

## PKC- $\delta$ and CaMKII- $\delta_2$ mediate ATP-dependent activation of ERK1/2 in vascular smooth muscle

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**Ginnan, Roman, Paul J. Pfeleiderer, Kevin Pumiglia, and Harold A. Singer.** PKC- $\delta$  and CaMKII- $\delta_2$  mediate ATP-dependent activation of ERK1/2 in vascular smooth muscle. *Am J Physiol Cell Physiol* 286: C1281–C1289, 2004. First published January 28, 2004; 10.1152/ajpcell.00202.2003.—ATP, a purinergic receptor agonist, has been shown to be involved in vascular smooth muscle (VSM) cell DNA synthesis and cell proliferation during embryonic and postnatal development, after injury, and in atherosclerosis. One mechanism that ATP utilizes to regulate cellular function is through activation of ERK1/2. In the present study, we provide evidence that ATP-dependent activation of ERK1/2 in VSM cells utilizes specific isoforms of the multifunctional serine/threonine kinases, PKC, and Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) as intermediates. Selective inhibition of PKC- $\delta$  activity with rottlerin, or adenoviral overexpression of kinase-negative PKC- $\delta$ , attenuated the ATP- and phorbol 12,13-dibutyrate (PDBu)-stimulated ERK1/2 activation. Inhibition of PKC- $\alpha$  activity with Gö-6976, or adenoviral overexpression of kinase-negative PKC- $\alpha$ , was ineffective. Alternatively, treatment with KN-93, a selective inhibitor of CaMKII activation, or adenoviral overexpression of kinase-negative CaMKII- $\delta_2$ , inhibited ATP-dependent activation of ERK1/2 but had no effect on PDBu- or PDGF-stimulated ERK1/2. In addition, adenoviral overexpression of dominant-negative *ras* (Ad.HA-Ras<sup>N17</sup>) partially inhibited the ATP- and PDBu-induced activation of ERK1/2 and blocked ionomycin- and EGF-stimulated ERK1/2, and inhibition of tyrosine kinases with AG-1478, an EGFR inhibitor, or the *src* family kinase inhibitor PP2 attenuated ATP-stimulated ERK1/2 activation. Taken together, these data indicate that PKC- $\delta$  and CaMKII- $\delta_2$  coordinately mediate ATP-dependent transactivation of EGF receptor, resulting in increased ERK1/2 activity in VSM cells.

protein kinase C- $\delta$ ; calcium/calmodulin-dependent protein kinase II- $\delta_2$ ; extracellular signal-regulated kinase 1/2; epidermal growth factor receptor transactivation; adenovirus

VASCULAR DISEASES INCLUDING atherosclerosis, restenosis, and hypertension are characterized by increases in migration and neointimal proliferation of vascular smooth muscle (VSM) cells. Migration and proliferation of VSM cells can be initiated or modulated by peptide growth factors acting on receptor tyrosine kinases such as PDGF, basic FGF, and heparin-binding EGF (4), as well as agonists for G protein-coupled receptors (GPCR), such as angiotensin II (17), thrombin (26), and ATP (6). A common feature of these stimuli is their ability to activate ERK1/2, a member of the MAPK family. Many reports have described important roles for ERK1/2 in VSM proliferation (5), migration (20), and gene transcription (33).

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Recent studies have linked GPCR-stimulated pathways with growth factor receptor-dependent activation of ERK1/2 through a pathway involving EGF receptor (EGFR) transactivation (22). This occurs either through direct phosphorylation of the EGFR by *src*, which, in turn, triggers EGFR autophosphorylation necessary for interaction with adaptor proteins (30), or a metalloprotease-dependent release of membrane-bound heparin-binding EGF, which then acts as a ligand for the EGFR (10). The nonreceptor tyrosine kinase proline-rich tyrosine kinase (PYK2) may also be a component of this EGFR transactivation pathway (34). PYK2 is known to be activated by Ca<sup>2+</sup>-dependent or PKC-dependent pathways (2).

It is not fully understood how GPCR agonists couple to the activation of nonreceptor tyrosine kinases such as the *src* family kinases (SFKs) or PYK2. One potential mechanism is via GPCR-dependent activation of PLC and subsequent generation of diacylglycerol (DAG) and inositol trisphosphate, leading to PKC activation and increased intracellular free Ca<sup>2+</sup> concentration, respectively. In VSM cells, angiotensin II has been reported to result in EGFR transactivation and ERK1/2 activation by activating nonreceptor tyrosine kinases by a PKC-dependent mechanism (11). Recently, we reported evidence that Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) mediates PYK2 and ERK1/2 activation in response to Ca<sup>2+</sup>-mobilizing stimuli in VSM cells and that ERK1/2 activation under these circumstances required a SFK and EGFR transactivation (15). Thus activation of PLC results in the generation of two intracellular signals (DAG and Ca<sup>2+</sup>) that can independently activate nonreceptor tyrosine kinases, EGFR transactivation, and ERK1/2 activation through the actions of intermediate multifunctional serine/threonine kinases. An alternative pathway has also been described that involves G protein  $\beta\gamma$ -subunit-mediated scaffolding of adaptor proteins and *ras*-activating proteins (32).

On the basis of knowledge that multiple signaling pathways may impinge on nonreceptor tyrosine kinases and ultimately ERK1/2, it appears likely that utilization of these pathways would be stimulus, cell type, and situation dependent. To discern the relative contribution of a given input pathway, experimental approaches capable of acutely inhibiting the activity of endogenous components are required. In the case of PKC-dependent signaling, this approach is confounded by the fact that PKCs are a large family of protein kinases consisting of conventional (cPKC) isozymes ( $\alpha$ ,  $\beta$ I,  $\beta$ II, and  $\gamma$ ) that require Ca<sup>2+</sup> and DAG for activation, novel (nPKC) isozymes ( $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$ ,  $\mu$ ) that require only DAG, and atypical PKCs ( $\zeta$  and

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$\nu/\lambda$ ) with mechanisms of activation that are  $\text{Ca}^{2+}$  and DAG independent (35). We have previously reported that PKC- $\alpha$  and - $\delta$  are abundant isozymes in cultured VSM cells (35) and that suppression of PKC- $\alpha$  expression with the use of antisense oligonucleotides had no effect on phorbol 12,13-dibutyrate (PDBu)-induced ERK1/2 activation in VSM cells (7). On this basis, we have hypothesized that PKC- $\delta$  mediates PDBu-induced ERK1/2 activation in VSM and propose that this would also extend to GPCR-induced ERK1/2 responses.

In the present study, we used specific pharmacological and molecular approaches to 1) assess the relative contribution of CaMKII- and PKC- $\delta$ -dependent pathways in mediating ATP-stimulated ERK1/2 and 2) determine the relative contributions of PKC- $\alpha$  and PKC- $\delta$  to phorbol ester- and ATP-stimulated ERK1/2 activation in cultured VSM cells. The results indicate important roles for both PKC and CaMKII as intermediates linking a GPCR agonist (ATP) to activation of tyrosine kinases and ras-dependent signaling pathways leading to ERK1/2 activation in VSM cells. Furthermore, we found that the PKC-dependent pathway mediating PDBu- and ATP-dependent ERK1/2 in VSM cells is specifically mediated by PKC- $\delta$ .

## METHODS

**Cell culture.** VSM cells were obtained from the medial layer of the thoracic aorta of 200- to 300-g Sprague-Dawley rats, as described earlier (14). After removal of the adventitial and endothelial layers, 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%  $\text{CO}_2$  and split twice a week. Before experimental use, confluent cultures were growth arrested for 16–24 h by exchanging the growth media with DMEM-F-12 without serum. The DMEM-F-12 without serum was replaced with Hanks' balanced salt solution with  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  and 10 mM HEPES, pH 7.4, 30–60 min before treatment.

**Immunoprecipitations and Western blotting.** Cells were lysed (0.5 ml/60 mm dish or 1 ml/100 mm dish) in a modified RIPA buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM  $\text{Na}_4\text{P}_2\text{O}_7$ , 2 mM  $\text{Na}_3\text{VO}_4$ , 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 10% glycerol, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, and 0.2 U/ml aprotinin). After lysis, the samples were centrifuged to clear the lysate of the insoluble debris and preincubated with 20  $\mu\text{g}$  protein A beads by rocking 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 addition of 20  $\mu\text{g}$  of 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 $\times$  SDS-PAGE sample buffer, and heated for 5 min at 95°C. Samples were resolved by using standard SDS-PAGE procedures, transferred to nylon-backed nitrocellulose (MSI), and immunoblotted. Following blocking 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 three times for 10 min with TBST (20 mM Tris, 150 mM NaCl, 0.2% Tween 20), and incubated for 1 h with appropriate secondary antibody (horseradish peroxidase conjugate; Amersham). The blots were then washed three times for 10 min with TBST, incubated in enhanced chemiluminescent substrate (Amersham), and exposed to X-ray film (E. M. Parker).

**Generation of dominant-negative ras adenovirus.** An adenovirus containing an  $\text{NH}_2$ -terminal hemagglutinin-tagged dominant-negative ras construct (Ad.HA-Ras<sup>N17</sup>) was constructed by using the AdEasy system, as previously described (27). This viral construct also encoded green fluorescent protein (GFP) under a separate cytomegalovirus promoter. Experiments were typically performed by using a multiplicity of infection (MOI) of 20, and under these conditions

nearly 100% of the cells were infected, as determined by the visualization of the coexpressed GFP in the recombinant virus (not shown).

**Cloning and generation of CaMKII- $\delta_2$  adenovirus.** Mutations to CaMKII- $\delta_2$  were engineered by using the Transformer Site Directed Mutagenesis kit (Clontech, Palo Alto, CA). Kinase-negative (KN)-CaMKII- $\delta_2$  was generated by replacement of the lysine 43 with an alanine (K43A). Adenoviral stocks encoding KN-CaMKII- $\delta_2$  were produced in collaboration with Dr. Michael Crow (Johns Hopkins, Baltimore, MD). Adenovirus encoding  $\beta$ -galactosidase (Ad.LacZ) was a gift from Dr. Rebecca Keller (Albany Medical College). All adenovirus stocks were propagated by adding small amounts of virus to human embryonic kidney-293 cells. When cells were ~50% lysed, cells along with media were collected, subjected to three freeze-thaw cycles, aliquoted, and stored at -80°C. Titer assays were performed by the method of O'Carroll et al. (29). All assays were performed by using  $\beta$ -galactosidase as an adenoviral control at matching MOI.

**PKC activity assay.** PKC- $\delta$  and PKC- $\alpha$  were immunoprecipitated from VSM cells and assayed as described earlier (23). After being washed three times with immunoprecipitation buffer and once in 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 phenylmethylsulfonyl fluoride), the protein A beads were incubated for 10 min at 30°C in 50 mM HEPES, pH 7.4, 10 mM  $\text{Mg}(\text{Ac})_2$ , 2 mM  $\text{CaCl}_2$ , 1 mM EGTA, 0.2 mg/ml histone III<sub>s</sub>, 1.4  $\mu\text{g}/\mu\text{l}$  phosphatidyl serine, 0.2  $\mu\text{g}/\mu\text{l}$  diolein, 1 mM ATP, and 2  $\mu\text{Ci}/\text{reaction}$  [ $^{32}\text{P}$ ]ATP. After incubation, 25  $\mu\text{l}$  of reaction were spotted onto P81 filter paper and washed five times in 75 mM phosphoric acid and once in ethanol. After drying,  $^{32}\text{P}$  incorporation was determined by scintillation counting by using a Beckman LS6500 scintillation counter.

**Materials.** KN-PKC- $\delta$  (AdKN-PKC- $\delta$ ) and KN-PKC- $\alpha$  adenovirus (AdKN-PKC- $\alpha$ ) were a gift from Dr. Trevor Biden (Baker Heart Research Institute, Melbourne, Australia). PKC- $\delta$  and PKC- $\alpha$  were rendered kinase negative by a point mutation in the ATP-binding

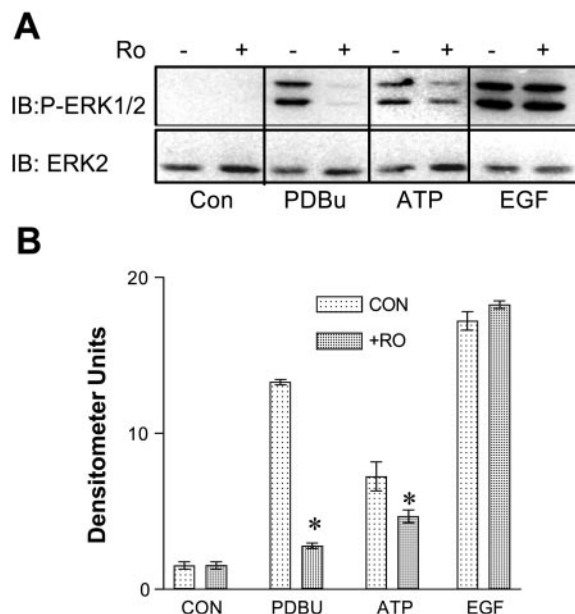


Fig. 1. PKC-dependent ERK1/2 activation in vascular smooth muscle (VSM) cells. A: VSM cells were pretreated with the isozyme nonselective PKC inhibitor Ro-31-8220 (RO; 2  $\mu\text{M}$ ) for 30 min before stimulation with phorbol 12,13-dibutyrate (PDBu; 0.3  $\mu\text{M}$ , 20 min), ATP (50  $\mu\text{M}$ , 5 min), or EGF (5 ng/ml, 5 min). Equal protein loadings of cell lysates were resolved by SDS-PAGE and immunoblotted (IB) with an antibody that recognizes the active phosphorylated (P) form of ERK1/2 (IB: P-ERK1/2) or total ERK1/2 (IB: ERK1/2). B: mean fold increases in P-ERK in 3 separate experiments. Values are means  $\pm$  SE. Con, control. \* $P < 0.05$ .

region of their kinase domain, and replication-deficient adenoviruses were generated as previously described (8).

Polyclonal antibodies to PKC- $\delta$ , PKC- $\alpha$ , and PKC- $\epsilon$  were purchased from Santa Cruz (Santa Cruz, CA). Monoclonal antibodies for PKC- $\delta$ , PKC- $\alpha$ , and ERK2 were purchased from Transduction Laboratories (Lexington, KY). The antibodies specific for active and total ERK1/2 and phosphorylated PKC (Ser<sup>660</sup>) were purchased from Cell Signaling Technology (Beverly, MA). All tissue culture media were purchased from GIBCO-BRL (Life Technologies), unless specifically stated. Tissue culture supplies (dishes, pipettes, etc.) were purchased from Fisher Scientific. Protein A-coated beads were purchased from Pierce 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**

*PKC-dependent activation of ERK1/2 in VSM.* Previous studies using a number of cell systems have demonstrated that diverse stimuli can variably activate PKC-dependent pathways leading to ERK1/2 activation. As shown in Fig. 1, phorbol ester activators of PKC are effective stimulants of ERK1/2 activation in VSM cells, and activation of ERK1/2 by PDBu is fully blocked by 2  $\mu$ M Ro-31-8220, an isozyme-nonspecific PKC inhibitor (3). In the case of the GPCR agonist, ATP, the

PKC inhibitor significantly, but incompletely, inhibited ERK1/2 activation (Fig. 1B), suggesting that the PKC-dependent pathway is one of multiple inputs into the ERK1/2 signaling cascade in response to this stimulus. Ro-31-8220 pretreatment had no inhibitory effects on EGF-stimulated ERK1/2 activation, indicating a lack of involvement of cPKCs and nPKCs or the activation of alternative pathways, coupling this stimulus to ERK1/2 activation.

*PKC- $\delta$  mediates activation of ERK1/2.* Western blotting (Fig. 2A) and previous RT-PCR approaches (35) have identified PKC- $\alpha$  and PKC- $\delta$  as the predominant PKC isozymes expressed in these cultures of rat aortic VSM cells. To determine the PKC isozyme(s) responsible for PDBu- and ATP-mediated ERK1/2 activation, rottlerin, a selective inhibitor of PKC- $\delta$  (19), and G $\delta$ -6976, a selective cPKC inhibitor (18), were used. To confirm the specificity of these inhibitors, we determined their effects on phosphorylation of PKC- $\delta$  on Ser<sup>662</sup> and PKC- $\alpha$  on Ser<sup>660</sup>, following activation of cells by PDBu (Fig. 2B). In the case of PKC- $\alpha$ , ionomycin (Iono) was added with PDBu to ensure that intracellular free Ca<sup>2+</sup> concentrations were not limiting. Phosphorylation of these conserved serine residues is reported to be via an autophosphorylation reaction consequent to activation (28). Rottlerin (3  $\mu$ M),

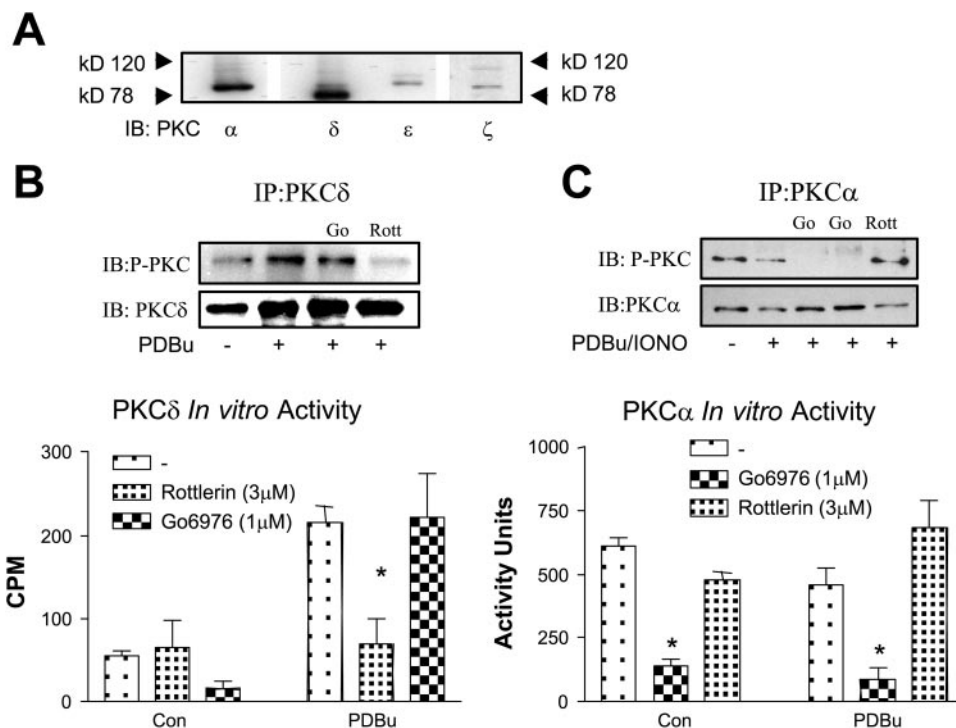


Fig. 2. Isozyme-selective inhibition of PKC in VSM cells. A: equal protein loadings (20  $\mu$ g) of VSM cell lysates were IB by using PKC isozyme-selective antibodies recognizing PKC- $\alpha$ , PKC- $\delta$ , PKC- $\epsilon$ , and PKC- $\zeta$ . B: *top*: to verify the efficacy and selectivity of isozyme-specific PKC inhibitors, VSM cells were treated with 3  $\mu$ M rottlerin (Rott) or 1  $\mu$ M G $\delta$ -6976 (Go) before addition of PDBu (0.3  $\mu$ M, 5 min), lysed, and immunoprecipitated (IP) with antibodies specific for PKC- $\delta$ . The immunoprecipitates were solubilized and IB with an antibody that recognizes the (auto)phosphorylated form of a conserved serine in PKC- $\delta$  (Ser<sup>662</sup>) (IB: P-PKC). Total PKC- $\delta$  in the immunoprecipitates was verified by immunoblotting with isozyme-selective antibody (IB: PKC- $\delta$ ). *Bottom*: PKC- $\delta$  was IP from VSM cell lysates under the conditions described above, and kinase activity was measured by in vitro kinase, as described in METHODS. Values are means  $\pm$  SE of 4 separate experiments. CPM, counts per minute. \* $P$  < 0.05. C: *top*: VSM cells were treated with 3  $\mu$ M Rott or 1  $\mu$ M Go before addition of PDBu plus ionomycin (Iono; 0.5  $\mu$ M, 5 min), lysed, and IP with antibodies specific for PKC- $\alpha$ . The immunoprecipitates were solubilized and IB with an antibody that recognizes the (auto)phosphorylated form of a conserved serine in PKC- $\alpha$  (Ser<sup>660</sup>) (IB: P-PKC). Total PKC- $\alpha$  in the immunoprecipitates was verified by immunoblotting with an isozyme-selective antibody. *Bottom*: PKC- $\alpha$  was IP from VSM cell lysates under the conditions described above and measured for their respective in vitro kinase activity as described in METHODS. Values are means  $\pm$  SE of 4 separate experiments. \* $P$  < 0.05.

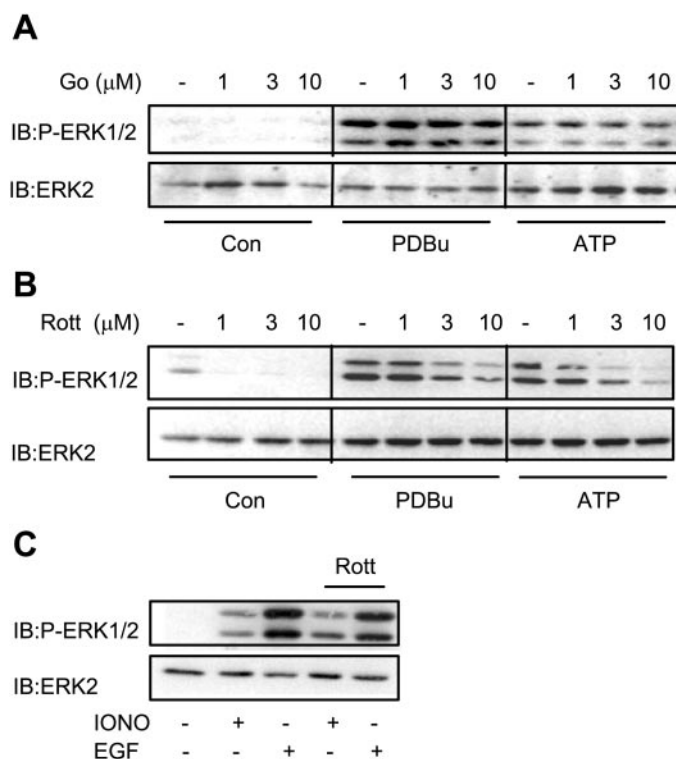


Fig. 3. Effect of PKC isozyme-selective inhibitors on ERK1/2 activation. *A*: VSM cells were pretreated with Gö, a selective PKC- $\alpha$  inhibitor, for 30 min before stimulation with 0.3  $\mu\text{M}$  PDBu or 50  $\mu\text{M}$  ATP for 5 min. ERK1/2 activity was assessed by immunoblotting with an antibody specific for phosphorylated and activated ERK1/2 (IB: P-ERK1/2) and total ERK1/2 (IB: ERK1/2). The immunoblots are representative of 3 experiments. *B*: cells were pretreated with Rott, a selective inhibitor of PKC- $\delta$ , and processed as in *A*. *C*: PKC-independent activation of ERK1/2 by stimulation with Iono (0.5  $\mu\text{M}$ , 5 min) and EGF (10 ng/ml, 5 min). Pretreatment with 3  $\mu\text{M}$  Rott for 30 min had no effect on ERK1/2 activation by these stimuli.

but not Gö-6976 (1  $\mu\text{M}$ ), inhibited autophosphorylation of PKC- $\delta$  on Ser<sup>662</sup> following activation by PDBu (Fig. 2*B*). Although resting phosphorylation of PKC- $\alpha$  Ser<sup>660</sup> was elevated and not further stimulated by addition of PDBu plus Iono, Gö-6976 blocked phosphorylation at this site, whereas rottlerin had no effect (Fig. 2*C*). To further test the specificity and efficacy of these PKC isozyme-selective inhibitors, *in vitro* kinase assays were performed on PKC- $\delta$  and PKC- $\alpha$  extracted from VSM cells treated as described above. Pretreatment of cells with rottlerin resulted in decreased PKC- $\delta$  kinase activity in PKC- $\delta$  immunoprecipitates, whereas Gö-6976 had no effect on PDBu-induced PKC- $\delta$  activation (Fig. 2*B*). Similar to the PKC- $\alpha$  autophosphorylation results above, treatment with PDBu did not enhance the measured PKC- $\alpha$  kinase activity in PKC- $\alpha$  immunoprecipitates. However, treatment with Gö-6976 selectively (compared with rottlerin) reduced both the basal activity of PKC- $\alpha$  and activity following PDBu stimulation. These control experiments suggest that, at the concentrations used, these drugs exerted the expected isozyme-selective inhibitory effects.

Pretreatment with Gö-6976 had no significant effects on either PDBu- or ATP-stimulated ERK1/2 activation at concentrations up to 10  $\mu\text{M}$  (Fig. 3*A*), indicating lack of involvement of PKC- $\alpha$ . In contrast, pretreatment with rottlerin inhibited both PDBu- and ATP-stimulated ERK1/2 activation in a dose-

dependent manner, consistent with its reported PKC- $\delta$  IC<sub>50</sub> of 6  $\mu\text{M}$  (19) (Fig. 3*B*). Three micromoles of rottlerin significantly inhibited PDBu- and ATP-induced ERK1/2 activity in VSM cells (Fig. 4). Activation of ERK1/2 in response to EGF or the Ca<sup>2+</sup> ionophore Iono, which we have previously shown to be dependent on activation of CaMKII (15), was unaffected by rottlerin pretreatment (Fig. 3*C*). These negative controls further establish the specificity of rottlerin and demonstrate a lack of nonspecific inhibitory effects on numerous tyrosine- and serine/threonine protein kinase intermediates in the MAPK cascade. Overall, these data indicate that PDBu- and ATP-dependent activation of ERK1/2 in VSM cells requires PKC- $\delta$  activation, with little or no contribution from PKC- $\alpha$ .

As an alternative to the pharmacological approaches, adenoviruses were used to deliver and overexpress KN-PKC constructs to obtain isozyme-selective, dominant-negative inhibition. Point mutations in the ATP-binding domain of PKC- $\delta$  render the enzyme inactive and unable to phosphorylate substrate (23). Furthermore, KN-PKC- $\delta$  competes with endogenous PKC- $\delta$  for substrate, thus acting as a dominant negative (23). Overexpression of KN-PKC- $\delta$  in VSM cells significantly attenuated PDBu- and ATP-induced ERK1/2 activity (Figs. 5*A* and 6) and had no effect on Iono-stimulated ERK1/2 (Fig. 5*A*), verifying previous findings using rottlerin, the PKC- $\delta$ -selective pharmacological inhibitor. Conversely, comparable overexpression of KN-PKC- $\alpha$  did not inhibit either PDBu- or ATP-induced activation of ERK1/2 (Figs. 5*C* and 6). Whereas expression of KN-PKC- $\delta$  alone partially inhibited ATP-induced ERK1/2 activity, addition of KN-93, a selective CaMKII inhibitor, in conjunction with KN-PKC- $\delta$ , completely inhibited ATP-induced activation of ERK1/2 (Fig. 5*B*). These results suggested a dual role for CaMKII and PKC- $\delta$  in the regulation of this ATP-dependent signaling pathway.

*CaMKII- $\delta$ -dependent activation of ERK1/2.* Previously, our laboratory reported that treatment with ATP results in increases in intracellular free Ca<sup>2+</sup> and activation of CaMKII in VSM cells (39). Using primarily pharmacological approaches, we established that CaMKII mediates the Ca<sup>2+</sup>-dependent activation of ERK1/2 (1), involving the nonreceptor tyrosine kinase PYK2, a SFK, and transactivation of EGFR (15). Pretreatment with KN-93 alone blocks Iono-induced activation of ERK1/2 and attenuates ATP-induced activation (Fig. 7*A*), consistent

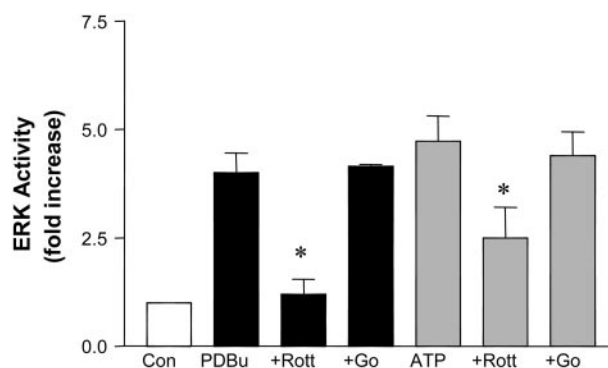


Fig. 4. Quantitation of effects of PKC isozyme-selective inhibitors on ERK1/2 activation. Values are mean ( $\pm$ SE) fold increases of PDBu- (0.3  $\mu\text{M}$ ) and ATP-induced (50  $\mu\text{M}$ ) ERK1/2 activity and the effects of pretreatment with Rott (3  $\mu\text{M}$ ) or Go (1  $\mu\text{M}$ ). ERK1/2 activity was assessed by immunoblotting with an antibody specific for phosphorylated and activated ERK1/2. Immunoblot signals were quantified by scanning densitometry. \* $P < 0.05$  by ANOVA.

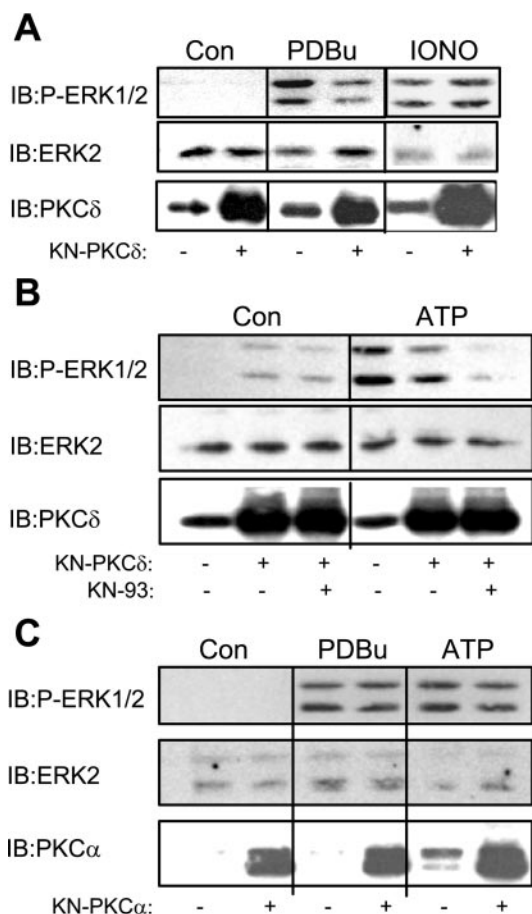


Fig. 5. Effect of kinase-negative (KN) PKC- $\delta$  and PKC- $\alpha$  on PDBu- and ATP-stimulated ERK1/2. **A:** VSM cells were infected with an adenovirus (Ad) encoding KN-PKC- $\delta$  [10 multiplicity of infection (MOI), 24 h] or as a Con adenovirus encoding  $\beta$ -galactosidase (Ad.LacZ) under the same conditions. The cells were then stimulated with 0.1  $\mu$ M PDBu for 5 min or 0.5  $\mu$ M Iono for 5 min, and ERK1/2 activation was determined by immunoblotting for active ERK1/2 (IB: P-ERK1/2). Immunoblotting for PKC- $\delta$  (IB: PKC- $\delta$ ) verified overexpression of KN-PKC- $\delta$ . The immunoblots shown are representative of 3 separate experiments. **B:** VSM cells were infected with 10 MOI AdKN-PKC- $\delta$  and stimulated with 50  $\mu$ M ATP for 5 min in the absence and presence of 30  $\mu$ M KN-93, a selective Ca<sup>2+</sup>/calmodulin (CaM)-dependent protein kinase II (CaMKII) inhibitor. ERK1/2 activation (P-ERK1/2) and KN-PKC- $\delta$  expression (PKC- $\delta$ ) were determined by immunoblotting. **C:** VSM cells were infected with 50 MOI of AdKN-PKC- $\alpha$ , encoding a KN-PKC- $\alpha$  construct before stimulation with 0.1  $\mu$ M PDBu for 10 min or 50  $\mu$ M ATP for 5 min. ERK1/2 activation and PKC- $\alpha$  expression were determined by immunoblotting. The immunoblots shown are representative of 3 separate experiments.

with a partial requirement for CaMKII in mediating the ATP response. To confirm the pharmacological approach and to establish CaMKII's role in ATP-induced activation of ERK1/2, we used a molecular approach to inhibit the endogenous VSM CaMKII isoforms, similar to the approach used to inhibit PKC isoforms. A KN-CaMKII- $\delta_2$  construct was produced by mutating a lysine in the ATP binding site (K43A) and recombined into an adenovirus (Ad-KN: $\delta_2$ ) (31). Adenoviral infection with Ad-KN: $\delta_2$  and overexpression of KN-CaMKII- $\delta_2$  in VSM cells has been recently shown by our laboratory (31) to inhibit CaMKII- $\delta_2$  catalytic activity, both in vitro and in intact cells. Expression of KN-CaMKII- $\delta_2$  inhibited Iono-stimulated ERK1/2 activation (Fig. 7, B and C) while having no effect on

the PDBu-stimulated response, indicating specificity with respect to the Ca<sup>2+</sup>-dependent pathways leading to ERK1/2 activation. With respect to physiological stimuli, ATP-induced activation of ERK1/2 was inhibited by KN-CaMKII- $\delta_2$  overexpression, whereas PDGF-stimulated responses were unaffected (Fig. 7C). Thus both pharmacological and molecular approaches indicate a role for CaMKII- $\delta_2$  in ATP-dependent activation of ERK1/2 in VSM cells, which appears to operate in parallel with a similar pathway utilizing PKC- $\delta$  as an intermediate.

*ATP-dependent ERK1/2 activation is ras dependent and involves EGFR transactivation.* Overexpression of a dominant-negative ras mutant (Ras<sup>N17</sup>) was utilized to determine the relative contribution of ras-dependent pathways to ATP-dependent ERK1/2 activation. High-efficiency infection of VSM cells with Ad.HA-Ras<sup>N17</sup> resulted in robust expression of the dominant-negative ras construct after 24 h (Fig. 8). The efficacy of Ras<sup>N17</sup> overexpression was confirmed by complete inhibition of EGF-stimulated ERK1/2 activation and near complete inhibition of Iono-stimulated ERK1/2 activation, which we have previously shown to depend on EGFR transactivation (15). ATP- or PDBu-stimulated ERK1/2 activation was partly inhibited by Ras<sup>N17</sup> overexpression, indicating the contribution of a ras-dependent pathway leading to ERK1/2 activation in response to this GPCR agonist and selective pharmacological activator of cPKCs and nPKCs.

Activation of nonreceptor tyrosine kinases, including SFK and Pyk2, and transactivation of the EGFR tyrosine kinase have been shown to mediate ERK1/2 signaling pathways in response to GPCR agonists (10). Furthermore, both Ca<sup>2+</sup>-dependent and PKC-dependent pathways have been reported to be proximal to nonreceptor tyrosine kinase activation, including PYK2 (11, 12). Pretreatment with the SFK inhibitor PP2 or the EGFR tyrosine kinase inhibitor AG-1478 significantly inhibited both PDBu (Fig. 9B) and ATP-induced ERK1/2 activation (Fig. 9). Taken with the ras dependency shown in Fig. 8, these pharmacological results imply the contribution of

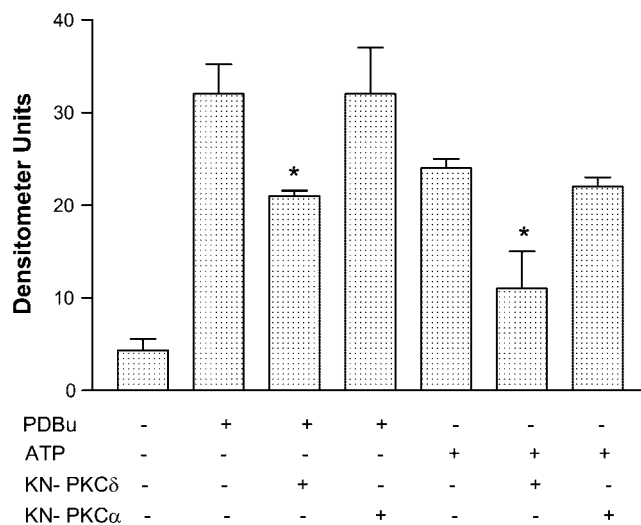


Fig. 6. Quantitation of effect of KN-PKC- $\delta$  and PKC- $\alpha$  on PDBu- and ATP-stimulated ERK1/2. Values are mean ( $\pm$ SE) fold increases of PDBu and ATP-induced ERK1/2 activation and the effect of KN-PKC- $\delta$  or KN-PKC- $\alpha$ , as described in Fig. 5, A and C. \* $P$  < 0.05 by ANOVA.

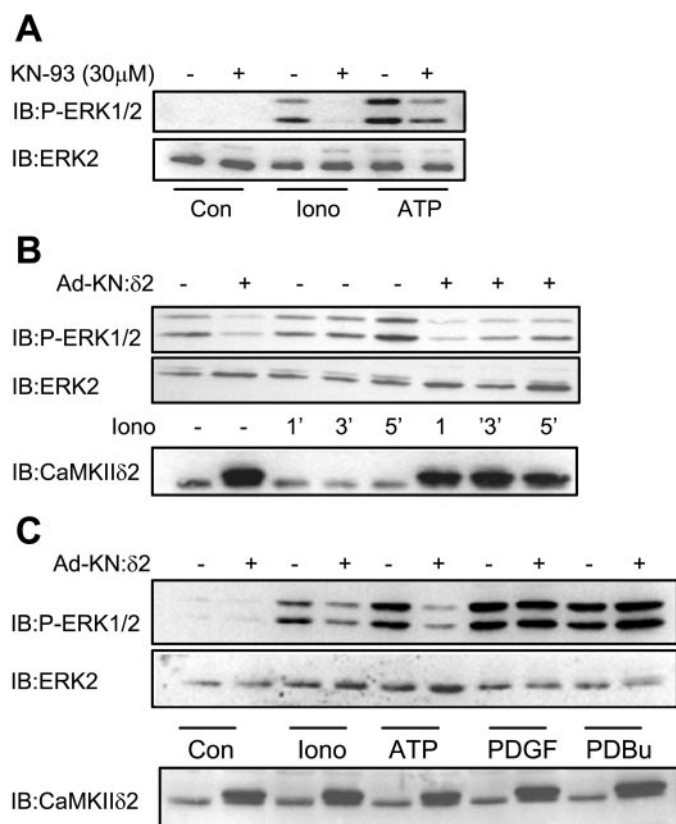


Fig. 7. Effect of CaMKII inhibition on ATP-induced ERK1/2 activation. *A*: VSM cells were treated with 30  $\mu$ M KN-93, a selective CaMKII inhibitor, before stimulation with Iono (0.5  $\mu$ M for 5 min) or ATP (50  $\mu$ M, 5 min). The cell lysates were then analyzed for ERK1/2 activation by immunoblotting (P-ERK1/2). *B*: cells were infected with an Ad encoding a KN-CaMKII- $\delta_2$  mutant (Ad-KN: $\delta_2$ ; 10 MOI) or Ad.LacZ as Con for 48 h before stimulation with 0.5  $\mu$ M Iono for the times indicated. ERK1/2 activation (P-ERK1/2) and KN-CaMKII- $\delta_2$  (CaMKII) expression were determined by immunoblotting. The CaMKII antibody specifically recognizes the unique COOH terminus of both the endogenous and overexpressed CaMKII- $\delta_2$  subunits. *C*: cells were infected with 10 MOI Ad-KN: $\delta_2$  or Ad.LacZ as Con for 48 h before stimulation with 0.5  $\mu$ M Iono (5 min), 50  $\mu$ M ATP (5 min), 10 ng/ml PDGF (5 min), or 0.3  $\mu$ M PDBu (5 min). The cell lysates were analyzed for activation of ERK1/2 (P-ERK1/2) and expression of endogenous and KN-CaMKII- $\delta_2$  (CaMKII- $\delta_2$ ). The immunoblots are representative of 3 separate experiments.

SFKs and EGFR transactivation as intermediates in ERK1/2 activation in response to PKC activators and ATP.

However, on the basis of the incomplete inhibition of PDBu-stimulated ERK1/2 activation by PP2 and AG-1478 (Fig. 9B), we cannot rule out other PKC- $\delta$ -dependent pathways that are SFK and EGFR transactivation independent. The model diagrammed in Fig. 10 incorporates both possibilities.

## DISCUSSION

In this study, we provide evidence indicating parallel roles for two multifunctional serine/threonine kinases, PKC and CaMKII, in ATP-dependent activation of ERK1/2 in rat aortic VSM cells. Both pharmacological and molecular approaches were used to inhibit the activity of endogenous PKC and CaMKII isoforms in these primary cells. The function of specific PKC isoforms as intermediates in signaling pathways leading to activation of ERK1/2 has not been well understood and, as discussed below, may depend on both the agonist and

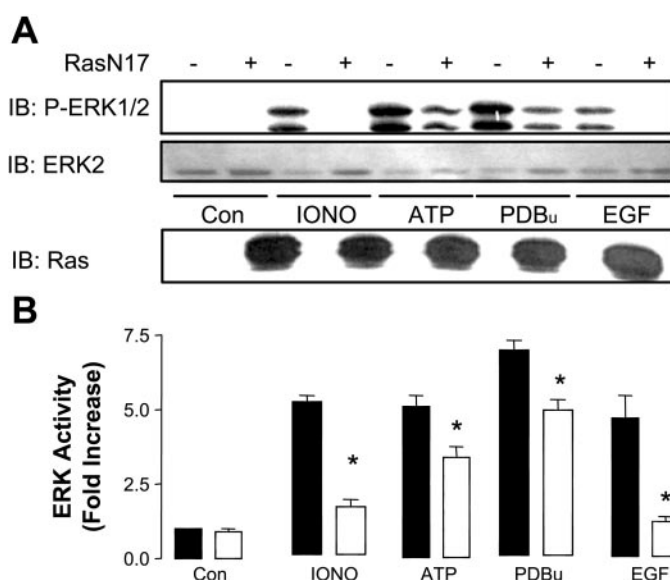


Fig. 8. Effect of ras inhibition on ERK1/2 activation. *A*: VSM cells were infected with 20 MOI of Ad.HA-Ras<sup>N17</sup>, a dominant-negative ras Ad construct, or 20 MOI Ad.GFP (green fluorescent protein) for 24 h before stimulation with 0.3  $\mu$ M Iono, 50  $\mu$ M ATP, 0.3  $\mu$ M PDBu, 5 ng/ml EGF, or 10 ng/ml PDGF-BB. Aliquots of lysates were IB for active ERK1/2 (IB: P-ERK1/2), total ERK (IB: ERK2), and ras protein (IB: Ras). *B*: values are mean ( $\pm$ SE) fold increases in P-ERK, with solid bars representing Ad.GFP-infected cells and open bars representing Ad.HA-Ras<sup>N17</sup> infected cells;  $n = 3$  separate experiments. \* $P < 0.05$ .

cell type studied. In the present study, we have provided evidence that PKC-dependent activation of ERK1/2 in VSM cells in response to PDBu and ATP specifically requires the PKC- $\delta$  isozyme. This finding is consistent with our labora-

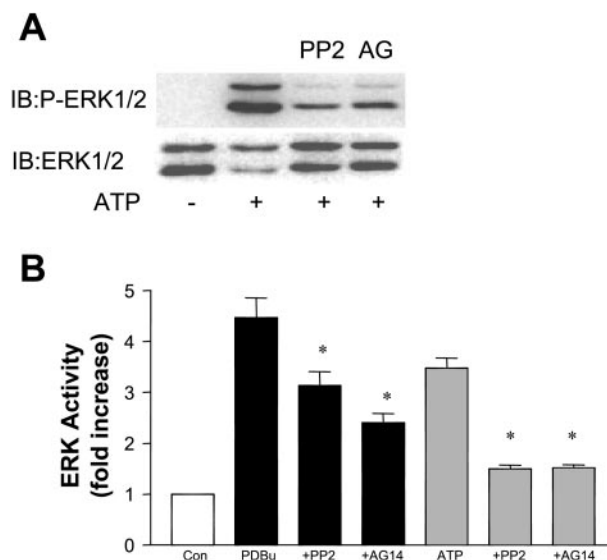


Fig. 9. Specific tyrosine kinase inhibitors attenuate ERK1/2 activation. *A*: VSM cells were pretreated with 10  $\mu$ M PP2, an inhibitor of src family kinases, or 0.3  $\mu$ M AG-1478 (AG14), a selective EGF receptor (EGFR) tyrosine kinase inhibitor, before addition of ATP (50  $\mu$ M) for 5 min. Equal protein loadings of cell lysates were resolved by SDS-PAGE and IB with an antibody that recognizes the active phosphorylated form of ERK1/2 (IB: P-ERK1/2) or total ERK1/2 (IB: ERK1/2). These immunoblots are representative of 4 separate experiments. *B*: values are mean ( $\pm$ SE) fold increases of PDBu- (0.3  $\mu$ M) and ATP-induced (50  $\mu$ M) ERK1/2 activity. \* $P < 0.05$  by ANOVA.

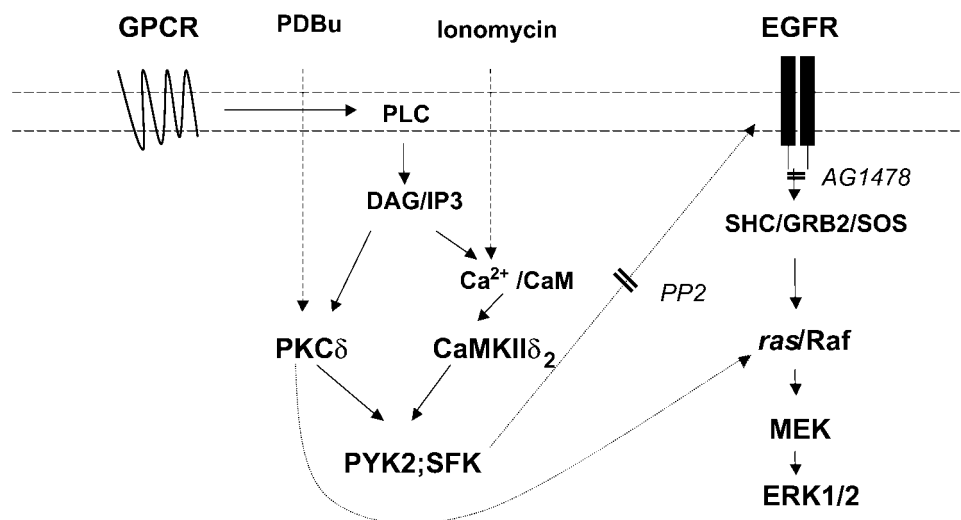


Fig. 10. Proposed model of PKC- $\delta$ - and CaMKII- $\delta_2$ -mediated ERK1/2 activation in VSM. DAG, diacylglycerol; IP<sub>3</sub>, inositol triphosphate; PYK2, proline-rich tyrosine kinase; src, src family kinase; SHC/GRB2/SOS, scaffolding and guanine nucleotide exchange proteins involved in ras and ERK1/2 activation; ras, p21 ras; Raf, Raf kinase; MEK, mitogen-activated protein kinase kinase; ERK1/2, extracellular signal-regulated kinase (p42/44 MAPK).

tory's earlier report that PDBu-dependent activation of ERK1/2 is not inhibited by antisense suppression of PKC- $\alpha$  in VSM cells (7). These data, along with our earlier study (15) implicating a SFK in Ca<sup>2+</sup>-dependent activation of ERK1/2, have resulted in a model that places the multifunctional serine/threonine protein kinases, PKC- $\delta$  and CaMKII- $\delta_2$ , proximal to nonreceptor tyrosine kinases and implicates them as key mediators of tyrosine phosphorylation events and ERK1/2 signaling stimulated in response to GPCR agonists (Fig. 10).

In recent studies, by using pharmacological approaches similar to those used here, it was concluded that PKC- $\delta$  mediated ERK1/2 activation in response to stimulation by angiotensin II in rat liver epithelial cells (24) and ERK1/2 activation and prostacyclin synthesis in endothelial cells in response to VEGF (16). On the other hand, studies using cardiomyocytes indicated that overexpression of a constitutively active PKC- $\delta$  mutant resulted in only small increases in ERK1/2 activation compared with constitutively active PKC- $\epsilon$  (21). In contrast, overexpression of constitutively active PKC- $\delta$  was found in that study to induce large increases in c-Jun NH<sub>2</sub>-terminal kinase and p38<sup>MAPK</sup> activation. PKC- $\delta$  has also been implicated in c-Jun NH<sub>2</sub>-terminal kinase activation in response to genotoxic agents in human U-937 myeloid leukemia cells (41). On the basis of the current study and literature, it is apparent that PKC isozymes can selectively couple to MAPK signaling pathways, although the particular isozymes and pathways may be cell-type specific.

The conclusions here rely, in part, on the specificities of PKC inhibitors, which have been questioned (9). However, control experiments indicated that rottlerin and Gö-6976 selectively inhibited PKC- $\delta$  and PKC- $\alpha$  autophosphorylation and in vitro kinase activity, respectively, clearly establishing their specificity relative to each other. Furthermore, the lack of effect of these inhibitors on ERK1/2 activation in response to other stimuli that do not depend on PKC (i.e., EGF, Iono) strongly suggests a lack of nonspecific effects of these agents on numerous intermediate scaffolding steps and protein kinases in the ERK activation cascade. Finally, the results obtained from overexpression of the KN-PKC- $\delta$  and -PKC- $\alpha$  constructs fully support the pharmacological approach and establish a selective function for PKC- $\delta$  in ERK1/2 activation in response to either PDBu or ATP.

Involvement of ras as an intermediate in PKC-dependent ERK1/2 activation stimulated by phorbol esters or GPCR agonists has been studied but is still a matter of controversy. There are a number of reports that conclude that ras-dependent pathways are not involved in, or only partially account for, ERK1/2 activation. Generally, the data supporting this conclusion are low levels of measurable ras activation stimulated by phorbol ester activators of PKC or stimuli acting through GPCRs and/or incomplete inhibition of ERK1/2 activation in response to these stimuli in cells overexpressing dominant-negative ras constructs. For example, angiotensin II-stimulated ERK activation in GN4 rat epithelial cells (24) and VSM cells (36) has been reported to be incompletely inhibited by overexpression of Ras<sup>N17</sup>. In the present study, PDBu- and ATP-induced ERK1/2 activation was partially inhibited by overexpressing HA-Ras<sup>N17</sup>, whereas EGF-induced ERK activation was completely blocked by this treatment. This result clearly indicates the efficacy of the Ras<sup>N17</sup> overexpression approach and supports the idea that at least part of the PDBu- and ATP-dependent activation of ERK1/2 in these cells is ras dependent. Similar conclusions have been reached by regarding a positive role for ras-dependent pathways in mediating angiotensin II-stimulated ERK1/2, Akt, and p70S6K activities (12).

To determine whether incomplete inhibition of PDBu- and ATP-dependent responses by overexpression of Ras<sup>N17</sup> reflects involvement of ras-independent pathways requires further investigation. Interestingly, overexpression of constitutively active PKC- $\delta$ , but not PKC- $\alpha$  or PKC- $\epsilon$ , has been reported to activate MEK1 and ERK1 in COS and NIH/3T3 cells, a response dependent on c-raf but only partially inhibited by Ras<sup>N17</sup> (37). These results in transformed cell lines, and the present results in primary cultures of VSM cells using approaches that modify endogenous PKC $\delta$  activity, suggest that PKC- $\delta$  may activate ras-independent pathways leading to ERK, perhaps by directly activating raf. Raf has been shown to directly interact with several PKC isozymes, including PKC- $\delta$  (16), - $\beta$  (13), and - $\zeta$  (38), and specific PKC phosphorylation sites have been identified in raf itself (38). However, phosphorylation of these sites alone is not sufficient to activate raf (25). PKC- $\zeta$ -raf interactions appear to require 14-3-3 proteins as intermediate binding proteins (38), and it has been recently

reported that 14-3-3 proteins are necessary for complete activation of raf (40). It is plausible that ras-independent activation of ERK1/2 involving PKC- $\delta$  may depend on similar interactions with raf, mediated by 14-3-3 proteins.

In addition to activation of PKC, many GPCR agonists stimulate increases in free intracellular  $\text{Ca}^{2+}$  in VSM cells, leading to activation of  $\text{Ca}^{2+}$ -dependent signaling pathways, including multifunctional  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinases. In previous studies, we have implicated CaMKII as an important mediator of ERK1/2 activation in response to  $\text{Ca}^{2+}$ -dependent stimuli in VSM cells. Similar conclusions have been reached in other cell systems, including PC12 cells and endothelial cells. However, all of the studies to date have been indirect and have relied on pharmacological approaches using calmodulin antagonists and CaMKII-selective inhibitors (KN-62, KN-93) and/or by overexpressing constitutively active CaMKII subunits. Recently, we have characterized the consequences of overexpressing a KN-CaMKII- $\delta_2$  construct in VSM cells and demonstrated that it functions as an inhibitor of endogenous CaMKII- $\delta_2$  activity with respect to substrate phosphorylation, both in vitro and in the intact cell (31). In the present study, we provide the first evidence using this dominant-negative approach to demonstrate involvement of CaMKII- $\delta_2$  in GPCR agonist-stimulated ERK1/2 activation.

On the basis of the ras dependency for Iono-dependent activation of ERK1/2 shown in this study and previous results indicating CaMKII-dependent activation of nonreceptor tyrosine kinases and EGFR transactivation, it appears that  $\text{Ca}^{2+}$  and PKC- $\delta$ -dependent signals activate common pathways leading to stimulation of ERK1/2. The combined contributions of PKC- $\delta$  and CaMKII- $\delta_2$  nearly completely account for ATP-induced activation of ERK1/2 and suggest a common downstream target at the level of the nonreceptor tyrosine kinases, as indicated in the proposed model (Fig. 10). This model is consistent with previous studies implicating nonreceptor tyrosine kinases and EGFR transactivation as intermediates in GPCR agonist stimulation of ERK1/2 in VSM cells (12, 34). However, the indirect pharmacological approaches used here to implicate involvement of nonreceptor tyrosine kinases do not rule out the possibility that other independent mechanisms may be involved in activating the EGFR receptor (10). Additional studies are warranted to determine the mechanisms by which PKC- $\delta$  and CaMKII- $\delta_2$  regulate nonreceptor tyrosine kinases and/or EGFR transactivation, as well as the functional implications of PKC- $\delta$ - and CaMKII- $\delta_2$ -dependent ERK1/2 activation in response to ATP and other GPCR agonists.

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