Am J Physiol Cell Physiol 288: C1193–C1201, 2005.
First published January 26, 2005; doi:10.1152/ajpcell.00499.2004.

TRANSLATIONAL PHYSIOLOGY

PKC-δ-dependent pathways contribute to PDGF-stimulated ERK1/2 activation in vascular smooth muscle

Roman Ginnan and Harold A. Singer

Center for Cardiovascular Sciences, Albany Medical College, Albany, New York

Submitted 12 October 2004; accepted in final form 18 January 2005

Ginnan, Roman, and Harold A. Singer. PKC-δ-dependent pathways contribute to PDGF-stimulated ERK1/2 activation in vascular smooth muscle. *Am J Physiol Cell Physiol* 288: C1193–C1201, 2005. First published January 26, 2005; doi:10.1152/ajpcell.00499.2004.—Platelet-derived growth factor (PDGF) is an important regulator of vascular smooth muscle (VSM) cell growth and migration and has been identified as a key mediator of neointima formation resulting from vascular injury. PDGF exerts its effects, in part, through activation of ERK1/2. Previously, we reported that PKC-δ, specifically compared with PKC-α, mediated phorbol ester- and ATP-dependent activation of ERK1/2 in VSM cells. The purpose of this study was to determine whether PKC-δ was involved in PDGF-dependent activation of ERK1/2 in VSM cells. The addition of PDGF resulted in the activation, and Src family kinase-dependent tyrosine phosphorylation, of PKC-δ. Treatment with rottlerin (0.1–10 μM), a selective PKC-δ inhibitor, or adenoviral overexpression of kinase-negative PKC-δ significantly attenuated PDGF-induced activation of ERK1/2. The effects of the PKC-δ inhibitors decreased with increasing concentrations of activator PDGF. Interestingly, treatment with Gö6976 (0.1–3 μM), a selective inhibitor of cPKCs, or adenoviral overexpression of kinase-negative PKC-α also inhibited PDGF-stimulated ERK1/2. Furthermore, inhibition of cPKC activity with Gö6976 or overexpression of kinase-negative PKC-α attenuated PKC-δ activation and tyrosine phosphorylation in response to PDGF. These studies indicate involvement of both PKC-δ and PKC-α isozymes in PDGF-stimulated signaling in VSM and suggest an unexpected role for PKC-α in the regulation of PKC-δ activity.

phospholipase C-γ; protein kinase C-δ; protein kinase C-α

INJURY TO BLOOD VESSELS in response to balloon angioplasty results in morphological changes, such as thickening of the medial layer and formation of neointima due, in part, to increases in vascular smooth muscle (VSM) cell growth and migration (48). Platelet-derived growth factor (PDGF) is an important mediator of injury-induced VSM cell proliferation and migration (7). PDGF-dependent activation of signaling pathways involving MAP kinases such as ERK1/2 has been implicated in regulation of cell proliferation and migration responses in VSM cells as well as other cell types (10). Numerous signaling intermediates, including phospholipase C-γ (PLC-γ) (1), Src family kinases (SFKs) (45), and phosphatidylinositol 3-kinase (PI3-kinase) (30) are activated in response to PDGF and have been shown to be coupled to ERK1/2 activation (14, 46, 55). Because of their dependence on the products of PLC activity (Ca^{2+} and diacylglycerol), protein kinase C (PKC) family members have also been proposed as signaling intermediates in PDGF-dependent pathways and subsequent cellular responses, including cell migration.

PKCs are subdivided into three groups based on requirements for their activation (42). The classic PKCs (α, β, γ) require both calcium and diacylglycerol for activation. Novel PKCs (δ, ε, η, θ, μ) lack a calcium-binding domain and thus only require diacylglycerol for activation. Atypical PKCs (ζ, ι, λ) lack both a calcium and diacylglycerol-binding domain and thus require neither for their activation. The most abundant PKC isozymes in cultured VSM cells appear to be PKC-α and PKC-δ, although PKC-ε and PKC-ζ have also been detected (9). In VSM cells, PKC-α and PKC-ε have been reported to translocate to focal adhesions on activation and regulate cell adhesion and migration (25). Furthermore, it was recently reported (49) that PKC-α and PKC-ε are involved in the PDGF-dependent tyrosine phosphorylation of GRB2-associated binding protein (GAB1), resulting in the formation of a GAB1/PI3-kinase/SHZ domain-containing phosphatase (SHP2) signaling complex in VSM cells that may be important for activation of ERK1/2. Similarly, PKC-δ has also been reported to translocate to newly formed focal adhesions in fibroblasts in response to serum or lysophosphatidic acid (2) and fibroblast contractility and motility stimulated by epidermal growth factor has been reported to be dependent on PKC-δ (28). In VSM, PKC-δ is activated by mechanical stress and VSM cells from PKC-δ-null mice migrate slower in response to scrape wounding (33). With the use of antisense oligonucleotides (9), isozyme-specific pharmacological inhibitors, and molecular methodologies (18), our previous studies indicate that PKC-δ, not PKC-α, mediates ATP, a G protein-coupled receptor agonist- and phorbol ester-induced activation of ERK1/2 in cultured VSM cells. It is unknown whether PKC-δ is a significant component of PDGF-dependent signaling pathways in VSM.

Besides differential requirements for allosteric regulators, PKC-δ differs from PKC-α in that PKC-δ can be tyrosine phosphorylated in response to stimulation by phorbol esters, hydrogen peroxide, and growth factors such as PDGF and EGF. Whereas tyrosine phosphorylation of PKC-δ is SFK dependent (22), the specific residues phosphorylated and specific SFK required appears to be stimulus dependent (20, 32). The functional consequences of PKC-δ tyrosine phosphorylation are not clear with some reports suggesting that tyrosine

Address for reprint requests and other correspondence: R. Ginnan, Center for Cardiovascular Sciences, Albany Medical College (MC8), 47 New Scotland Ave., Albany, NY 12208 (E-mail: ginnanr@mail.amc.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

 0363-6143/05 $8.00 Copyright © 2005 the American Physiological Society

Downloaded from http://ajpcell.physiology.org/ by 10.220.33.6 on May 26, 2017

phosphorylation mediates or enhances the catalytic activity of PKC-δ (3, 22), and others suggesting PKC-δ tyrosine phosphorylation is a consequence of, but not a requirement for, catalytic activity (35). Ultimately, some of these discrepancies might be related to the specific tyrosine residues phosphorylated in PKC-δ. Recently, we (18, 19) and others (16, 50) have reported a requirement for SFKs in the EGF receptor transactivation-dependent activation of ERK1/2 in VSM cells. While the role of SFKs in PDGF-dependent tyrosine phosphorylation events and DNA synthesis (45) is well established, their role in PDGF-dependent activation of ERK1/2 is a matter of controversy. A recent study (31) in SYF cells (embryonic fibroblasts deficient in the SFKs Src, Yes, and Fyn) reported that the SFKs are not required for PDGF-dependent activation of ERK1/2. Furthermore, SU-6656, a selective inhibitor of SFKs, was reported to block PDGF-dependent tyrosine phosphorylation of known SFK substrates, including PKC-δ, but the drug had little effect on PDGF-dependent activation of ERK1/2 (5).

The primary purpose of this study was to determine whether PKC-δ, an isozyme that we have specifically associated with phorbol ester- and ATP-dependent activation of ERK1/2, is involved in the regulation of PDGF-induced ERK1/2 activity in VSM cells. The results of the study indicate that both PKC-δ and PKC-α are involved in PDGF-induced activation of ERK1/2, dependent on the concentration of PDGF used to activate the pathway. Furthermore, our studies suggest a novel and unexpected interaction between PKC-α and PKC-δ, such that PKC-α modulates PKC-δ catalytic activity and tyrosine phosphorylation in response to PDGF.

EXPERIMENTAL PROCEDURES

Cell culture. VSMs were obtained from the medial layer of the thoracic aorta of 200–300 g Sprague-Dawley rats, as described earlier (17). After the adventitial and endothelial layers were removed, medial smooth muscle cells were enzymatically dispersed and cultured in DMEM/F-12 + 10% fetal bovine serum (Hyclone). The VSM cells were maintained at 37°C with 5% CO_2 and split twice per week. Before experimental use, confluent cultures were growth arrested for 16–24 h by exchanging the growth media with DMEM/F-12 that was serum free. The serum-free media was replaced with Hanks' balanced salt solution containing Mg^{2+} and Ca^{2+} and 10 mM HEPES, pH 7.4, for 30–60 min before treatment.

Immunoprecipitations and Western blot analysis. Cells were lysed (0.5 ml/60 mm dish or 1 ml/100 mm dish) in a modified RIPA buffer composed of 10 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 10% glycerol, 1 mM DTT, 0.1 mM PMSF, and 0.2 U/ml aprotinin. After lysis, the samples were centrifuged to clear the lysate of the insoluble debris and preincubated with 20 μg protein A beads by being rocked for 30 min at 4°C, followed by centrifugation and transfer to a fresh 1.5 mm tube. Primary antibody was incubated for 90 min before the addition of 20 μg protein A beads to capture the immune complexes. The pelleted beads were then washed three times with 0.5-ml RIPA buffer, dissolved in 3× SDS-PAGE sample buffer and heated for 5 min at 95°C. The samples were resolved with the use of standard SDS-PAGE procedures, transferred to nylon-backed nitrocellulose (MSI), and immunoblotted. After being blocked in 5% nonfat dry milk or 3% BSA, the immunoblots were incubated for either 1 h at room temperature or overnight at 4°C, washed 3 × 10 min with 20 mM Tris-150 mM NaCl-0.2% Tween 20 (TBST), and incubated for 1 h with appropriate secondary antibody (HRP conjugate, Amersham). The blots were then washed 3 × 10 min with TBST, incubated in enhanced chemiluminescent substrate (Amersham), and exposed to X-ray film (Parker).

PKC activity assay. PKC-δ was immunoprecipitated from VSM cells and assayed as described earlier (36). After being washed three times in immunoprecipitation buffer and once in a sucrose buffer (10 mM MOPS, pH 7.4, 250 mM sucrose, 2.5 mM EGTA, 2 mM EDTA, 0.2 U/ml aprotinin, and 0.2 mM PMSF), the protein A beads were incubated in a buffer for 10 min at 30°C that was composed of 50 mM HEPES, pH 7.4, 10 mM Mg $(Ac)_2$, 2 mM $CaCl_2$, 1 mM EGTA, 0.2 mg/ml histone IIIS, 1.4 μg/μl phosphatidyl serine, 0.2 μg/μl diolein, 1 mM ATP, and 2 μCi/reaction ^{32}P-ATP. After incubation, 25 μl of reaction were spotted onto P81 filter paper, washed five times in 75 mM phosphoric acid, and once in ethanol. After drying, ^{32}P incorporation was determined with the use of a scintillation counter (model LS6500, Beckman).

Materials. Kinase-negative PKC-δ (AdKN-PKC-δ) and kinase-negative PKC-α (AdKN-PKC-α) adenoviruses (Ad) were gifts from Dr. Trevor Biden (Baker Heart Research Institute, Melbourne, Australia). The PKCs were rendered kinase negative by a point mutation in the ATP-binding region of the kinase domain, and replication-deficient adenoviruses were generated as previously described (11). An adenovirus-containing constitutively active PKC-δ (AdCa-PKC-δ) was a gift from Allen Sameral (Loyola University, Chicago, IL). All adenovirus stocks were propagated by the addition of small amounts of virus to human embryonic kidney HEK-293 cells. When cells were ~50% lysed, cells and media were collected, subjected to 3× freeze/thaw cycles, aliquotted, and stored at −80°C. Titer assays were performed by the method of O'Carroll et al. (43). All assays were performed with the use of an adenovirus-containing β-galactosidase as a control at matching multiplicity of infection (MOI).

Polyclonal antibodies to PKC-δ, -α, and -ε were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody for ERK2 was purchased from Transduction Laboratories (Lexington, KY). The antibodies specific for active and total ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA). Antibody selective for $p21^{ras}$ was purchased from Oncogene research products. The inhibitors of PKC-δ, PKC-α, PI3-kinase, and MEK1 were purchased from Calbiochem (La Jolla, CA). All tissue culture media were purchased from GIBCO-BRL (Life Technologies) unless specifically stated otherwise. Tissue culture supplies (dishes, pipettes, etc.) were purchased from Fisher Scientific. SDS-PAGE and Western blotting supplies were purchased from Bio-Rad unless otherwise stated. All other chemicals were purchased from Sigma (St. Louis, MO).

RESULTS

PLC-dependent regulation of PDGF signaling. Phospholipase C-γ (PLC-γ) is activated in response to growth factors, including PDGF (30), and has been shown to mediate PDGF-dependent activation of PKCs (40). In VSM cells, PLC-γ has been reported to have an important role in mitogenic responses, including cell migration and proliferation (54). Treatment of VSM cells with U-73122, a pharmacological inhibitor of PLCs, inhibited PDGF-induced increases in ERK1/2 activity in a concentration-dependent manner (Fig. 1*A*), confirming earlier studies and indicating a potential role for PKC isozymes in mediating PDGF-dependent increases in ERK1/2 activity. As a negative control, U-73122 had no effect on ERK1/2 activation in response to phorbol 12,13-dibutyrate (PDBu), a direct activator of classic (c)PKC and novel (n)PKC isozymes (Fig. 1*A*). Treatment of VSM cells with U-73433, an inactive analog of U-73122, also had no effect on PDGF-stimulated ERK1/2 activation (Fig. 1*B*).

PDGF-dependent regulation of PKC-δ. In addition to allosteric activation by Ca^{2+} and/or diacylglycerol, a series of

Downloaded from http://ajpcell.physiology.org/ by 10.220.33.6 on May 26, 2017

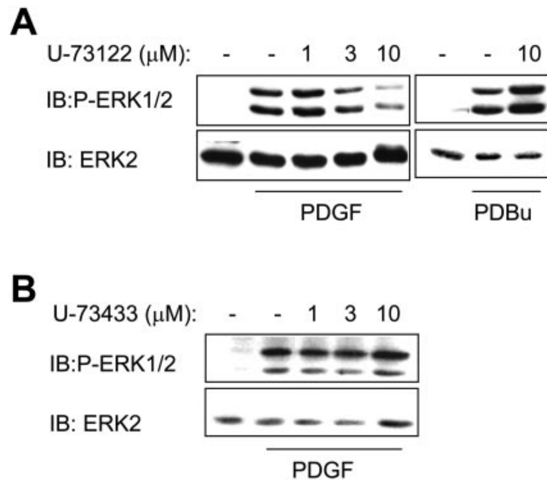

Fig. 1. Phospholipase C (PLC)-dependent regulation of platelet-derived growth factor (PDGF) induced ERK1/2 activation. *A*: vascular smooth muscle (VSM) cells were stimulated with 40 ng/ml PDGF-BB for 5 min or 0.3 μM phorbol 12,13-dibutyrate (PDBu) for 10 min after a 30-min pretreatment with U-73122, a selective PLC inhibitor. VSM whole cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membrane. ERK1/2 activity was assessed by immunoblotting (IB) with antibody specific for activated ERK1/2 (IB:P-ERK1/2). The membranes were also immunoblotted with antibody selective for ERK2 (IB:ERK2) to verify equal protein loading. *B*: VSM cells were treated with U-73433, an inactive analog of U-73122, 30 min before PDGF-BB stimulation, and ERK1/2 activity was determined as described above.

"priming" phosphorylations (29), association with membranes, and protein-protein interactions (42) can affect PKC activity and function. PKC-δ activity may also be modulated by Src family kinase (SFK)-dependent tyrosine phosphorylations (20). Treatment of VSM cells with PDGF resulted in phosphorylation of PKC-δ at Thr^{505}, a phosphorylation event reported to be consequent to PKC-δ activation (29) (Fig. 2*A*). Immunoprecipitated PKC-δ from PDGF-stimulated VSM cells had enhanced activity assayed in vitro compared with PKC-δ immunoprecipitated from unstimulated cells (Fig. 2*B*). Treatment with PDGF also resulted in robust tyrosine phosphorylation of PKC-δ (Fig. 2*C*), a response previously shown to require PKC-δ activation (22) and to be dependent on a SFK (5). Interestingly, treatment with EGF did not result in PKC-δ tyrosine phosphorylation, as has been shown in other cells types (13), suggesting a unique functional relationship between PKC-δ and PDGF in VSM.

PKC-δ involvement in PDGF-stimulated activation of ERK1/2. Given the evidence above that PDGF activates PKC-δ and our previous study (18), indicating that PKC-δ mediates ERK1/2 activation in response to ATP and PDBu, involvement of PKC-δ in PDGF-dependent activation of ERK1/2 was tested. Pretreatment of VSM cells with rottlerin (0.1–10 μM)-inhibited PDGF (10 ng/ml) stimulated ERK1/2 activation, consistent with its reported efficacy as a selective PKC-δ inhibitor (23) (Fig. 3). As a negative control, rottlerin had no effect on ionomycin-induced activation of ERK1/2 (Fig. 3), a response previously shown to depend on increases in free intracellular Ca^{2+} and activation of CaM kinase II in these cells (15).

Rottlerin (5 μM) effectively blocked PDGF-dependent activation of PKC-δ as reflected by Thr^{505} phosphorylation, regardless of the level of PDGF stimulation (Fig. 4*A*). However, the inhibitory effect of rottlerin on PDGF-induced ERK1/2 activation diminished with increasing concentrations of PDGF (Fig. 4*B*). These results indicate involvement of PKC-δ in PDGF-dependent activation of ERK1/2 along with the recruitment of additional, redundant pathways leading to ERK1/2 activation with increasing levels of PDGF stimulation.

To further verify PKC-δ involvement in PDGF-dependent activation of ERK1/2, a complementary molecular approach was used. Kinase-negative PKC-δ (KN-PKC-δ^{K376A}) has been shown to be catalytically inactive but able to compete with wild-type PKC-δ for substrate binding, thus acting as a dominant-negative enzyme (36). Overexpression of KN-PKC-δ utilizing an adenovirus expression system (AdKN-PKC-δ) (39), attenuated PDGF (10 ng/ml)-stimulated ERK1/2 activation while having no effect on ionomycin-stimulated activation (Ca^{2+} dependent) (Fig. 5*A*), or as previously shown, EGF-dependent activation of ERK1/2 (18).

PKC-α involvement in PDGF-stimulated activation of ERK1/2. Saito et al. (49) reported that PKC-α regulates PDGF-dependent activation of GAB1, a component of a PDGF-

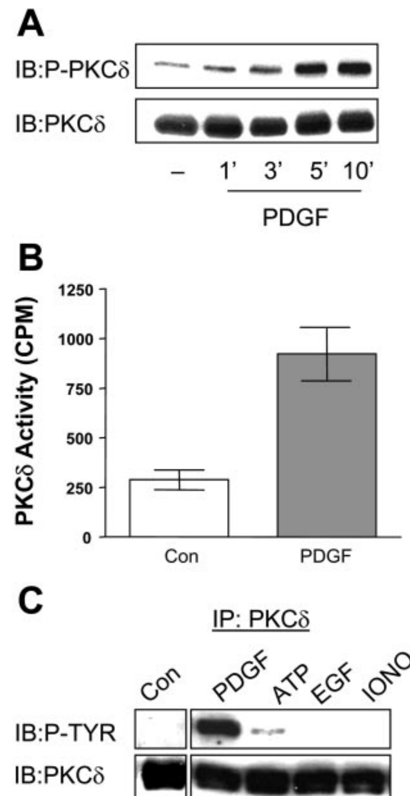

Fig. 2. PDGF-dependent regulation of PKC-δ. *A*: VSM cells were stimulated with 10 ng/ml PDGF-BB for the indicated times. Equal protein loads were immunoblotted with antibody recognizing phosphorylated PKC-δ (Thr^{505}, IB: P-PKC-δ) and total PKC-δ (IB:PKC-δ). *B*: PKC-δ was immunoprecipitated (IP) from quiescent VSM cells or VSM cells stimulated with 10 ng/ml PDGF-BB for 5 min. In vitro kinase assays were performed on the immunoprecipitates, as described in EXPERIMENTAL PROCEDURES. The graphs represent quantification of four separate experiments. Values shown are means ± SE. *C*: PKC-δ was immunoprecipitated (IP:PKC-δ) from control VSM cells or cells treated with 10 ng/ml PDGF-BB (5 min), 50 μM ATP (5 min), 5 ng/ml EGF (3 min), or 0.5 μM ionomycin (IONO, 5 min). Tyrosine phosphorylation of PKC-δ was determined by immunoblotting with antibody specific for tyrosine-phosphorylated proteins (IB:P-TYR, PY20). Total PKC-δ was determined using antibody specific for PKC-δ (IB:PKC-δ).

Downloaded from http://ajpcell.physiology.org/ by 10.220.33.6 on May 26, 2017

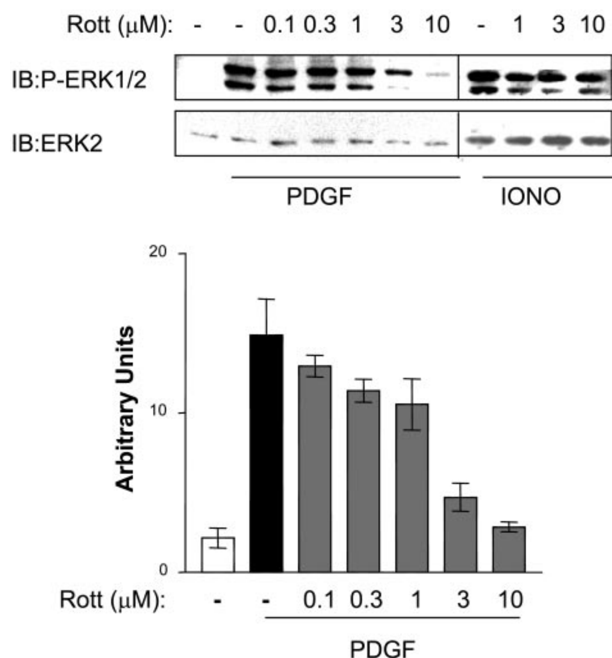

Fig. 3. Role of PKC-δ in PDGF-dependent activation of ERK1/2. VSM cells were pretreated with the indicated concentrations of rottlerin (Rott), a selective inhibitor of PKC-δ, for 30 min before stimulation with 10 ng/ml PDGF-BB or 0.5 μM ionomycin (Iono) for 5 min. ERK1/2 activity was assessed by immunoblotting as described earlier. The graph represents quantification of three experiments ± SE.

stimulated signaling pathway resulting in activation of ERK1/2. In agreement with this earlier study, pretreatment of VSM cells with Gö6976, a selective inhibitor of cPKCs, attenuated PDGF (10 ng/ml)-induced ERK1/2 activation in a concentration-dependent manner, consistent with its reported IC_{50} (21) (Fig. 6*A*). As a negative control, pretreatment with Gö6976 had no effect on PDBu-induced ERK1/2 activity, a response previously shown to be dependent on PKC-δ in VSM cells (18). Unlike the effects of the PKC-δ inhibitor, inhibition of ERK1/2 with Gö6976 was independent of the level of PDGF stimulation (Fig. 6*B*). To verify PKC-α involvement in PDGF-dependent activation of ERK1/2, KN-PKC-α was overexpressed in VSM cells as described earlier using AdKN-PKC-α (27). Similar to Gö6976 pretreatment, overexpression of KN-PKC-α attenuated PDGF-dependent activation of ERK1/2 while having little effect on PDBu-stimulated ERK1/2 activity (Fig. 5*B*), the latter in agreement with previous studies (9, 18).

PKC-α-dependent regulation of PKC-δ activity. We have previously shown that treatment with rottlerin but not Gö6976 inhibited phorbol ester- or ATP-dependent activation of PKC-δ measured by PKC-δ Thr^{505} phosphorylation (18), indicating the relative specificity of these PKC inhibitors. Similarly, immunoprecipitated PKC-δ catalytic activity from PDBu-stimulated VSM cells was unaffected by pretreating the cells with Gö6976, but blocked by rottlerin pretreatment (Fig. 7*A*). In contrast, pretreatment with either rottlerin or Gö6976 attenuated PDGF-stimulated increases in PKC-δ catalytic activity in PKC-δ immunoprecipitates (Fig. 7*A*). A similar pattern of effects was observed with the use of complementary molecular approaches. Overexpression of KN-PKC-α inhibited PDGF-stimulated increases in PKC-δ activity in PKC-δ immunoprecipitates (Fig. 7*B*). Conversely, PKC-δ inhibitors had no significant effect on PDGF-stimulated PKC-α activity in PKC-α immunoprecipitates (Fig. 7*C*). Taken together, these results indicate a role for PKC-α in the PDGF-dependent regulation of PKC-δ.

Involvement of SFKs in PDGF-stimulated ERK1/2 activation and tyrosine phosphorylation of PKC-δ. Recently, it was reported that SU-6656 is a useful tool for determining the role of SFKs in PDGF-dependent signaling because of its relative specificity for SFK inhibition compared with the PDGF receptor tyrosine kinase itself (5). Treatment of VSM cells with SU-6656 or PP2, an alternative SFK inhibitor, attenuated PDGF-induced ERK1/2 activation (Fig. 8*A*), consistent with previous reports (45) suggesting roles for SFKs in PDGF-dependent signaling. However, the function of SKFs in PDGF signaling is likely to be complex and at multiple levels. As shown in Fig. 2, stimulation of VSM cells with PDGF results in the tyrosine phosphorylation of PKC-δ. The addition of SU-6656 (Fig. 7*B*) or PP2, another selective SFK tyrosine kinase inhibitor, inhibited PDGF-stimulated tyrosine phosphorylation of PKC-δ (Fig. 7*B*). These data are consistent with previous reports indicating a role for SFKs in catalyzing the tyrosine phosphorylation of PKC-δ (12, 52) and suggest one

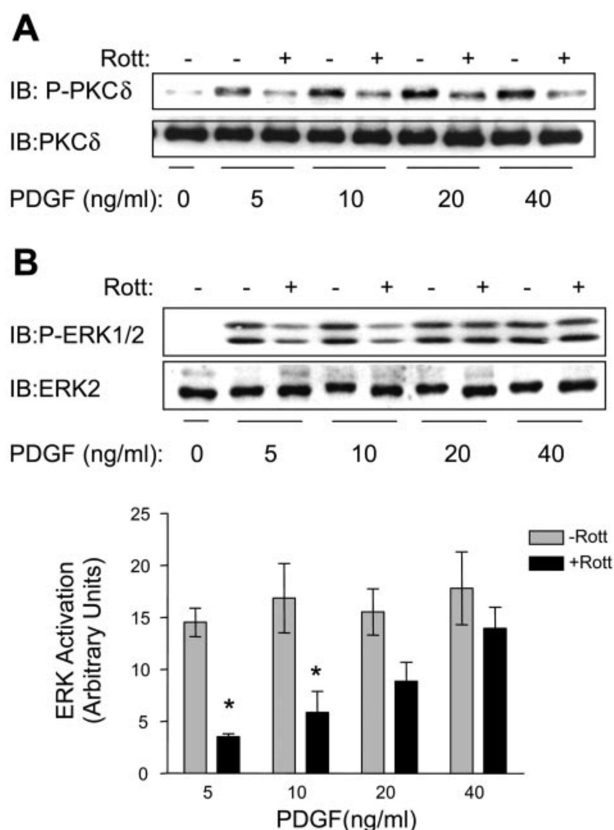

Fig. 4. Effect of PDGF concentration on the role of PKC-δ in ERK1/2 activation. *A*: VSM cells were pretreated with 5 μM rottlerin 30 min before stimulation with the indicated concentrations of PDGF-BB for 5 min. PDGF induced PKC-δ phosphorylation on Thr^{505} (IB:P-PKC-δ) and total PKC-δ (IB:PKC-δ) was assessed by immunoblotting. *B*: ERK1/2 activity (IB:P-ERK1/2) in VSM cells treated as described above was determined by immunoblotting. Films from 3 separate experiments were subjected to scanning densitometry and the results were quantified as shown on the graph ± SE. $*P < 0.05$ as determined by ANOVA.

Downloaded from http://ajpcell.physiology.org/ by 10.220.33.6 on May 26, 2017

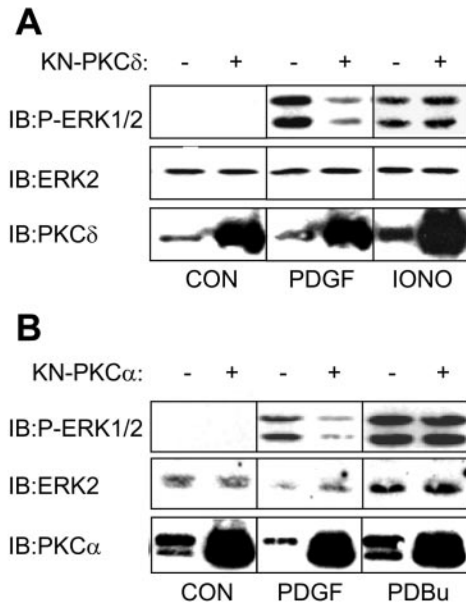

Fig. 5. Effects of kinase-negative PKC-δ (KN-PKC-δ) and kinase-negative PKC-α (KN-PKC-α) on PDGF-dependent activation of ERK1/2. *A*: VSM cells were infected with 10 multiplicity of infection (MOI) AdKN-PKC-δ. After 48 h, the cells were then stimulated with 10 ng/ml PDGF-BB or 0.5 μM ionomycin for 5 min and ERK1/2 activation was determined by immunoblotting for active ERK1/2 (P-ERK1/2). Immunoblotting for PKC-δ was carried out to verify overexpression of KN-PKC-δ. *B*: VSM cells were infected for 48 h with 50 MOI AdKN-PKC-α before stimulation with 10 ng/ml PDGF-BB or 0.1 μM phorbol 12,13-dibutyrate (PDBu) as indicated. ERK1/2 activity (IB:P-ERK1/2) and overexpression of KN-PKC-α (IB:PKC-α) was determined by immunoblotting.

potential mechanism for SFK involvement in PDGF signaling in these cells.

PKC-α-dependent regulation of PKC-δ tyrosine phosphorylation and catalytic activity. In VSM cells, PDBu stimulates PKC-δ catalytic activity (Fig. 7) (18) and results in the tyrosine phosphorylation of PKC-δ (Fig. 9*A*). Pretreatment with rottlerin effectively blocks both PKC-δ catalytic activity (Fig. 7) (18) and PKC-δ tyrosine phosphorylation stimulated by the direct phorbol ester activator PDBu (Fig. 9*A*), suggesting that PKC-δ must be activated to be tyrosine phosphorylated and/or that activation of the SKF catalyzing this phosphorylation is a consequence of PKC-δ activation. Interestingly, treatment with 5 μM rottlerin inhibited PKC-δ tyrosine phosphorylation stimulated by lower concentrations (5 ng/ml) of PDGF but not higher concentrations (10–40 ng/ml) (Fig. 9*A*). These results are significant in that under conditions of higher levels of PDGF stimulation, PKC-δ catalytic activity is dissociated from PKC-δ tyrosine phosphorylation, suggesting activation of a SFK that is independent of PKC-δ activation. Given the involvement of PKC-α in PDGF-stimulated ERK1/2 activation and PKC-δ activation, we tested the hypothesis that PKC-δ tyrosine phosphorylation may be similarly dependent on PKC-α. Consistent with the hypothesis, Gö6976 dose-dependently attenuated PDGF-induced PKC-δ tyrosine phosphorylation (Fig. 9*B*), irrespective of the level of PDGF stimulation (Fig. 9*C*).

DISCUSSION

PDGF-dependent signaling has been well studied as an archetypal pathway for activation of ERK1/2 (1, 4, 38, 44, 46). Classically, this pathway involves PDGF receptor dimerization, tyrosine autophosphorylation, and recruitment of adapter proteins resulting in the Ras-dependent sequential activation of Raf, MEK, and ERK1/2. However, PDGF also stimulates additional signaling molecules, including PLC-γ and PI3-kinase. A recent study (49) in VSM cells, confirmed here, implicates PLC-γ as a key mediator of PDGF-induced ERK1/2 activation.

Activation of cPKC and nPKC isozymes is secondary to activation of PLC and generation of diacylglycerol. PKCs have been identified as key mediators of signaling pathways leading to ERK1/2 activation (20, 41, 51), although most of these studies have been in the context of G protein-coupled receptor stimuli that couple to PLC-β and phospholipase D (6, 8, 37,

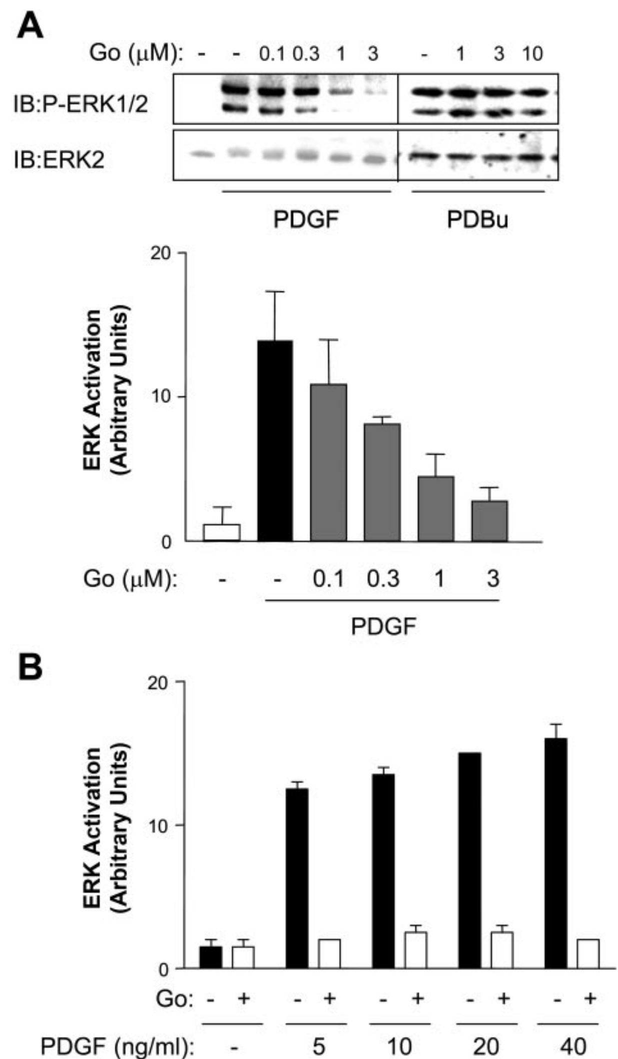

Fig. 6. Role of PKC-α in PDGF-dependent activation of ERK1/2. *A*: VSM cells were pretreated with the indicated concentrations of Gö6976, a selective inhibitor of PKC-α, for 30 min before stimulation with 10 ng/ml PDGF-BB or 0.1 μM PDBu for 5 min. ERK1/2 activity was assessed by immunoblotting with an antibody specific for activated ERK1/2 (IB: P-ERK1/2) and total ERK1/2 (IB: ERK1/2). The graph represents quantification of three experiments ± SE. *B*: VSM cells were pretreated with 1 μM Gö6976 for 30 min before stimulation with increasing concentrations of PDGF-BB as indicated. ERK1/2 activation was determined by immunoblotting for active ERK1/2 (P-ERK1/2) and the blots quantified by scanning densitometry. The values shown are means ± SE from 3 experiments.

Downloaded from http://ajpcell.physiology.org/ by 10.220.33.6 on May 26, 2017

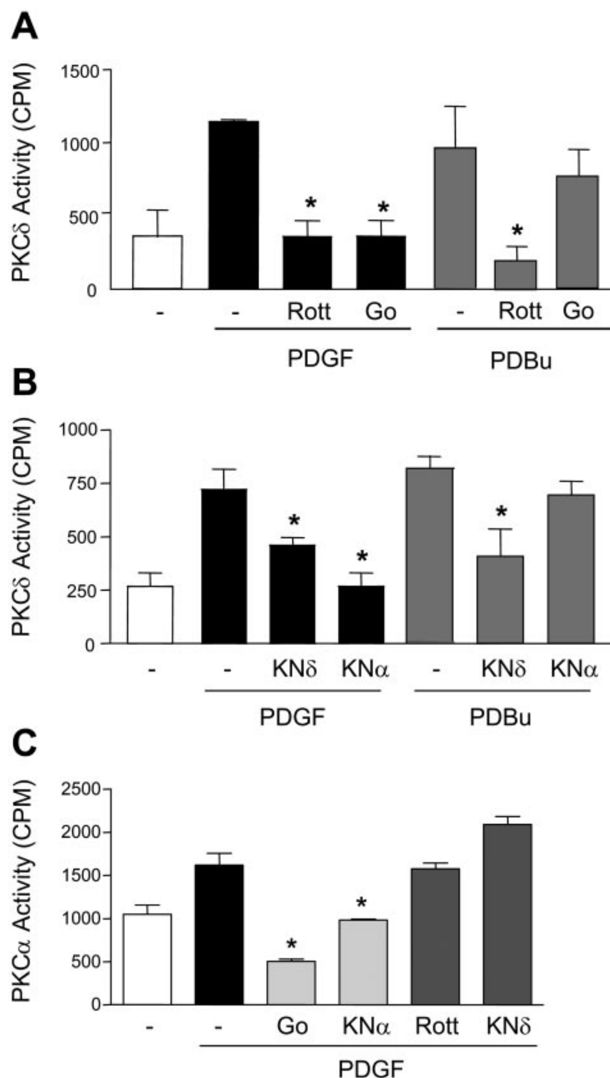

Fig. 7. Isozyme-selective inhibition of PKC-δ in VSM cells. *A*: VSM cells were pretreated with 5 μM rottlerin (Rott) or 2 μM Gö6976 (Go) before stimulation with10 ng/ml PDGF-BB or 0.3 μM PDBu. PKC-δ was immunoprecipitated from the cell lysates and PKC kinase activity was measured as described in EXPERIMENTAL PROCEDURES. The graphs represent quantification of four separate experiments. *B*: VSM cells were infected with 10 MOI kinase-negative PKC-δ (KNδ) or 50 MOI kinase-negative PKC-α (KNα) for 48 h before stimulation with PDGF-BB or PDBu as described above. PKC-δ was immunoprecipitated and kinase activity measured as described above. The graph represents quantitation of four separate experiments. *C*: VSM cells were treated with 2 μM Gö6976, 5 μM rottlerin, 50 MOI KNα, or 10 MOI KNδ, as described above. After treatment with 10 ng/ml PDGF-BB for 5 min, PKC-α was immunoprecipitated and kinase activity measured, as described above. All values shown are means ± SE. $*P < 0.05$.

53) activation. Recently, we (18) reported that PKC-δ, specifically compared with PKC-α, was responsible for mediating G protein-coupled receptor- and phorbol ester-dependent activation of ERK1/2 in cultured rat VSM cells. Other studies have generally excluded PKC-δ from growth factor and PLC-γ-dependent pathways leading to ERK1/2 activation in smooth muscle (49) and heart (27) in favor of PKC-α and -ε isozymes (47, 49). Consistent with those studies, we demonstrate here that PDGF stimulation of ERK1/2 in VSM cells depend strongly on PKC-α, as inferred through the effects of both a pharmacological inhibitor of cPKCs (Gö6976) or by overexpressing a kinase-negative PKC-α mutant. However, we also show for the first time that PDGF is a strong activator of PKC-δ in VSM, and that PKC-δ is involved in regulating ERK1/2 in response to low levels of PDGF stimulation. Apparently, additional pathways dependent on PKC-α are recruited with increasing concentrations of PDGF, perhaps explaining the apparent lack of dependence on PKC-δ reported in the earlier studies (47, 49). Previous studies (32) reporting coimmunoprecipitation of PKC-δ with the PDGF receptor suggest that this signaling pathway may be compartmentalized and possibly localized with a subset of PDGF receptors most sensitive to PDGF activation. In a similar manner, complementary roles for PI3-kinase (14) and FAK (26) in PDGF-dependent ERK1/2 have been discovered, suggesting a hierarchy of PDGF signaling that may be linked to specific functional outcomes.

In the present study, we measured increases in PKC-δ catalytic activity, assayed under conditions of saturating lipid activators, in PKC-δ immunoprecipitates from PDGF- and PDBu-stimulated cells. The molecular basis for this increase in specific activity may be secondary to autophosphorylation after lipid-dependent activation (29), or, as discussed below, secondary to tyrosine phosphorylation of active PKC-δ. Consistent with either idea, PKC-δ activation stimulated by PDBu was inhibited specifically by pretreating with rottlerin, the PKC-δ selective inhibitor, or by overexpressing kinase-negative PKC-δ. However, PKC-δ activation in response to stimulation by PDGF was inhibited by either PKC-δ or PKC-α inhibitors. This raises the possibility that the co-requirement for the two isozymes in the activation of ERK1/2 by low concentrations of PDGF results from a hierarchy of PKC signaling with PDGF-induced PKCδ activation

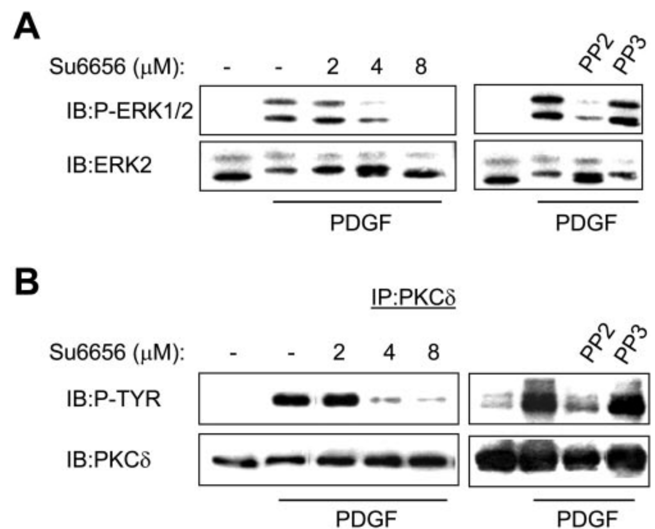

Fig. 8. Role of Src family kinases in PDGF-dependent activation of ERK1/2 and tyrosine phosphorylation of PKC-δ. *A*: VSM cells were treated with the indicated concentrations of SU-6656 or 1 μM PP2, selective inhibitors of Src family kinases, before stimulation with 10 ng/ml PDGF. PP3 (1 μM), an inactive analog of PP2, was used as a control for PP2 treatment. ERK1/2 activation was assessed by immunoblotting as described previously. *B*: VSM cells were treated as described above. PKC-δ was immunoprecipitated from cell lysates with PKC-δ selective antibody (IP:PKC-δ) and immunoblotted with an anti-P-tyr antibody to determine the extent of PKC-δ tyrosine phosphorylation (IB:P-TYR). Total PKC-δ was determined by immunoblotting with a PKC-δ-specific antibody (IB:PKC-δ).

Downloaded from http://ajpcell.physiology.org/ by 10.220.33.6 on May 26, 2017

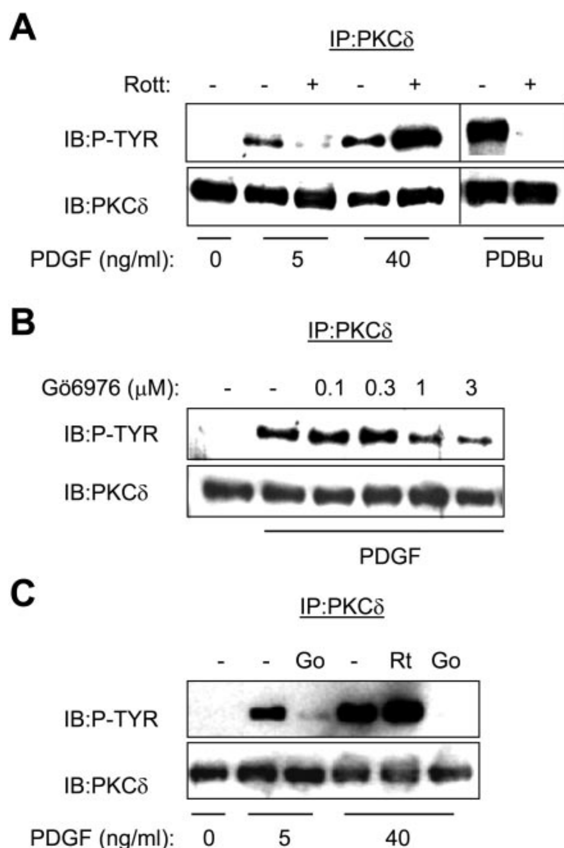

Fig. 9. Role of PKC-α in PDGF-dependent tyrosine phosphorylation of PKC-δ. *A*: VSM cells were pretreated with 5 μM rottlerin 30 min before stimulation with PDGF-BB for 5 min or 0.3 μM PDBu for 10 min. Tyrosine phosphorylation of PKC-δ was determined by immunoblotting with antibody specific for tyrosine phosphorylated proteins (IB:P-TYR). Total PKC-δ was determined using antibody specific for PKC-δ (IB:PKC-δ). *B*: VSM cells were treated with the indicated concentrations of Gö6976 for 30 min before stimulation with 10 ng/ml PDGF-BB for 5 min. PKC-δ was immunoprecipitated (IP:PKC-δ) from the lysates and immunoblotted for tyrosine phosphorylation (IB:P-TYR) and total PKC-δ (IB:PKC-δ). *C*: VSM cells were treated with 2 μM Gö6976 (Go) or 5 μM Rottlerin (Rt) for 30 min before stimulation with 5 or 40 ng/ml PDGF as indicated. PDGF-dependent tyrosine phosphorylation of PKC-δ in PKC-δ immunoprecipitates was determined by immunoblotting as described earlier.

dependent on PKC-α activation. One potential mechanism to explain this unexpected hierarchy is through PKC-α-dependent regulation of PKC-δ access to membrane lipid activators and/or membrane-bound SFKs. Although speculative at this point, this mechanism is consistent with a previously proposed role for PKC-α in regulating PDGF receptor-dependent scaffolding events (49). Thus regulation of protein-protein interactions by PKC-α may also play an important role in mediating PDGF-induced activation of PKC-δ and subsequent cellular functions (41).

It is well established that PDGF stimulation in several cell types results in tyrosine phosphorylation of PKC-δ catalyzed by a SFK (3, 5, 20, 22) and a recent study (34) indicated that PDGF or phorbol ester-stimulated PKCδ Tyr^{187} phosphorylation is specifically catalyzed by Fyn. Previous studies (22) using inhibitors of PKC activity suggested that tyrosine phosphorylation of PKC-δ requires PKC-δ activation. Consistent with this, we observed nearly complete inhibition of PDBu-stimulated PKC-δ tyrosine phosphorylation by the PKC-δ-selective inhibitor rottlerin. Similarly, tyrosine phosphorylation of PKC-δ stimulated by low concentrations of PDGF was inhibited by rottlerin. However, in response to higher levels of PDGF stimulation, the tyrosine phosphorylation response was insensitive to rottlerin indicating that PKC-δ activation per se was not required for its tyrosine phosphorylation. This finding, and the fact that PDGF-stimulated tyrosine phosphorylation of PKC-δ was inhibited by Gö6976, strongly suggests that under these conditions, PKC-α activation is required for activation of a SFK, or, as suggested above, for controlling access of PKC-δ to an active SFK. PKC-dependent regulation of SFK activity could occur through either direct regulation of SFKs or by mediating a protein tyrosine phosphatase (PTP), such as SHP2 (49), PTP-α (52), or PTP phosphates enriched in prolines, glutamic acid, serines, and threonines (PEST) (24), which in turn modulates SFK activity.

Although we observed consistent correlations in the patterns of PKC-δ activation and PKC-δ tyrosine phosphorylation, the functional outcome of PKC-δ tyrosine phosphorylation with respect to catalytic activity is controversial with some reports (35) indicating enhancement of activity and other reports (20) showing inhibition of activity. These conflicting results may ultimately be explained, at least in part, by the stimulus used to induce PKC-δ tyrosine phosphorylation and the specific residues phosphorylated. The functional significance of PKC-δ tyrosine phosphorylation from the standpoint of the ERK1/2 end points in this system is also not yet understood. SFK inhibitors, including SU-6656 and PP1, inhibited both PKC-δ tyrosine phosphorylation and PDGF-stimulated ERK1/2 activation, consistent with a positive function for PKC-δ tyrosine phosphorylation in activation of ERK1/2. However, SFKs could be involved at multiple levels in the complex PDGF-stimulated ERK1/2 signaling network (45, 49). Therefore, we cannot yet rule out the possibility that tyrosine phosphorylation of PKC-δ is unrelated to, or perhaps even negatively regulates, PKC-δ-dependent activation of ERK1/2. Additional studies, perhaps using site-specific mutation of specific PKC-δ tyrosine residues would be required to definitively assess the function of PKC-δ tyrosine phosphorylation in mediating PDGF-stimulated increases in PKC-δ activity and ERK/1/2 activation.

In summary, in the present study we demonstrate the differential involvement of two PKC isozymes in the regulation of ERK1/2 activation in VSM cells in response to stimulation by PDGF. On the basis of both pharmacological and molecular inhibitory approaches, PKC-α plays an important role in PDGF stimulation of ERK1/2, consistent with previously published studies (49). Interestingly, a PKC-δ-dependent pathway appears to be selectively involved in regulating ERK1/2 activation only in response to low levels of PDGF stimulation. An unexpected hierarchy of PKC signaling was discovered, with PKC-α regulating PKC-δ activity and PKC-δ tyrosine phosphorylation after stimulation by PDGF but not PDBu. A potential mechanism is proposed whereby PKC-α regulates access of PKC-δ to membrane lipid activators and/or Src family kinases. Future studies are required to test this mechanism and to clarify the functional significance of the PKC-α/PKC-δ interaction and PKC-δ tyrosine phosphorylation with respect to PDGF signaling and function in VSM cells.

Downloaded from http://ajpcell.physiology.org/ by 10.220.33.6 on May 26, 2017

ACKNOWLEDGMENTS

The authors thank Virginia Foster for technical assistance and Wendy Hobb for assistance in preparing this manuscript.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants R01-HL-40992 and R01-HL-49426.

REFERENCES

1. **Alimandi M, Heidaran MA, Gutkind JS, Zhang J, Ellmore N, Valius M, Kazlauskas A, Pierce JH, and Li W.** PLC-γ activation is required for PDGF-βR-mediated mitogenesis and monocytic differentiation of myeloid progenitor cells. *Oncogene* 15: 585–593, 1997.
2. **Barry ST and Critchley DR.** The RhoA-dependent assembly of focal adhesions in Swiss 3T3 cells is associated with increased tyrosine phosphorylation and the recruitment of both pp125FAK and protein kinase C-δ to focal adhesions. *J Cell Sci* 107: 2033–2045, 1994.
3. **Benes C and Soltoff SP.** Modulation of PKCδ tyrosine phosphorylation and activity in salivary and PC-12 cells by Src kinases. *Am J Physiol Cell Physiol* 280: C1498–C1510, 2001.
4. **Berk BC.** Vascular smooth muscle growth: autocrine growth mechanisms. *Physiol Rev* 81: 999–1030, 2001.
5. **Blake RA, Broome MA, Liu X, Wu J, Gishizky M, Sun L, and Courtneidge SA.** SU6656, a selective src family kinase inhibitor, used to probe growth factor signaling. *Mol Cell Biol* 20: 9018–9027, 2000.
6. **Booz GW and Baker KM.** Protein kinase C in angiotensin II signalling in neonatal rat cardiac fibroblasts. Role in the mitogenic response. *Ann NY Acad Sci* 752: 158–167, 1995.
7. **Bornfeldt KE, Raines EW, Graves LM, Skinner MP, Krebs EG, and Ross R.** Platelet-derived growth factor. Distinct signal transduction pathways associated with migration versus proliferation. *Ann NY Acad Sci* 766: 416–430, 1995.
8. **Burnstock G.** Purinergic signaling and vascular cell proliferation and death. *Arterioscler Thromb Vasc Biol* 22: 364–373, 2002.
9. **Busuttil SJ, Morehouse DL, Youkey JR, and Singer HA.** Antisense suppresssion of protein kinase C-α and -δ in vascular smooth muscle. *J Surg Res* 63: 137–142, 1996.
10. **Cantley LC.** Growth factors bind receptor tyrosine kinases to stimulate cell survival, cell division, cell growth, and cytoskeletal rearrangement. *Sci STKE* 2003: tr8, 2003.
11. **Carpenter L, Cordery D, and Biden TJ.** Inhibition of protein kinase Cδ protects rat INS-1 cells against interleukin-1beta and streptozotocin-induced apoptosis. *Diabetes* 51: 317–324, 2002.
12. **Crosby D and Poole AW.** Physical and functional interaction between protein kinase Cδ and Fyn tyrosine kinase in human platelets. *J Biol Chem* 278: 24533–24541, 2003.
13. **Denning MF, Dlugosz AA, Threadgill DW, Magnuson T, and Yuspa SH.** Activation of the epidermal growth factor receptor signal transduction pathway stimulates tyrosine phosphorylation of protein kinase Cδ. *J Biol Chem* 271: 5325–5331, 1996.
14. **Duckworth BC and Cantley LC.** Conditional inhibition of the mitogen-activated protein kinase cascade by wortmannin. Dependence on signal strength. *J Biol Chem* 272: 27665–27670, 1997.
15. **Eguchi S, Iwasaki H, Ueno H, Frank GD, Motley ED, Eguchi K, Marumo F, Hirata Y, and Inagami T.** Intracellular signaling of angiotensin II-induced p70 S6 kinase phosphorylation at Ser^{411} in vascular smooth muscle cells. Possible requirement of epidermal growth factor receptor, Ras, extracellular signal-regulated kinase, and Akt. *J Biol Chem* 274: 36843–36851, 1999.
16. **Eguchi S, Numaguchi K, Iwasaki H, Matsumoto T, Yamakawa T, Utsunomiya H, Motley ED, Kawakatsu H, Owada KM, Hirata Y, Marumo F, and Inagami T.** Calcium-dependent epidermal growth factor receptor transactivation mediates the angiotensin II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. *J Biol Chem* 273: 8890–8896, 1998.
17. **Geisterfer AA, Peach MJ, and Owens GK.** Angiotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells. *Circ Res* 62: 749–756, 1988.
18. **Ginnan R, Pfleiderer PJ, Pumiglia K, and Singer HA.** PKC-δ and CaMKII-δ_2 mediate ATP-dependent activation of ERK1/2 in vascular smooth muscle. *Am J Physiol Cell Physiol* 286: C1281–C1289, 2004.
19. **Ginnan R and Singer HA.** CaM kinase II-dependent activation of tyrosine kinases and ERK1/2 in vascular smooth muscle. *Am J Physiol Cell Physiol* 282: C754–C761, 2002.
20. **Gschwendt M.** Protein kinase Cδ. *Eur J Biochem* 259: 555–564, 1999.
21. **Gschwendt M, Dieterich S, Rennecke J, Kittstein W, Mueller HJ, and Johannes FJ.** Inhibition of protein kinase Cμ by various inhibitors. Differentiation from protein kinase C isoenzymes. *FEBS Lett* 392: 77–80, 1996.
22. **Gschwendt M, Kielbassa K, Kittstein W, and Marks F.** Tyrosine phosphorylation and stimulation of protein kinase Cδ from porcine spleen by Src in vitro. Dependence on the activated state of protein kinase Cδ. *FEBS Lett* 347: 85–89, 1994.
23. **Gschwendt M, Muller HJ, Kielbassa K, Zang R, Kittstein W, Rincke G, and Marks F.** Rottlerin, a novel protein kinase inhibitor. *Biochem Biophys Res Commun* 199: 93–98, 1994.
24. **Habib T, Herrera R, and Decker SJ.** Activators of protein kinase C stimulate association of Shc and the PEST tyrosine phosphatase. *J Biol Chem* 269: 25243–25246, 1994.
25. **Haller H, Lindschau C, Maasch C, Olthoff H, Kurscheid D, and Luft FC.** Integrin-induced protein kinase Cα and Cε translocation to focal adhesions mediates vascular smooth muscle cell spreading. *Circ Res* 82: 157–165, 1998.
26. **Hauck CR, Hsia DA, and Schlaepfer DD.** Focal adhesion kinase facilitates platelet-derived growth factor-BB-stimulated ERK2 activation required for chemotaxis migration of vascular smooth muscle cells. *J Biol Chem* 275: 41092–41099, 2000.
27. **Heidkamp MC, Bayer AL, Martin JL, and Samarel AM.** Differential activation of mitogen-activated protein kinase cascades and apoptosis by protein kinase Cε and δ in neonatal rat ventricular myocytes. *Circ Res* 89: 882–890, 2001.
28. **Iwabu A, Smith K, Allen FD, Lauffenburger DA, and Wells A.** Epidermal growth factor induces fibroblast contractility and motility via a protein kinase Cδ-dependent pathway. *J Biol Chem* 279: 14551–14560, 2004.
29. **Keranen LM, Dutil EM, and Newton AC.** Protein kinase C is regulated in vivo by three functionally distinct phosphorylations. *Curr Biol* 5: 1394–1403, 1995.
30. **Klinghoffer RA, Duckworth B, Valius M, Cantley L, and Kazlauskas A.** Platelet-derived growth factor-dependent activation of phosphatidylinositol 3-kinase is regulated by receptor binding of SH2-domain-containing proteins which influence Ras activity. *Mol Cell Biol* 16: 5905–5914, 1996.
31. **Klinghoffer RA, Sachsenmaier C, Cooper JA, and Soriano P.** Src family kinases are required for integrin but not PDGFR signal transduction. *EMBO J* 18: 2459–2471, 1999.
32. **Konishi H, Yamauchi E, Taniguchi H, Yamamoto T, Matsuzaki H, Takemura Y, Ohmae K, Kikkawa U, and Nishizuka Y.** Phosphorylation sites of protein kinase Cδ in H_2O_2-treated cells and its activation by tyrosine kinase in vitro. *Proc Natl Acad Sci USA* 98: 6587–6592, 2001.
33. **Li C, Wernig F, Leitges M, Hu Y, and Xu Q.** Mechanical stress-activated PKCδ regulates smooth muscle cell migration. *FASEB J* 17: 2106–2108, 2003.
34. **Li W, Chen XH, Kelley CA, Alimandi M, Zhang J, Chen Q, Bottaro DP, and Pierce JH.** Identification of tyrosine 187 as a protein kinase C-δ phosphorylation site. *J Biol Chem* 271: 26404–26409, 1996.
35. **Li W, Mischak H, Yu JC, Wang LM, Mushinski JF, Heidaran MA, and Pierce JH.** Tyrosine phosphorylation of protein kinase C-δ in response to its activation. *J Biol Chem* 269: 2349–2352, 1994.
36. **Li W, Yu JC, Shin DY, and Pierce JH.** Characterization of a protein kinase C-δ (PKC-δ) ATP binding mutant. An inactive enzyme that competitively inhibits wild type PKC-δ enzymatic activity. *J Biol Chem* 270: 8311–8318, 1995.
37. **Li X, Lee JW, Graves LM, and Earp HS.** Angiotensin II stimulates ERK via two pathways in epithelial cells: protein kinase C suppresses a G-protein coupled receptor-EGF receptor transactivation pathway. *EMBO J* 17: 2574–2583, 1998.
38. **Lokker NA, Sullivan CM, Hollenbach SJ, Israel MA, and Giese NA.** Platelet-derived growth factor (PDGF) autocrine signaling regulates survival and mitogenic pathways in glioblastoma cells: evidence that the novel PDGF-C and PDGF-D ligands may play a role in the development of brain tumors. *Cancer Res* 62: 3729–3735, 2002.
39. **Matassa AA, Kalkofen RL, Carpenter L, Biden TJ, and Reyland ME.** Inhibition of PKCα induces a PKCδ-dependent apoptotic program in salivary epithelial cells. *Cell Death Differ* 10: 269–277, 2003.

Downloaded from http://ajpcell.physiology.org/ by 10.220.33.6 on May 26, 2017

40. **Moriya S, Kazlauskas A, Akimoto K, Hirai S, Mizuno K, Takenawa T, Fukui Y, Watanabe Y, Ozaki S, and Ohno S.** Platelet-derived growth factor activates protein kinase Cε through redundant and independent signaling pathways involving phospholipase Cγ or phosphatidylinositol 3-kinase. *Proc Natl Acad Sci USA* 93: 151–155, 1996.
41. **Nakashima S.** Protein kinase Cα (PKCα): regulation and biological function. *J Biochem (Tokyo)* 132: 669–675, 2002.
42. **Newton AC.** Protein kinase C: structure function and regulation. *J Biol Chem* 270: 28495–28498, 1995.
43. **O'Carroll SJ, Hall AR, Myers CJ, Braithwaite AW, and Dix BR.** Quantifying adenoviral titers by spectrophotometry. *Biotechniques* 28: 408–410, 412, 2000.
44. **Owens GK.** Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev* 75: 487–517, 1995.
45. **Parsons JT and Parsons SJ.** Src family protein tyrosine kinases: cooperating with growth factor and adhesion signaling pathways. *Curr Opin Cell Biol* 9: 187–192, 1997.
46. **Pearson G, Robinson F, Beers GT, Xu BE, Karandikar M, Berman K, and Cobb MH.** Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* 22: 153–183, 2001.
47. **Robin P, Boulven I, Bôle-Feysot C, Tanfin Z, and Leiber D.** Contribution of PKC-dependent and independent processes in the temporal regulation of ERK by ET-1, EGF and PDGF in rat myometrial cells: role in proliferation. *Am J Physiol Cell Physiol* 286: C798–C806, 2004.
48. **Ross R, Masuda J, and Raines EW.** Cellular interactions, growth factors, and smooth muscle proliferation in atherogenesis. *Ann NY Acad Sci* 598: 102–112, 1990.
49. **Saito Y, Hojo Y, Tanimoto T, Abe J, and Berk BC.** Protein kinase C-α and protein kinase C-ε are required for Grb2-associated binder-1 tyrosine phosphorylation in response to platelet-derived growth factor. *J Biol Chem* 277: 23216–23222, 2002.
50. **Shah BH, Farshori MP, Jambusaria A, and Catt KJ.** Roles of Src and epidermal growth factor receptor transactivation in transient and sustained ERK1/2 responses to gonadotropin-releasing hormone receptor activation. *J Biol Chem* 278: 19118–19126, 2003.
51. **Singer HA.** Protein kinase C. In: *Biochemistry of Smooth Muscle Contraction*, edited by Bárány M. New York: Academic, 1996, chapt. 12, p. 155–166.
52. **Stetak A, Csermely P, Ullrich A, and Keri G.** Physical and functional interactions between protein tyrosine phosphatase α, PI 3-kinase, and PKCδ. *Biochem Biophys Res Commun* 288: 564–572, 2001.
53. **Takahashi T, Kawahara Y, Okuda M, Ueno H, Takeshita A, and Yokoyama M.** Angiotensin II stimulates mitogen-activated protein kinases and protein synthesis by a Ras-independent pathway in vascular smooth muscle cells. *J Biol Chem* 272: 16018–16022, 1997.
54. **Tallquist MD, Klinghoffer RA, Heuchel R, Mueting-Nelsen PF, Corrin PD, Heldin CH, Johnson RJ, and Soriano P.** Retention of PDGFR-β function in mice in the absence of phosphatidylinositol 3′-kinase and phospholipase Cγ signaling pathways. *Genes Dev* 14: 3179–3190, 2000.
55. **Van Dijk MC, Hilkmann H, and van Blitterswijk WJ.** Platelet-derived growth factor activation of mitogen-activated protein kinase depends on the sequential activation of phosphatidylcholine-specific phospholipase C, protein kinase C-zeta and Raf-1. *Biochem J* 325: 303–307, 1997.

Downloaded from http://ajpcell.physiology.org/ by 10.220.33.6 on May 26, 2017