnover in a dose-

and time-dependent manner (Figure 3.2 and Figure 3.3). Extended treatment with Ang II led to desensitization of inositol phospholipid hydrolysis, which is a measurement of PLC-β mediated signaling (Figure 3.4). PKC activation by PMA also inhibited Ang II-induced inositol phospholipid hydrolysis in HASMC (Figure 3.5 and Figure 3.6). In addition, 4αPDD (an inactive analogue of PMA) did not exert any influence on Ang II-induced inositol phospholipid hydrolysis (Figure 3.5), and GF109203X (a PKC specific inhibitor) inhibited PMA-induced inhibition in Ang II-induced inositol phospholipid hydrolysis (Figure 3.6). These results confirm that PKC activation, an event following PLC-β activation, may negatively regulate Ang II-induced inositol phospholipid signaling in HASMC, similar to regulation of GPCR agonist-stimulated inositol phospholipid signaling in other cell types (Ali *et al.*, 1998; Filtz *et al.*, 1999; Oppermann *et al.*, 1996).

4.3 Potential substrates for PKC-induced desensitization in HASMC

4.3.1 AT1 receptors and G protein coupled receptor kinase (GRK)

Proposed candidates for PKC phosphorylation substrates which correlate with desensitization are the AT1 receptor, PLC-β enzymes, and membrane signaling proteins which interfere in the agonist-mediated signaling, *e.g.* RGS proteins. We found that AlF_4^--stimulated inositol phospholipid signaling in

HASMC is tending to be inhibited by PKC activation (Figure 3.7 and Figure 3.8). Since AlF_4^- directly activates the G protein (Yu *et al.*, 1995), involvement of the AT1 receptor is bypassed by stimulation of inositol phospholipid signaling by AlF_4^- instead of Ang II. PMA pretreatment tended to inhibit AlF_4^--stimulated inositol phospholipid signaling to roughly the same extent as PMA-induced inhibition of Ang II-induced inositol phospholipid hydrolysis (Figure 3.7 and Figure 3.8). A less robust effect of AlF_4^--stimulated inositol phospholipid hydrolysis, compared to Ang II, made AlF_4^--induced activation more difficult to quantitate. Therefore, tendencies, but not statistical significance, are noted. 4αPDD did not affect AlF_4^--stimulated inositol phospholipid signaling (Figure 3.7). GF109203X blocked PMA-induced inhibition of AlF_4^--stimulated inositol phospholipid signaling (Figure 3.8), similar to Ang II-induced inositol phospholipid hydrolysis (Figure 3.5 and Figure 3.6). These results suggest that the majority of PKC-induced inhibition of Ang II-induced inositol phospholipid signaling occurs downstream of the Ang II receptor in HASMC.

4.3.2 G-proteins

G protein subunits are not very likely PKC substrate candidates. Therefore, other candidates such as PLC-β isoenzymes and RGS proteins are of greater

interest as PKC substrates correlated with Ang II-induced desensitization in HASMC.

4.3.3 Phospholipase C-β

Differences in phosphorylation levels among PLC-β isoenzymes following whole cell treatment with vehicle, Ang II, PMA, or GF109203X were not clearly observed, even though PLC-β isoenzymes were phosphorylated (Figure 3.9). PLC-β1 was especially highly phosphorylated under basal condition and did not display any significant difference in phosphorylation between the control (vehicle treatment) and other drug treatments (Figure 3.9a). A putative PKC phosphorylation site of PLC-β1 is at residue serine 887 (Ser^{887}) (Ryu *et al.*, 1990; Xu A *et al.*, 2001). The phosphorylation residue Ser^{887} of PLCβ1 is located in the carboxy-terminal (C-terminal) region, which is not important for catalytic activity, but is important for localization to the membrane (Jenco *et al.*, 1997, Kim *et al.*, 1996), nuclear activity (Kim *et al.*, 1996), and activation by Gα protein subunits (Jenco *et al.*, 1997; Kim *et al.*, 1996; Park *et al.*, 1993, Wu *et al*, 1993). The fact that PLC-β1 was phosphorylated independently of PKC activation may support other studies suggesting that PLC-β1 is not a good substrate *in vitro* for PKC (Filtz *et al.*, 1999; Strassheim *et al.*, 1998). However, the site of basal phosphorylation of PLC-β1 in intact HASMC is not known, and whether basal phosphorylation of PLC-β1 results from PKC activity is also unknown. Furthermore, whether

phosphorylation affects PLC-β1 membrane association is still not known. Therefore, further investigation would be worthwhile.

Differences in phosphorylation levels of PLC-β3 in HASMC following drug treatment seem to be related to differences in immunoreactive protein levels (Figure 3.9b), suggesting that PLC-β3, like PLC-β1, was phosphorylated independent of drug treatment in HASMC. Although other studies have shown that PLC-β3 phosphorylation by PKC inhibited Gα protein-mediated signaling (but not basal or Gβγ -mediated signaling) (Yue *et al.*, 2000; Strassheim, et al., 1998; Strassheim and Williams, 2000), our results are not consistent with PKC-mediated PLC-β3 phosphorylation. This suggests that PLC-β phosphorylation by PKC may not be a direct trigger for desensitization of Ang II-induced inositol phospholipid signaling in HASMC. The putative PKC phosphorylation site in PLC-β3 has been reported as Ser^{1105} (Yue *et al.*, 2000). Similar to the putative PKC phosphorylation site in PLC-β1, Ser^{1105} of PLC-β3 is also located in the C-terminal region of the protein, relating to membrane association but not to catalytic activity. However, phosphorylation of PLC-β3 occurs at Ser^{1105} by not only PKC, but also by PKA and PKG (Yue *et al.*, 2000; Yue *et al.*, 1998; Xia C *et al.*, 2001). These findings suggest that phosphorylation of Ser^{1105} in PLC-β3 may be a general target for a variety of kinases in heterologous pathways to modulate PLC-β3 activity. For example, Strassheim *et al.*, (1998) suggested that PKC phosphorylation of PLC-β3 is not essential for opioid receptor desensitization. The site of basal

phosphorylation of PLC-β3 in HASMC is not known, and whether PLC-β3 phosphorylation by PKC affects membrane association in HASMC is also unknown. In future studies, it will be necessary to examine; 1) the phosphorylation site of PLC-β in HASMC, 2) the membrane association of phosphorylated PLC-β in HASMC, 3) the kinase responsible for basal phosphorylation of PLC-β in HASMC, 4) whether kinases other than PKC induce desensitization of Ang II-induced inositol phospholipid signaling through PLC-β3 phosphorylation, and 5) whether G protein or RGS proteins are phosphorylated following PKC activation.

It is speculated that PLC-β1 and PLC-β3 might have different roles or characteristics in HASMC, and that the roles and regulation of PLC-β isoenzymes might be different in tissues or experimental conditions. Filtz *et al.* (1999) reported that recombinant purified PLC-βT and mammalian PLC-β2, but not mammalian PLC-β1, were phosphorylated *in vitro* by PKC. Other reports have shown that a selective PKC-mediated negative feedback targets PLC-β3, but not PLC-β1 (Strassheim and Williams, 2000). Additionally, native PKC phosphorylation of PLC-β1 has also been reported (Litosch, 1996; Ryu *et al.*, 1990; Xu A *et al.* 2001). Furthermore, each PKC isoform has different phosphorylation specificities for PLC-β isoenzymes; PLC-β1: PKCα >> PKCε; not PKCζ, and PLC-β3: no phosphorylation by PKCα (Litosch, 1997). These reports emphasize the hypothesis that PKC phosphorylation of PLC-β isoenzyme may depend on the amount and isotype of PLC-β and PKC isoenzymes expressed in individual tissues.

4.3.4 Regulators of regulators of G-protein signaling (RGS) proteins

Other possible phosphorylation substrates for PKC are regulators of G protein signaling (RGS) proteins. RGS proteins are GAP and modulate the AT1 receptors/Gαq/PLC-β interaction (Grant *et al.*, 2000; Cunningham *et al.*, 2001; Dowal *et al*, 2001). Of all RGS proteins, RGS2 and RGS4 have been reported to interact with the Gαq subunit (Cunningham *et al.*, 2001; Grant *et al.*, 2000; Xu X *et al.*, 1999), which mediates Ang II-induced inositol phospholipid signaling in vascular smooth muscle (Ushio-Fukai *et al.*, 1998b). Particularly, RGS2 is specific for Gαq (Heximer *et al.*, 1997) and is phosphorylated by PKC (Cunningham *et al.*, 2001) to decrease its GAP activity, which should result in an increase in Gαq activity. However, mRNA levels of RGS2 in rat vascular smooth muscle cells are increased by PKC activation (Grant *et al.*, 2000), resulting in a decrease in Gαq activity. Therefore, PKC may regulate RGS2 in the agonist-stimulated inositol phospholipid signaling by two opposed ways, direct RGS2 attenuation by phosphorylation and indirect RGS2 potentiation by an increase in mRNA levels of RGS2.

RGS4 shows more diverse characteristics than RGS2 and binds to not only activated Gαq but also to Gβγ (Dowal *et al.*, 2001). Interestingly, Gβγ bound to RGS4 increases its activity in HEK293 cells (Bunemann and Hosey, 1998; Camps

et al., 1992; Katz *et al.*, 1992). Because Gβγ stimulates PLC-β3 activity (Park *et al.*, 1993), RGS4 bound to Gβγ may increase PLC-β3 activity. Functional consequences of RGS phosphorylation on PLC-β activity in whole cells remain under investigation, since there is no evidence of RGS4 phosphorylation by PKC. In further studies, it is important to examine 1) whether RGS4 is phosphorylated by PKC activation and 2) whether PKC phosphorylation of RGS4 affects Ang II-induced inositol phospholipid signaling in HASMC through Gα and/or Gβγ.

4.4 Protein kinase C in signaling pathways

PKC isoenzyme substrates discussed above have highly diverse characteristics. As stated in the introduction, PKC, one of the first activated enzymes following inositol phospholipid hydrolysis, may be involved in the down-regulation of PLC-β signaling pathway via phosphorylation of a variety of cell signaling proteins. PKC, a serine/threonine kinase, includes at least ten isoforms in mammals, classified into three major subfamilies; the conventional (cPKC), novel (nPKC), and atypical (aPKC) (Newton, 1995; Ventura and Maioli 2001; Hug and Sarre, 1993). The cPKC subfamily (α, βI, βII, and γ) is characterized by its regulation by means of Ca^{2+} and a putative Ca^{2+}-binding site in C2 domain. The C2 domain is important for membrane binding activity as Ca^{2+} increases its affinity to negatively charged membranes (Nalefski and Falke, 1996; Rizo and Sudhof,

1998; Newton, 2001; Murray and Honig, 2002). The nPKC subfamily (δ, ε, η/L, θ, and μ) is structurally similar to the cPKC subfamily but with no putative Ca^{2+}-binding site in the C2 domain. Unlike other subfamilies, the aPKC subfamily (ζ and ι/λ) contains neither the C2 domain nor binding activity to DAG or phorbol ester in the C1 domain (Nishizuka, 1988; Ono *et al.*, 1989; Hommel *et al.*, 1994; Zhang, *et al.*, 1995). In vascular smooth muscle cells, at least four different PKC isozymes, α, δ, ε and ζ, have been identified (Dixon *et al.*, 1994; Liou and Morgan, 1994; Liao *et al.*, 1997) and Ang II leads to the membrane-binding activity of some PKCs (Dixon *et al.*, 1994, Greco *et al.*, 2002). PKCα, from the cPKC subfamily, translocates from cytosol to membrane upon Ang II stimulation leading to an increase in both DAG and intracellular Ca^{2+} (Greco *et al.*, 2002; Haller *et al.*, 1994; Natarajan *et al.*, 1994). PKCδ and PKCε, from the nPKC subfamily, also translocates from cytosol to membrane upon Ang II stimulation (Greco *et al.*, 2002; Maloney *et al.*, 1998; Natarajan *et al.*, 1994). PKCζ, of the aPKC subfamily, does not translocate from the cytosol upon Ang II stimulation (Damron *et al.*, 1998; Greco *et al.*, 2002; Liao *et al.*, 1997). Although Ang II activates all four PKCs, PKCζ cannot translocate to the membrane, where major proteins for Ang II-induced signal transduction are assembled. Translocation of PKCs to the membrane may be important for proximity to substrates and may also affect concentration. This suggests that PKCζ may be the least likely to be directly involved in negative feedback of Ang II-induced inositol phospholipid signaling.

Ang II increases intracellular Ca^{2+} ($[Ca^{2+}]i$) by an initial release of Ca^{2+} from intracellular stores (endoplasmic reticulum), followed by a sustained Ca^{2+} influx from extracellular fluid (Griendling *et al.*, 1991; Van Breemen and Saida, 1989; Somlyo AP and Somlyo AV, 1994). We observed that treatment with 2mM EGTA to chelate extracellular Ca^{2+} inhibited Ang II-induced inositol phospholipid signaling in HASMC (data not shown), suggesting that extracellular Ca^{2+} may play an important part in Ang II-induced inositol phospholipid signaling in HASMC. The importance of changes in Ca^{2+} levels on PLC-β and PKC were not explored but are of potential importance for membrane binding affinity of PKC and the proximity between PKCs and substrates, including PLC-β on the plasma membrane. Following Ang II stimulation, PKCα-Ca^{2+} may translocate and bind more tightly to the membrane than PKCε and PKCζ. Therefore, PKCs, especially PKCα-Ca^{2+} localized on the membrane, may be concentrated enough to phosphorylate proteins in Ang II-induced inositol phospholipid signaling in HASMC.

4.5 Conclusion

Attempts to elucidate the mechanism of Ang II-induced desensitization of inositol phospholipid signaling have revealed a complicated system. Proteins in the signal transduction pathway transfer to specific locations, in different tissues, with different time courses, following agonist stimulation. In future studies, it will be

important to investigate individual protein expression and localization and their interactions with other proteins, in an appropriate time course, to elucidate the regulation of Ang II-induced desensitization.

This work was supported by U.S. Public Health Service Grant GM-29536, and an American Heart Association beginning Grant-in-Aid to T.M.F

I thank Ryan Millimaki for his excellent technical assistance in Western blot assay technique, used as part of the phosphorylation assay.

BIBLIOGRAPHY

Abdellatif MM, Neubauer CF, Lederer WJ and Rogers TB (1991) Angiotensin-induced desensitization of the phosphoinositide pathway in cardiac cells occurs at the level of the receptor. *Circ Res* **69**:800-809.

Ali H, Fisher I, Haribabu B, Richardson RM and Snyderman R (1997) Role of phospholipase Cβ3 phosphorylation in the desensitization of cellular responses to platelet-activating factor. *J Biol Chem* **272**:11706-11709.

Ali H, Sozzani S, Fisher I, Barr AJ, Richardson RM, Haribabu B and Snyderman R (1998) Differential regulation of formyl peptide and platelet-activating factor receptors. Role of phospholipase Cβ3 phosphorylation by protein kinase A. *J Biol Chem* **273**:11012-11016.

Anborgh PH, Seachrist JL, Dale LB and Ferguson SS (2000) Receptor/β-arrestin complex formation and the differential trafficking and resensitization of β2-adrenergic and angiotensin II type 1A receptors. *Mol Endocrinol* **14**:2040-2053.

Balmforth AJ, Shepherd FH, Warburton P and Ball SG (1997) Evidence of an important and direct role for protein kinase C in agonist-induced phosphorylation leading to desensitization of the angiotensin AT1A receptor. *Br J Pharmacol* **122**:1469-1477.

Berk BC, Vekshtein V, Gordon HM and Tsuda T (1989) Angiotensin II-stimulated protein synthesis in cultured vascular smooth muscle cells. *Hypertension* **13**:305-314.

Berridge MJ and **Irvine** RF (1984) Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* **312**:315-321.

Berridge MJ (1993) Inositol trisphosphate and calcium signalling. *Nature* **361**:315-325.

Bihoreau C, Monnot C, Davies E, Teutsch B, Bernstein KE, Corvol P and Clauser E (1993) Mutation of Asp74 of the rat angiotensin II receptor confers changes in antagonist affinities and abolishes G-protein coupling. *Proc Natl Acad Sci USA* **90**:5133-5137.

Blayney L, Gapper P and Rix C (1998) Identification of phospholipase C β isoforms and their location in cultured vascular smooth muscle cells of pig, human and rat. *Cardiovasc Res* **40**:564-572.

Bouvier M, Hausdorff WP, De Blasi A, O'Dowd BF, Kobilka BK, Caron MG and Lefkowitz RJ (1988) Removal of phosphorylation sites from the β2-adrenergic receptor delays onset of agonist-promoted desensitization. *Nature* **333**:370-373.

Brock TM, Rittenhouse SE, Powers CW, Ekstein LS, Gimbrone MA and Alexander RW (1985) Phorbol ester and 1-oleoyl-2-acetylglycerol inhibit Angiotensin activation of phospholipase C cultured vascular smooth muscle cells. *J. Biolo. Chem.* **260**:14158-14162.

Bunemann M and **Hosey** MM (1998) Regulators of G protein signaling (RGS) proteins constitutively activate Gβγ-gated potassium channels. *J Biol Chem* **273**:31186-31190.

Camps M, Carozzi A, Schnabel P, Scheer A, Parker PJ and Gierschik P (1992) Isozyme-selective stimulation of phospholipase C-β2 by G protein βγ-subunits. *Nature* **360**:684-686.

Chuang TT, LeVine H 3rd and De Blasi A (1995) Phosphorylation and activation of β-adrenergic receptor kinase by protein kinase C. *J Biol Chem* **270**:8660-18665.

Cockcroft S and **Thomas** GM (1992) Inositol-lipid-specific phospholipase C isoenzymes and their differential regulation by receptors. *Biochem J* **288**:1-14.

Cunningham ML, Filtz TM and Harden TK (1999) Protein kinase C-promoted inhibition of Gα11-stimulated phospholipase C-β activity. *Mol Pharmacol* **56**:265-271.

Cunningham ML, Waldo GL, Hollinger S, Hepler JR and Harden TK (2001) Protein kinase C phosphorylates RGS2 and modulates its capacity for negative regulation of Gα11 signaling. *J Biol Chem* **276**:5438-5444.

Damron DS, Nadim HS, Hong SJ, Darvish A and Murray PA (1998) Intracellular translocation of PKC isoforms in canine pulmonary artery smooth muscle cells by ANG II. *Am J Physiol* **274**:L278-L288.

Dixon BS, Sharma RV, Dickerson T and Fortune J (1994) Bradykinin and angiotensin II: activation of protein kinase C in arterial smooth muscle. *Am J Physiol* **266**:C1406-C1420.

Doss RC, Perkins JP and Harden TK (1981) Recovery of β-adrenergic receptors following longterm exposure of astrocytoma cells to catecholamine. *J Biol Chem* **256**:12281-12286.

Dowal L, Elliott J, Popov S, Wilkie TM and Scarlata S (2001) Determination of the contact energies between a regulator of G protein signaling and G protein subunits and phospholipase C β1. *Biochemistry* **40**:414-421.

Ferguson SS (2001) Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol Rev* **53**:1-24.

Filtz TM, Li Q, Boyer JL, Nicholas RA and Harden TK (1994) Expression of a cloned P2Y purinergic receptor that couples to phospholipase C. *Mol Pharmacol* **46**:8-14.

Filtz TM, Cunningham ML, Stanig KJ, Paterson A and Harden TK (1999) Phosphorylation by protein kinase C decreases catalytic activity of avian phospholipase C-β. *Biochem J* **338**:257-264.

Fisher SK (1995) Homologous and hetelogous regulation of receptor-stimulateed phosphoinoside hydolysis. *Eur J Pharmacol.* **288**:231-250.

Geisterfer AA, Peach MJ and Owens GK (1988) Angiotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells. *Circ Res* **62**:749-756.

Gerfen CR, Choi WC, Suh PG, and Rhee SG (1988) Phospholipase C I and II brain isozymes: immunohistochemical localization in neuronal systems in rat brain. *Proc Natl Acad Sci USA* **85**:3208-3212.

Gilman AG (1995) Nobel Lecture. G proteins and regulation of adenylyl cyclase *Biosci Rep* **15**:65-97.

Goodman OB Jr, Krupnick JG, Santini F, Gurevich VV, Penn RB, Gagnon AW, Keen JH and Benovic JL (1996) β-arrestin acts as a clathrin adaptor in endocytosis of the β2-adrenergic receptor. *Nature* 383:447-450.

Grant SL, Lassegue B, Griendling KK, Ushio-Fukai M, Lyons PR and Alexander RW (2000) Specific regulation of RGS2 messenger RNA by angiotensin II in cultured vascular smooth muscle cells. *Mol Pharmacol* **57**:460-467.

Greco S, Muscella A, Elia MG, Salvatore P, Storelli C and Marsigliante S (2002) Activation of angiotensin II type I receptor promotes protein kinase C translocation and cell proliferation in human cultured breast epithelial cells. *J Endocrinol* **174**:205-214.

Griendling KK, Taubman MB, Akers M, Mendlowitz M and Alexander RW. (1991) Characterization of phosphatidylinositol-specific PLC from cultured vascular smooth muscle cells. *J Biol Chem* **266**:15498-15504.

Griendling KK, Lassegue B and Alexander RS (1996) Angiotensin receptors and their therapeutic implications. *Annu Rev Pharmacol Toxicol* **36**:281-306.

Griendling KK, Ushio-Fukai M, Lassègue B and Alexander RW (1997) Angiotensin II signaling in vascular smooth muscle: New concepts. *Hypertension* **29**:366-373.

Hadcock JR and **Malbon** CC (1988) Down-regulation of β-adrenergic receptors: agonist-induced reduction in receptor mRNA levels. *Proc Natl Acad Sci USA* **85**:5021-5025.

Haller H, Quass P, Lindschau C, Luft FC and Distler A (1994) Platelet-derived growth factor and angiotensin II induce different spatial distribution of protein kinase C-α and -β in vascular smooth muscle cells. *Hypertension* **23**:848-852.

Hansen CA, Schroering AG and Robishaw JD (1995) Subunit expression of signal transducing G proteins in cardiac tissue: implications for phospholipase C-β regulation. *J Mol Cell Cardiol* **27**:471-484.

Hausdorff WP, Bouvier M, O'Dowd BF, Irons GP, Caron MG and Lefkowitz RJ (1989) Phosphorylation sites on two domains of the β2-adrenergic receptor are involved in distinct pathways of receptor desensitization. *J Biol Chem* **264**:12657-12665.

Helin K, Stoll M, Meffert S, Stroth U and Unger T (1997) The role of angiotensin receptors in cardiovascular diseases. *Ann Med* **29**:23-29.

Hepler JR and **Gilman** AG (1992) G proteins. *Trends Biochem Sci* **17**:383-387.

Hepler JR. (1999) Emerging roles for RGS proteins in cell signalling. *Trends Pharmacol Sci* **20**:376-382.

Hermans E, Vanisberg MA, Geurts M and Maloteaux JM (1997) Down-regulation of neurotensin receptors after ligand-induced internalization in rat primary cultured neurons. *Neurochem Int* **31**:291-299.

Heximer SP, Watson N, Linder ME, Blumer KJ and Hepler JR (1997) RGS2/G0S8 is a selective inhibitor of Gqα function. *Proc Natl Acad Sci USA* **94**:14389-14393.

Homma Y, Takenawa T, Emori Y, Sorimachi H, and Suzuki K (1989) Tissue- and cell type-specific expression of mRNAs for four types of inositol phospholipid-specific phospholipase C. *Biochem Biophys Res Commun* **164**:406-412.

Hommel U, Zurini M and Luyten M (1994) Solution structure of a cysteine rich domain of rat protein kinase C. *Nat Struct Biol* **1**:383-387.

Hug H and **Sarre** T (1993) Protein kinase C isoenzymes: divergence in signal transduction? *Biochem J* **291**:329-343.

Huwiler A, Briner VA, Fabbro D and Pfeilschifter J (1997) Feedback regulation of extracellular ATP-stimulated phosphoinositide hydrolysis by protein kinase C-α in bovine glomerular endothelial cells. *Kidney Int* **52**:329-337.

Iglesias AG, Suarez C, Feierstein C, Diaz-Torga G and Becu-Villalobos D (2001) Desensitization of angiotensin II: effect on. *Am J Physiol Endocrinol Metab* **280**:E462- E470.

Ishizaka N, Alexander RW, Laursen JB, Kai H, Fukui T, Oppermann M, Lefkowitz RJ, Lyons PR and Griendling KK (1997) G protein-coupled receptor kinase 5 in cultured vascular smooth muscle cells and rat aorta: Regulation by angiotensin II and hypertension. *J Biol Chem* **272**:32482-32488.

Jenco JM, Becker KP and Morris AJ (1997) Membrane-binding properties of phospholipase C-β1 and phospholipaseC-β2: role of the C-terminus and effects of polyphosphoinositides, G-proteins and Ca^{2+}. *Biochem J* **327**:431-437.

Jhon DY, Lee HH, Park D, Lee CW, Lee KH, Yoo OJ, and Rhee SG (1993) Cloning, sequencing, purification, and G_q-dependent activation of phospholipase C-β3. *J Biol Chem* **268**:6654-6661.

Jockers R, Angers S, Da Silva A, Benaroch P, Strosberg AD, Bouvier M and Marullo S (1999) β2-adrenergic receptor down-regulation. Evidence for a pathway that does not require endocytosis. *J Biol Chem* **274**:28900-28908.

Johnston CI (1992) Franz Volhard Lecture. Renin-angiotensin system: a dual tissue and hormonal system for cardiovascular control. *J Hypertens Suppl* **10**:S13-S26.

Ji H, Leung M, Zhang Y, Catt KJ and Sandberg K (1994) Differential structural requirements for specific binding of nonpeptide and peptide antagonists to the AT1 angiotensin receptor. Identification of amino acid residues that determine binding of the antihypertensive drug losartan. *J Biol Chem* **269**:16533-16536.

Jiang H, Wu D, and Simon MI (1994) Activation of phospholipase C β4 by heterotrimeric GTP-binding proteins. *J Biol Chem* **269**:7593-7596.

Ju H, Zhao S, Tappia PS, Panagia V and Dixon IM (1998) Expression of Gqα and PLC-β in scar and border tissue in heart failure due to myocardial infarction. *Circulation* **97**:892-899.

Kai H, Fukui T, Lassegue B, Shah A, Minieri CA and Griendling KK (1996) Prolonged exposure to agonist results in a reduction in the levels of the Gq/G11α subunits in cultured vascular smooth muscle cells. *Mol Pharmacol* **49**:96-104.

Katz A, Wu D and Simon MI (1992) Subunits βγ of heterotrimeric G protein activate β2 isoform of phospholipase C. *Nature* **360**:686-689.

Kim CG, Park D and Rhee SG (1996) The role of carboxyl-terminal basic amino acids in Gqα-dependent activation, particulate association, and nuclear localization of phospholipase C-β1 *J Biol Chem* **271**:21187-21192.

Kim S and Iwao H (2000) Molecular and cellular mechanisms of angiotensin II-mediated cardiovascular and renal diseases. *Pharmacol Rev* **52**:11-34.

Kozasa T and **Gilman** AG (1996) Protein kinase C phosphorylates G12α and inhibits its interaction with Gβγ. *J Biol Chem* **271**:12562-12567.

Lajat S, Harbon S and Tanfin Z (1998) Carbachol-induced desensitization of PLC-β pathway in rat myometrium: downregulation of Gqα/G11α. *Am J Physiol* **275**:C636-C645.

Lassegue B, Alexander RW, Nickeng G, Clark M, Murphy TJ and Griendling KK (1995) Angiotensin II down-regulates the vascular smooth muscle AT1 receptor by transcriptional and post transcriptional mechanisms: evidence for homologous and hetelogous regulation. *Mol Pharmacol* **48**:601-609.

Lee CW, Lee KH, Lee SB, Park D and Rhee SG (1994) Regulation of phospholipase C-β4 by ribonucleotides and the α subunit of Gq. *J Biol Chem* **269**:25335-25338.

Lee SB and **Rhee** SG (1995) Significance of PIP2 hydrolysis and regulation of phospholipase C isozymes. *Curr Opin Cell Biol* **7**:183-189.

Lee SB, Rao AK, Lee KH, Yang X, Bae YS, and Rhee SG (1996) Decreased expression of phospholipase C-β2 isozyme in human platelets with impaired function. *Blood* **88**:1684-1691.

Liao DF, Monia B, Dean N and Berk BC (1997) Protein kinase C-ζ mediates angiotensin II activation of ERK1/2 in vascular smooth muscle cells. *J Biol Chem* **272**:6146-6150.

Liou YM and **Morgan** KG (1994) Redistribution of protein kinase C isoforms in association with vascular hypertrophy of rat aorta. *Am J Physiol* **267**:C980-C989.

Litosch I (1996) Protein kinase C inhibits the Ca^{2+}-dependent stimulation of phospholipase C- β1 *in vitro*. *Recept Signal Transduct* **6**:87-98.

Litosch I (1997) G-protein βγ subunits antagonize protein kinase C-dependent phosphorylation and inhibition of phospholipase C-β1. *Biochem J* **326**:701-707.

Liu S, Carrillo JJ, Pediani JD and Milligan G (2002) Effective information transfer from the α1b-adrenoceptor to Gα11 requires both β/γ interactions and an aromatic group four amino acids from the C terminus of the G protein. *J Biol Chem* **277**:25707-25714.

Liu WW, Mattingly RR and Garrison JC (1996) Transformation of Rat-1 fibroblasts with the v-src oncogene increases the tyrosine phosphorylation state and activity of the α subunit of Gq/G11. *Proc Natl Acad Sci USA* **93**:8258-8263.

Lohse MJ, Benovic JL, Caron MG and Lefkowitz RJ (1990a) Multiple pathways of rapid β2-adrenergic receptor desensitization. Delineation with specific inhibitors. *J Biol Chem* **265**:3202-3211.

Lohse MJ, Benovic JL, Codina J, Caron MG and Lefkowitz RJ (1990b) β-Arrestin: a protein that regulates β-adrenergic receptor function. *Science* **248**:1547-1550.

Lopez I, Mak EC, Ding J, Hamm HE and Lomasney JW (2001) A novel bifunctional phospholipase c that is regulated by Gα12 and stimulates the Ras/mitogen-activated protein kinase pathway. *J Biol Chem* **276**:2758-2765.

Lounsbury KM, Schlegel B, Poncz M, Brass LF and Manning DR (1993) Analysis of Gzα by site-directed mutagenesis. Sites and specificity of protein kinase C-dependent phosphorylation. *J Biol Chem* **268**:3494-3498.

Macrez N, Morel JL, Kalkbrenner F, Viard P, Schultz G and Mironneau J (1997) A βγ dimer derived from G13 transduces the angiotensin AT1 receptor signal to stimulation of Ca^{2+} channels in rat portal vein myocytes. *J Biol Chem* **272**:23180-23185.

Macrez-Lepretre N, Kalkbrenner F, Morel JL, Schultz G and Mironneau J (1997) G protein heterotrimer Gα13β1γ3 couples the angiotensin AT1A receptor to increases in cytoplasmic Ca^{2+} in rat portal vein myocytes. *J Biol Chem* **272**:10095-10102.

Maloney JA, Tsygankova O, Szot A, Yang L, Li Q and Williamson JR (1998) Differential translocation of protein kinase C isozymes by phorbol esters, EGF, and ANG II in rat liver WB cells. *Am J Physiol* **274**:C974- C982.

Meszaros JG, Raphael R, Lio FM and Brunton LL (2000) Protein kinase C contributes to desensitization of ANG II signaling in adult rat cardiac fibroblasts. *Am J Physiol Cell Physiol* **279**:C1978-C1985.

Min DS, Kim DM, Lee YH, Seo J, Suh PG and Ryu SH (1993a) Purification of a novel phospholipase C isozyme from bovine cerebellum. *J Biol Chem* **268**:12207-12212.

Min DS, Kim Y, Lee YH, Suh PG, and Ryu SH (1993b) A G-protein-coupled 130 kDa phospholipase C isozyme, PLC-β4, from the particulate fraction of bovine cerebellum. *FEBS Lett* **331**:38-42.

Mizuguchi M, Yamada M, Kim SU, and Rhee SG (1991) Phospholipase C isozymes in neurons and glial cells in culture: an immunocytochemical and immunochemical study. *Brain Res* **548**:35-40.

Murray D and **Honig** B (2002) Electrostatic control of the membrane targeting of C2 domains. *Mol Cell* **9**:145-154.

Nalefski EA and **Falke** JJ. (1996). The C2 domain calcium-binding motif: structural and functional diversity. *Protein Sci* **5**, 2375-2390.

Natarajan R, Lanting L, Xu L and Nadler J (1994) Role of specific isoforms of protein kinase C in angiotensin II and lipoxygenase action in rat adrenal glomerulosa cells. *Mol Cell Endocrinol* **101**:59-66.

Newton AC (1995) Protein kinase C: structure, function, and regulation. *J Biol Chem* **270**:28495-28498.

Newton AC (2001) Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. *Chem Rev* **101**:2353-2364.

Nishizuka Y (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* **334**:661-665.

Noda K, Saad Y, Kinoshita A, Boyle TP, Graham RM, Husain A nd Karnik SS (1995) Tetrazole and carboxylate groups of angiotensin receptor antagonists bind to the same subsite by different mechanisms. *J Biol Chem* **270**:2284-2289

Noh DY, Shin SH and Rhee SG (1995) Phosphoinositide-specific phospholipase C and mitogenic signaling. *Biochim Biophys Acta* **1242**:99-113.

Oakley RH, Laporte SA, Holt JA, Barak LS and Caron MG (1999) Association of β-arrestin with G protein-coupled receptors during clathrin-mediated endocytosis dictates the profile of receptor resensitization. *J Biol Chem* 274:32248-3257.

Ohyama K, Yamano Y, Chaki S, Kondo T and Inagami T (1992) Domains for G-protein coupling in angiotensin II receptor type I: studies by site-directed mutagenesis. *Biochem Biophys Res Commun* **189**:677-683.

Olivares-Reyes JA, Smith RD, Hunyady L, Shah BH and Catt KJ (2001) Agonist-induced signaling, desensitization, and internalization of a phosphorylation-deficient AT1A angiotensin receptor. *J Biol Chem* **276**:37761-37768.

Ono Y, Fujii T, Igarashi K, Kuno T, Tanaka C, Kikkawa U and Nishizuka Y (1989) Phorbol ester binding to protein kinase C requires a cysteine-rich zinc-finger-like sequence. *Proc Natl Acad Sci USA* **86**:4868-4871.

Oppermann M, Freedman NJ, Alexander RW and Lefkowitz RJ (1996) Phosphorylation of the type 1A angiotensin II receptor by G protein-coupled receptor kinases and protein kinase C. *J Biol Chem* **271**:13266-13272.

Ozawa K, Yamada K, Kazanietz MG, Blumberg PM, Beaven and MA (1993) Different isozymes of protein kinase C mediate feedback inhibition of phospholipase C and stimulatory signals for exocytosis in rat RBL-2H3 cells. *J Biol Chem* **268**:2280-2283.

Pak Y, O'Dowd BF, Wang JB and George SR (1999) Agonist-induced, G protein-dependent and -independent down-regulation of the mu opioid receptor. The receptor is a direct substrate for protein-tyrosine kinase. *J Biol Chem* **274**:27610-27616.

Park D, Jhon DY, Lee CW, Lee KH and Rhee SG (1993) Activation of phospholipase C isozymes by G protein βγ subunits. *J Biol Chem* **268**:4573-4576.

Paterson A, Boyer JL, Watts VJ, Morris AJ, Price EM and Harden TK (1995) Concentration of enzyme-dependent activation of PLC-β1 and PLC-β2 by Gα11 and βγ-subunits. *Cell Signal* **7**:709-720.

Pronin AN and **Benovic** JL (1997) Regulation of the G protein-coupled receptor kinase GRK5 by protein kinase C. *J Biol Chem* **272**:3806-3812.

Qian H, Pipolo L and Thomas WG (1999) Identification of protein kinase C phosphorylation sites in the angiotensin II (AT1A) receptor. *Biochem J* **343**:637-644.

Rebecchi MJ and **Pentyala** SN (2000) Structure, function, and control of phosphoinositide-specific phospholipase C. *Physiol Rev* **80**:1291-1335.

Rhee SG and **Bae** YS (1997) Regulation of phosphoinositide-specific phospholipase C isozymes. *J Biol Chem* **272**:15045-15048.

Rhee SG and **Choi** KD (1992) Regulation of inositol phospholipid-specific phospholipase C isozymes. *J Biol Chem* **267**:12393-12396.

Rhee SG (2001) Regulation of phosphoinositide-specific phospholipase C. *Annu Rev Biochem* **70**:281-312.

Rizo J and **Sudhof** TC (1998) C2-domains, structure and function of a universal Ca^{2+}-binding domain. *J Biol Chem* **273**:15879-15882.

Ryu SH, Kim UH, Wahl MI, Brown AB, Carpenter G, Huang KP and Rhee SG. (1990). Feedback regulation of phospholipase C-β by protein kinase C. *J Biol Chem* **265**:17941-17945.

Sasaki K, Yamano Y, Bardhan S, Iwai N, Murray JJ, Hasegawa M, Matsuda Y and Inagami T (1991) Cloning and expression of a complementary DNA encoding a bovine adrenal angiotensin II type-1 receptor. *Nature* **351**:230-233.

Schambye HT, Hjorth SA, Weinstock J and Schwartz TW (1995) Interaction between the nonpeptide angiotensin antagonist SKF-108,566 and histidine 256 (HisVI:16) of the angiotensin type 1 receptor. *Mol Pharmacol* **47**:425-431.

Siderovski DP, Strockbine B and Behe CI (1999) Whither goest the RGS proteins? *Crit Rev Biochem Mol Biol* **34**:215-251.

Smith RD, Hunyady L, Olivares-Reyes JA, Mihalik B, Jayadev S and Catt KJ (1998) Agonist-induced phosphorylation of the angiotensin AT1a receptor is localized to a serine/threonine-rich region of its cytoplasmic tail. *Mol Pharmacol* **54**:935-941.

Smrcka AV and **Sternweis** PC (1993) Regulation of purified subtypes of phosphatidylinositol-specific phospholipase Cβ by G protein α and βγ subunits. *J Biol Chem* **268**:9667-9674.

Somlyo AP and **Somlyo** AV (1994) Signal transduction and regulation in smooth muscle. *Nature* **372**:231-236.

Song C, Hu CD, Masago M, Kariyai K, Yamawaki-Kataoka Y, Shibatohge M, Wu D, Satoh T and Kataoka T (2001) Regulation of a novel human phospholipase C, PLCε, through membrane targeting by Ras. *J Biol Chem* **276**:2752-2757.

Sorensen SD, Linseman DA and Fisher SK (1998) Down-regulation of phospholipase C-β1 following chronic musacarinic receptor activation. *Eur J Pharmacol* **346**:R1-R2.

Spat A, Enyedi P, Hajnoczky G and Hunyady L (1991) Generation and role of calcium signal in adrenal glomerulosa cell. *Exp Physiol* **76**:859-885.

Strassheim D, Law PY and Loh HH (1998). Contribution of phospholipase C-β3 phosphorylation to the rapid attenuation of opioid-activated phosphoinositide response. *Mol Pharmacol* **53**:1047-1053.

Strassheim D and **Williams** CL (2000) P2Y2 purinergic and M3 muscarinic acetylcholine receptors activate different phospholipase C-β isoforms that are uniquely susceptible to protein kinase C-dependent phosphorylation and inactivation. *J Biol Chem* **275**:39767-39772.

Strathmann M, Wilkie TM and Simon MI (1989) Diversity of the G-protein family: sequences from five additional α subunits in the mouse. *Proc Natl Acad Sci USA* **86**:7407-7409.

Tanaka O and **Kondo** H (1994) Localization of mRNAs for three novel members (β3, β4 and γ2) of phospholipase C family in mature rat brain. *Neurosci Lett* **182**:17-20.

Thomas WG, Motel TJ, Kule CE, Karoor V, and Baker KM (1998) Phosphorylation of the angiotensin II (AT1A) receptor carboxyl terminus: a role in receptor endocytosis. *Mol Endocrinol* **12**:1513-1524.

Timmermans PB, Wong PC, Chiu AT, Herblin WF, Benfield P, Carini DJ, Lee RJ, Wexler RR, Saye JA and Smith RD (1993) Angiotensin II receptors and angiotensin II receptor antagonists. *Pharmacol Rev* **45**:205-251.

Touyz RM and **Schiffrin** EL (2000) Signal transduction mechanisms mediating the physiological and pathophysiological actions of angiotensin II in vascular smooth muscle cells. *Pharmacol Rev* **52**:639-672.

Trejo J and **Coughlin** SR (1999) The cytoplasmic tails of protease-activated receptor-1 and substance P receptor specify sorting to lysosomes versus recycling. *J Biol Chem* **274**:2216-2224.

Tsuda T, Griendling KK, Ollerenshaw JD, Lassegue B and Alexander RW (1993) Angiotensin-II-and endothelin-induced protein phosphorylation in cultured vascular smooth muscle cells. *J Vasc Res* **30**:241-249.

Ullian ME and **Linas** SL (1990) Angiotensin II surface receptor coupling to inositol triphosphate formation in vascular smooth muscle cells. *J Biol Chem* **265**:195-200.

Umemori H, Inoue T, Kume S, Sekiyama N, Nagao M, Itoh H, Nakanishi S, Mikoshiba K and Yamamoto T (1997) Activation of the G protein Gq/11 through tyrosine phosphorylation of the α subunit. *Science* **276**:1878-1881.

Umemori H, Hayashi T, Inoue T, Nakanishi S, Mikoshiba K and Yamamoto T (1999) Involvement of protein tyrosine phosphatases in activation of the trimeric G protein Gq/11. *Oncogene* **18**:7399-7402.

Ushio-Fukai M, Alexander RW, Akers M and Griendling KK (1998a) p38 Mitogen-activated protein kinase is a critical component of the redox-sensitive signaling pathways activated by angiotensin II. Role in vascular smooth muscle cell hypertrophy. *J Biol Chem* **273**:15022-15029.

Ushio-Fukai M, Griendling KK, Akers M, Lyons PR and Alexander RW (1998b) Temporal dispersion of activation of phospholipase C-β1 and -γ isoforms by angiotensin II in vascular smooth muscle cells: Role of $\alpha_{q/11}$, α_{12}, and $\beta\gamma$ G protein subunits. *J Biol Chem* **273**:19772-19777.

Ushio-Fukai M, Alexander RW, Akers M, Lyons PR, Lassegue B and Griendling KK (1999) Angiotensin II receptor coupling to phospholipase D is mediated by the $\beta\gamma$ subunits of heterotrimeric G proteins in vascular smooth muscle cells. *Mol Pharmacol* **55**:142-149.

Valiquette M, Bonin H, Hnatowich M, Caron MG, Lefkowitz RJ and Bouvier M (1990) Involvement of tyrosine residues located in the carboxyl tail of the human β2-adrenergic receptor in agonist-induced down-regulation of the receptor. *Proc Natl Acad Sci USA* **87**:5089-5093.

Valiquette M, Parent S, Loisel TP and Bouvier M (1995) Mutation of tyrosine-141 inhibits insulin-promoted tyrosine phosphorylation and increased responsiveness of the human β2-adrenergic receptor. *EMBO J* **14**:5542-5549.

van Breemen C and **Saida** K (1989) Cellular mechanisms regulating $[Ca^{2+}]i$ smooth muscle. *Annu Rev Physiol* **51**:315-329.

Ventura C and **Maioli** M. (2001). Protein kinase C control of gene expression. *Crit Rev Eukaryot Gene Expr* **11**:243-267.

Wieland T, Nurnberg B, Ulibarri I, Kaldenberg-Stasch S, Schultz G and Jakobs KH (1993) Guanine nucleotide-specific phosphate transfer by guanine nucleotide-binding regulatory protein β-subunits. Characterization of the phosphorylated amino acid. *J Biol Chem* **268**:18111-18118.

Winstel R, Freund S, Krasel C, Hoppe E and Lohse MJ (1996) Protein kinase cross-talk: membrane targeting of the β-adrenergic receptor kinase by protein kinase C. *Proc Natl Acad Sci USA* **93**:2105-2109.

Wu D, Jiang H, Katz A and Simon MI (1993) Identification of critical regions on phospholipase C-β1 required for activation by G-proteins. *J Biol Chem* **268**:3704-3709.

Xia C, Bao Z, Yue C, Sanborn BM and Liu M (2001) Phosphorylation and regulation of G-protein-activated phospholipase C-β3 by cGMP-dependent protein kinases. *J Biol Chem* **276**:19770-19777.

Xu A, Wang Y, Xu LY and Gilmour RS (2001) Protein kinase C α-mediated negative feedback regulation is responsible for the termination of insulin-like growth factor I-induced activation of nuclear phospholipase C β1 in Swiss 3T3 cells. *J Biol Chem* **276**:14980-14986.

Xu X, Zeng W, Popov S, Berman DM, Davignon I, Yu K, Yowe D, Offermanns S, Muallem S and Wilkie TM (1999) RGS proteins determine signaling specificity of Gq-coupled receptors. *J Biol Chem* **274**:3549-3556.

Yu P, Chen Q, Harnett KM, Amaral J, Biancani P and Behar J (1995) Direct G protein activation reverses impaired CCK signaling in human gallbladders with cholesterol stones. *Am J Physiol* **269**:G659-G665.

Yue C, Dodge KL, Weber G and Sanborn BM (1998) Phosphorylation of serine 1105 by protein kinase A inhibits phospholipase Cβ3 stimulation by Gαq. *J Biol Chem* **273**:18023-18027.

Yue C, Ku CY, Liu M, Simon MI and Sanborn BM (2000) Molecular mechanism of the inhibition of phospholipase C β3 by protein kinase C. *J Biol Chem* **275**:30220-30225.

Zhang G, Kazanietz MG, Blumberg PM and Hurley JH (1995) Crystal structure of the cys2 activator-binding domain of protein kinase C δ in complex with phorbol ester. *Cell* **81**:917-924.