Protein Kinase C Regulates Integrin-induced Activation of the Extracellular Regulated Kinase Pathway Upstream of Shc*

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Adhesion of fibroblasts to extracellular matrices via integrin receptors is accompanied by extensive cytoskeletal rearrangements and intracellular signaling events. The protein kinase C (PKC) family of serine/threonine kinases has been implicated in several integrin-mediated events including focal adhesion formation, cell spreading, cell migration, and cytoskeletal rearrangements. However, the mechanism by which PKC regulates integrin function is not known. To characterize the role of PKC family kinases in mediating integrin-induced signaling, we monitored the effects of PKC inhibition on fibronectin-induced signaling events in Cos7 cells using pharmacological and genetic approaches. We found that inhibition of classical and novel isoforms of PKC by down-regulation with 12-O-tetradecanoyl-phorbol-13-acetate or overexpression of dominant-negative mutants of PKC significantly reduced extracellular regulated kinase 2 (Erk2) activation by fibronectin receptors in Cos7 cells. Furthermore, overexpression of constitutively active PKCα, PKCθ, or PKCε was sufficient to rescue 12-O-tetradecanoyl-phorbol-13-acetate-mediated down-regulation of Erk2 activation, and all three of these PKC isoforms were activated following adhesion. PKC was required for maximal activation of mitogen-activated kinase kinase 1, Raf-1, and Ras, tyrosine phosphorylation of Shc, and Shc association with Grb2. PKC inhibition does not appear to have a generalized effect on integrin signaling, because it does not block integrin-induced focal adhesion kinase or paxillin tyrosine phosphorylation. These results indicate that PKC activity enhances Erk2 activation in response to fibronectin by stimulating the Erk/mitogen-activated protein kinase pathway at an early step upstream of Shc.

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‡ The abbreviations used are: MAPK, mitogen-activated protein kinase; PKC, protein kinase C; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; Erk2, extracellular regulated kinase 2; MEK, mitogen-activated kinase kinase; FAK, focal adhesion kinase; PI 3-K, phosphatidylinositol 3-kinase; PLC, phospholipase C; EGF, epidermal growth factor; PTP, phosphotyrosine phosphatase; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; RBD, Ras-binding domain; HA, hemagglutinin; IP, immunoprecipitation(s); GAP, GTPase-activating protein.
pharmacological agents blocks cell adhesion and cell spreading (21, 22) and have been reported to inhibit cell migration (23), FAK phosphorylation (24, 25), and focal adhesion formation (26). Second, several PKC isoforms have been implicated in adhesion-dependent events. PKCa and PKCc are associated with focal adhesions (27, 28), and PKCα and PKCe translocate to the membrane following integrin activation (29, 30). Third, integrin engagement leads to increased phospholipase C (PLC) activity, increased diacylglycerol levels, and arachidonic acid production, pathways involved in PKC activation (21, 29, 31, 32).

Although the specific PKC isoforms involved in integrin-mediated events are beginning to be defined, how PKCs regulate integrin-induced signaling events and what their targets are have not been fully explored. To characterize the role of PKC in integrin-dependent signaling, we monitored the effects of inhibiting PKC on fibronectin-induced signaling events in Cos7 cells using pharmacological and molecular genetic approaches. Down-regulating classical and novel PKC isoforms with TPA or over-expression of dominant-negative mutants of PKC in Cos7 cells did not block integrin-induced FAK or paxillin tyrosine phosphorylation; however, fibroactin-induced Erk2 activation was significantly reduced. Inhibition of PKC also greatly reduced fibronectin-induced MEK1, Raf-1, and Ras activation as well as Shc tyrosine phosphorylation and Grb2 association. These results indicate that fibroactin-induced PKC activation plays a role in modulating the MAPK pathway by regulating early events upstream of Shc.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Adhesion Assays—**Cos7 cells were maintained in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 50 units penicillin, 50 μg/ml streptomycin, and 2 mM glutamine. For adhesion assays, tissue culture plates were coated with 5 μg/ml fibronectin (Collaborative Biomedical) in PBS overnight at 4 °C and blocked with 1% bovine serum albumin prior to use. Near confluent plates of cells were serum starved overnight in DMEM containing 0.1% fetal bovine serum. Cells were washed with PBS and trypsinized in 0.01% trypsin containing 5 mM EDTA for 5 min at 37 °C. Trypsinization was terminated by Dounce homogenization. The soluble cytosolic fraction (S30) were run in parallel and visualized under UV light. The percentage of PKC from amino acid residues 1–385 and is tagged with Hisa/T70 tag at the N terminus. The kinase domain truncation mutant of mouse PKCε (pSRD-PKCε KD) encodes amino acid residues 348–674 and is preceded by Met. The kinase domain truncation mutant of rabbit PKCε (pSRD-RH-PKCε KD) encodes amino acid residues 386–736 preceded by Met. The kinase domain truncation mutant of rabbit PKCα (pSRD-PKCα KD) encodes amino acid residues 298–672. Cos7 cells were transfected at 2 × 10⁶ cells/10-cm plate with 8–10 μg of total DNA using the LipofectAMINE procedure as described by the manufacturer (Life Technologies, Inc.). 48 h after transfection, cells were used in adhesion assays as described above.

**Kinase Assays—**Erk2, MEK1, and Raf-1 kinase assays were performed as described previously (36, 37). Extracts were clarified by centrifugation at 13,000 × g for 10 min, and protein concentrations were determined using Coomassie Blue Reagent (Bio-Rad). Erk2, MEK1, and Raf-1 substrates were 0.25 mg/ml myelin basic protein (Life Technologies, Inc.), 50 μg/ml kinase dead GST-MAPK (Upstate Biochemical), and 12.5 μg/ml kinase dead GST-MEK1 (Upstate Biochemical), respectively. Reactions were incubated at 30 °C for 15–30 min and terminated with 40 μl of 2 × SDS Sample buffer by heating to 95 °C for 5 min and analyzed by SDS-polyacrylamide gel electrophoresis. Gels were transferred to polyvinylidene difluoride (Bio-Rad), and after autoradiography and quantitation by phosphoimaging (Fuji) blots were subjected to immunoblotting for the respective kinases.

**Immunoprecipitations—**Cells adherent to fibroactin or left in suspension were lysed in RIPA buffer (10 mM Tris, pH 7.2, 158 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% sodium deoxycholate, 1% Triton-X, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 100 units/ml aprotinin, and 10 μg/ml leupeptin), passed through a 25ga needle, and clarified by centrifugation at 13,000 × g for 10 min. Protein concentrations were determined using the BCA assay (Pierce). Immunoprecipitations (IP) were incubated for 2–4 h at 4 °C with protein A-conjugated agarose beads (Pierce) to capture the complexes. All IP were washed three times with RIPA, resuspended in 2 × SDS sample buffer, boiled, and analyzed by SDS-polyacrylamide gel electrophoresis. Gels were transferred to polyvinylidene difluoride membrane and probed by immunoblotting. After blocking in 5% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 and incubating with primary antibody, blots were incubated with horseradish peroxidase-conjugated secondary antibody and visualized with chemiluminescence reagent (NECN Life Science Products). Blots were stripped in 2% SDS at 65 °C for 30 min, rinsed extensively, and reprobed as indicated in the figure legends.

**Lysates—**Cells were lysed in hypotonic cytosolic buffer (10 mM Tris, pH 7.4, 0.5 mM EDTA, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 100 units/ml aprotinin) for 20 min on ice after washing with PBS. Cells were collected and broken open by Dounce homogenization. The soluble cytosolic fraction (S30) was collected after centrifugation at 30,000 × g for 30 min. The pellets (P30) were further fractionated into membrane and Triton X-100-insoluble pellets by solubilization with detergent buffer containing 1% Triton X-100. The soluble membrane fraction (P30Mem) was recovered after centrifugation at 30,000 × g for 30 min. The Triton X-100-insoluble pellets were resuspended in RIPA, and supernatants (P30Ins) were collected after centrifugation at 14,000 × g. The ratio of total cytosolic protein:membrane protein:Triton-insoluble was calculated, and that ratio was maintained when extracts were loaded onto SDS gels. Both soluble and insoluble membrane extracts were pooled for immunoprecipitation of PKCε from membrane fractions.

**Ras GTP Loading Assay—**The amount of GTP bound to Ras was measured according to the protocol of Vailancourt et al. (38), which was modified as described by Zheng et al. (39) and Clark and Hynes (40). Briefly, confluent, serum-starved cells were washed with phosphate-free DMEM and incubated with 0.25 mM of [32P]GTP/plate for 4–5 h. Cells were washed, trypsinized, and placed in suspension as described above, except that TBS was substituted for PBS. Suspended cells were incubated in phosphate-free DMEM and 1 mM [32P]GTP for 1 h at 37 °C. Cells were plated onto fibroactin coated plates for 20–30 min, until cells were adherent and beginning to spread. Plated cells were washed twice with PBS, lysed, processed, and immunoprecipitated as described above. GTPGDP was eluted from immunoprecipitates with 25 μl 0.75 M KH2PO4 (pH 3.4) at 68 °C for 10 min. TLC was carried out on polyethyleneimine-cellulose plates. GTP and GDP unlabeled standards were run in parallel and visualized under UV light. The percentage of GTP bound relative to the ratio of GTPGDP was quantitated on a Fuji phosphoimaging system. The ability of GTP-Ras to bind the effector region in Raf-1 was monitored as described previously (41).
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Fig. 1. Expression of different PKC isoforms in Cos7 cells and effect of long term TPA treatment. A, whole cell lysates from Cos7 cells were probed by immunoblotting with isoform-specific antibodies to the different classes of PKC (lanes Cos). Rat brain extracts (lanes c) were used as positive controls for antibody recognition, except for PKC7 and PKC6, which were MDCK and Jurkat cell extracts, respectively. The asterisks denote the PKC-specific bands present in Cos cells. B, Cos7 cells were left untreated or treated with 100 ng/ml TPA for 24 h, placed in suspension (S), or plated on fibronectin (FN) for various times. Whole cell lysates were analyzed by immunoblotting for the presence of different PKC isoforms. The change in mobility shift observed for PKC6 is due to a tear in the gel and does not represent a mobility shift due to fibronectin-mediated adhesion.

RESULTS

PKC Expression in Cos7 Cells—As a first step toward defining the role of PKC in fibronectin-induced signaling in Cos7 cells, we examined which PKC isoforms are expressed in Cos7 cells. Immunoblot analysis of Cos7 cell extracts with antibodies specific for eleven different PKC isoforms revealed that Cos7 cells express eight PKC isoforms (Fig. 1A): classical PKCα, PKCβII, and PKCβIII, novel PKCδ and PKCe, atypical PKCε and PKCζ as well as PKCμ.

Prolonged treatment of cells with TPA results in degradation and loss of expression of TPA-responsive PKCs, effectively resulting in a cell that is null for those PKCs. Of the PKC isoforms in Cos7 cells, only PKCα, β, δ, and ε should be affected by prolonged TPA exposure. Treatment of Cos7 cells for 24 h with 100 ng/ml TPA eliminated PKCα, βII, and δ but only reduced PKCε by 70% as monitored by immunoblotting of whole cell lysates (Fig. 1B). Higher doses or longer TPA treatment did not further reduce PKC levels (data not shown). As expected, atypical PKC isoforms and PKCμ were unaffected by long term TPA treatment. Plating cells on fibronectin did not change the level of PKC expression either before or after TPA-mediated down-regulation (Fig. 1B).

Stimulation of PKC Following Adhesion—The best characterized mechanism leading to in vivo activation of classical and novel PKCs following receptor stimulation involves an increase in intracellular diacylglycerol levels, which is mediated by PLC isoymes (42). The observations that PLCs translocate to integrin complexes and that adhesion of epithelial cells to collagen induces PLC activation through β1 integrin indicate that activation of PLC may be important for integrin signaling events (31, 32, 43). To determine whether PLCγ can be activated by fibronectin in Cos7 cells, PLCγ1 was immunoprecipitated from cell extracts following adhesion of Cos7 cells to fibronectin, and the levels of tyrosine phosphorylation were monitored by immunoblotting with anti-phosphotyrosine antibody, because tyrosine phosphorylation of PLCγ1 activates it. Plating cells on fibronectin resulted in a rapid increase in tyrosine phosphorylation of PLCγ1 (Fig. 2A). No tyrosine phosphorylation of PLCγ1 was detected in suspension cells. Thus adhesion of Cos7 cells to fibronectin activates PLCγ1, which could lead to activation of PKC through diacylglycerol production.

TPA-responsive PKCs translocate from the cytosolic fraction into detergent-soluble membrane fractions during activation. To determine whether adhesion of Cos7 cells to fibronectin causes an increase in membrane-associated PKCs, cells were subjected to biochemical fractionation as outlined under “Experimental Procedures.” Cells were fractionated into cytosolic (S30) and membrane fractions (P30). The membrane fraction was further fractionated into Triton X-100-soluble (P30Mem) and Triton X-100-insoluble (P30Ins) fractions, and each was analyzed by immunoblotting with different PKC isoform antibodies. Adhesion to fibronectin resulted in translocation of a small fraction of PKCα and PKCβII to the soluble membrane fraction, both of which were predominately cytosolic in suspension cells (Fig. 2B). A corresponding increase in soluble membrane-associated PKC kinase activity was also observed (data not shown). Unlike PKCβII, PKCβIII was found to be primarily in the P30 pellet and fractionated equally between the soluble membrane and detergent-insoluble fraction in suspension cells. This distribution did not change following adhesion to fibronectin (Fig. 2B). Approximately a third of total PKCs was associated with the P30 pellet in suspension cells. Following adhesion to fibronectin a small but reproducible increase in the amount of PKCe in the soluble membrane fraction (P30Mem) occurred concurrently with a reduction in mobility (Fig. 2B). Like PKCe, approximately a third of the total PKCδ was found in the P30 pellet, but PKCδ distribution did not change following adhesion. Growth factors and TPA have been shown to induce tyrosine phosphorylation of PKCδ (44, 45). Adhesion of Cos7 cells to fibronectin also resulted in inducible tyrosine phosphorylation of membrane-associated PKCδ, as seen by immunoprecipitation of PKCδ and immunoblotting with antiphosphotyrosine antibody (Fig. 2C). Thus adhesion of Cos7 cells to fibronectin induces activation of PLCγ1, membrane association of PKCα, β, and ε, and tyrosine phosphorylation of membrane-associated PKCδ.

Effect of PKC Inhibition on Adhesion and Spreading—TPA-induced PKC down-regulation did not adversely affect the ability of Cos7 cells to adhere to fibronectin (Fig. 3A). However, prolonged TPA treatment did result in delayed spreading on fibronectin (Fig. 3B). Spreading in untreated cells could be seen as early as 10 min after plating on fibronectin (60%), but TPA-treated cells were poorly spread at 10 min (5%). By 45 min TPA-treated cells had reached the same level of spreading as untreated cells at 10 min (58%). At 60 min after plating only 64% of the TPA-treated cells had spread compared with 90% for untreated cells. Thus inhibition of PKC function in Cos7 cells by long term TPA treat-
ment affects cell spreading but not cell adhesion.

Effect of PKC Inhibition on Tyrosine Phosphorylation—We next investigated which integrin-induced downstream signaling events are affected by loss of novel or classical PKC isoform function. Adhesion to extracellular matrices induces tyrosine phosphorylation of two focal adhesion-associated proteins, FAK and paxillin (46). To determine whether inhibition of PKC affects fibronectin-induced FAK or paxillin phosphorylation, FAK and paxillin were immunoprecipitated from cells plated on fibronectin for various times and probed by immunoblotting with anti-phosphotyrosine antibody. Adhesion to fibronectin induced robust tyrosine phosphorylation of both FAK and paxillin (Fig. 4A, P-tyr blot). TPA-induced PKC down-regulation did not block fibronectin-induced FAK or paxillin phosphorylation, although it did reduce tyrosine phosphorylation at very early (10 min) stages of cell adhesion.

Previous studies demonstrated that inhibition of PKC blocked integrin-induced FAK phosphorylation (22, 25). Because TPA-induced PKC down-regulation did not completely inhibit PKCε expression, we examined the effect of inhibiting PKCε on integrin-induced FAK phosphorylation. Either of the two dominant-negative mutants of PKCε (PKCε KD or PKCε RD) was co-expressed with HA-tagged FAK, and the ability of fibronectin to induce FAK tyrosine phosphorylation was monitored. The PKCε mutants were expressed at levels greater than 10-fold over endogenous levels (Fig. 4B, PKC blot) as determined by immunoblotting of whole cell extracts with PKCε antibody. The kinase inactive PKCε mutant (PKCε K−) and the regulatory domain of PKCε (PKCε RD) have previously been shown to act as a dominant-negative mutants (14, 35) and in Cos7 cells inhibited fibronectin-induced Erk2 activation (Fig. 5).

Analysis of immunoprecipitated HA-tagged FAK by immunoblotting with anti-phosphotyrosine antibody following 30 min of adhesion of transfected Cos7 cells to fibronectin indicated that co-expression of either dominant-negative mutant of PKCε had no effect on the ability of fibronectin to induce FAK phosphorylation (Fig. 4B). Similar results were observed with dominant-negative PKCδ and PKCζ mutants (data not shown). Furthermore, overexpression of the constitutively active kinase domain of PKCε (PKCε KD) did not induce or enhance FAK tyrosine phosphorylation (Fig. 4B). Expression of the dominant-negative PKCε (PKCε RD) in combination with TPA-mediated down-regulation of PKCε also did not block fibronectin-induced FAK tyrosine phosphorylation (data not shown). These data indicate that members of the classical and novel PKCs are not likely to be involved in regulating integrin-induced FAK tyrosine phosphorylation in Cos7 cells.

Effect of PKC Inhibition on Erk2 Activation—Erk2 is activated following integrin-mediated adhesion (1). Because TPA-induced activation of PKC has been shown to stimulate Erk2 activation, we examined whether PKCε plays a role in fibronectin-induced Erk2 activation. Adhesion of Cos7 cells to fibronectin results in a 14-fold stimulation of Erk2 activity, which peaks at 20–30 min after adhesion, as measured in an immune complex kinase assay with myelin basic protein (Fig. 5A; see also Fig. 7A). Inhibition of PKC by TPA-induced down-regulation reduced fibronectin-induced Erk2 activity by 60% (see Fig. 7A). These results suggest that maximal integrin-induced activation of Erk2 requires PKC.

To determine whether the effects of TPA-induced PKC down-regulation were due to specific effects on PKC and not due to nonspecific effects of TPA, we over-expressed dominant-negative mutants of PKC and monitored their effect on Erk2 activation in response to fibronectin. Cos7 cells were transiently transfected with plasmids expressing PKC mutants and HA-tagged Erk2, and Erk2 activation was measured in an immune complex kinase assay after immunoprecipitating with HA tag antibody. Over-expression of the regulatory domain of PKCζ (PKCζ RD) and full-length kinase inactive PKCε (PKCε K−) inhibited fibronectin-induced HA-Erk2 activation by 50 and 65%, respectively (Fig. 5, B and C; see also Fig. 7B). All PKC

**Fig. 2.** Fibronectin-induced regulation of PLCγ and different PKC isoforms in Cos7 cells. A, Cos7 cells were plated in suspension (S) or plated on fibronectin (FN) for various times. PLCγ1 was immunoprecipitated (PLCγ IP) from whole cell lysates, and fibronectin-induced tyrosine phosphorylation was monitored by immunoblotting with anti-phosphotyrosine antibody (P-tyr blot). PI, preimmune serum. Total levels of PLCγ1 in the IP were measured by reprobing the blot with anti-PLCγ1 antibody (PLCγ blot). B, Cos7 cells were placed in suspension or plated on fibronectin for 30 min. Cells were fractionated into cytosolic (S30), detergent-soluble membrane (P30Mem), and detergent-insoluble (P30Ins) fractions as described under "Experimental Procedures." Lysates from each fraction were probed by immunoblotting with isoform-specific PKC antibodies (PKC blot). C, Cos7 cells were stimulated for various times and probed by immunoblotting with anti-phosphotyrosine antibody following 30 min of adhesion of transfected Cos7 cells to fibronectin (P-tyr blot). Total levels of PKCδ in the IP were detected by reprobing the blot with anti-PKCδ antibody (PKCδ blot).
mutants were expressed greater than 10-fold over endogenous levels as measured by immunoblotting with PKC antibody (Fig. 5, B and C, PKC blot). Over-expression of truncated PKCδ (PKCδ RD) also inhibited fibronectin-induced Erk2 activation by 45% (Fig. 5C). Additionally, over-expression of constitutively active forms of PKCα or PKCe, mutants expressing only the catalytic domain (PKCα KD or PKCe KD), were able to rescue Erk2 activation in cells where Erk2 activation was inhibited by TPA-mediated PKC down-regulation (Fig. 5D). Similar results were obtained with a PKCδ KD mutant (data not shown). Thus inhibition of PKC significantly blocks fibronectin-induced Erk2 activation, indicating that PKC is an important regulator of integrin-mediated Erk2 activation in Cos7 cells and that several PKC isoforms are capable of regulating Erk2 activation.

Transfection of Cos7 cells with either wild type PKCe (PKCe wild type) or constitutively active forms of PKC (PKCα KD or PKCe KD) did not induce a significant enhancement of fibronectin-induced Erk2 activation over that seen with vector

FIG. 3. Effect of PKC inhibition on cell adhesion and spreading. A, Cos7 cells were left untreated or treated with 100 ng/ml TPA for 24 h then placed in suspension or plated on fibronectin (FN) for various times. The percentage of adhesion was measured by assaying the amount of protein in adherent cells relative to that in suspension. Results presented are from three independent assays. B, Cos7 cells were treated as described above, except that at various times after spreading cells were photographed under 40× magnification and the amount of cell spreading was quantitated by counting the percentage of spread cells versus round cells in several fields. Cells were considered spread if they lost nuclear refractility and membrane blebbing. Graph legends are fibronectin (○) and TPA-treated on fibronectin (●).

FIG. 4. Effect of PKC inhibition on fibronectin-induced FAK and paxillin tyrosine phosphorylation. A, Cos7 cells left untreated or treated with 100 ng/ml TPA for 24 h then placed in suspension (S) or plated on fibronectin (FN) for various times. Paxillin or FAK was immunoprecipitated (Paxillin IP and FAK IP) from cell lysates, and the level of tyrosine phosphorylation analyzed by immunoblotting with anti-phosphotyrosine antibody (P-tyr blots). PI, preimmune serum. B, Cos7 cells were transfected with 3 μg of plasmid expressing HA-tagged FAK and 8 μg of empty vector (vector) or plasmids expressing various PKCe mutants. Cells were left in suspension (S) or plated on fibronectin (FN) for 20 min. FAK was immunoprecipitated (HA IP) with HA antibody, and its phosphorylation was monitored by immunoblotting with anti-phosphotyrosine antibody (P-tyr blots). Whole cell extracts were probed by immunoblotting with PKC antibodies to measure the level of overexpressed PKC mutants (PKC blot). *, PKC bands.
alone. Nor did overexpression of active PKC significantly increase basal levels of Erk2 activity. Co-expression of two or more constitutively active PKCs also failed to enhance Erk2 activation (data not shown). Overexpression of the active catalytic domain of PKCα (PKCα KD) inhibited cell growth and greatly reduced the ability of the transfected cells to readhere to fibronectin. Nevertheless, it was still able to rescue Erk2 activity in those cells able to readhere (data not shown). Together these data indicate that PKC is important for fibronectin-induced Erk2 activation but that overexpression of constitutively active PKC is not sufficient to induce Erk2 activation in suspension. In addition, overexpression of PKC does not enhance fibronectin-induced Erk2 activation. These data suggest that stimulation of PKC alone is not sufficient for Erk2 activation and that downstream targets of PKC in the Erk signaling pathway may be limiting such that overexpression of PKC in the presence of fibronectin is not further stimulatory.

**Effect of PKC Inhibition on MEK1 and Raf-1 Activation**—To
Ras-binding domain (RBD) in Raf-1 requires that GTP be bound to Ras to bind its effector Raf-1. The ability of Ras to bind to Raf-1 is also measured by monitoring the ability of Ras to bind to Raf-1. The ability of Ras to bind to Raf-1 is also measured by monitoring the ability of Ras-GTP (1.67-fold) to bind to Raf (Fig. 8, Ras-RBD Assay) without reducing the total level of Ras (data not shown). Thus, TPA-induced PKC down-regulation suppresses the ability of fibronectin to induce Ras activation. These results indicate that PKC is able to regulate fibronectin-induced Erk2 activation by interceding in the MAPK signaling pathway at a point upstream of Ras.

Effect of PKC Inhibition on Shc Phosphorylation—Activation of Erk2 through integrin receptors involves tyrosine phosphorylation of the adaptor protein Shc (47–49). Phosphorylated Shc recruits the Grb2-SOS complex, which enhances Ras-GTP binding through the GTP exchange activity of SOS (50, 51). To determine whether inhibition of PKC affects fibronectin-induced Shc phosphorylation, Shc was immunoprecipitated from cells plated on fibronectin for various times and probed by immunoblotting with anti-phosphotyrosine antibody. Adhesion to fibronectin induced robust tyrosine phosphorylation of Shc (Fig. 9A). Long term treatment of cells with TPA did not affect the total levels of Shc (Fig. 9A, Shc blot) but dramatically reduced fibronectin-induced Shc phosphorylation. The reduction in Shc tyrosine phosphorylation observed in PKC down-regulated cells similarly resulted in reduced association of Grb2 with immunoprecipitated Shc (Fig. 9B, Grb2 blot). Thus, Shc tyrosine phosphorylation represents at least one PKC-dependent event upstream of Ras that is responsible for integrin-induced Erk2 activation.

DISCUSSION

Using both pharmacological and genetic approaches, we have demonstrated that maximal Erk2 activation by fibronectin receptors in Cos7 cells is dependent on activation of classical or novel PKCs. Constitutively active mutants of three different PKC isoforms failed to induce agonist-independent Erk2 activation, but activated mutants of PKCα, PKCβ, or PKCε were able to rescue Erk2 activation after TPA-induced PKC-downregulation, suggesting that several PKC isoforms are capable of regulating integrin-induced Erk2 activation. Maximal activation of MEK1, Raf-1, and Ras was also dependent on PKC, indicating that PKC regulates Erk2 activation at a step upstream of Ras. Integrin-induced tyrosine phosphorylation of Shc and Grb2 association was also dependent on PKC. Thus, PKC acts to stimulate Erk2 activation in response to fibronectin by stimulating the Erk/MAPK pathway at a step upstream of Ras by regulating Shc phosphorylation.

PKC has been linked to activation of the Erk/MAPK pathway through plasma membrane receptors in several cell types. G-protein coupled receptors such as the M1 muscarinic receptor that couple to Gq and Go activate Erk in a PKC-dependent manner (52). EGFr-induced Erk activation in keratinocytes also requires PKC (14). However, in other cell types, such as Rat-1 cells, PKC is not involved in EGFr-induced Erk activation (53). In Swiss 3T3 cells stimulated with platelet-derived growth factor, the dose of platelet-derived growth factor influences the extent to which PKC is required for Erk activation (54). Thus, the nature of the stimulus and strength of the signal may determine the requirement for PKC in Erk activation.

The exact step in the Erk/MAPK pathway where PKC is required to stimulate Erk activation also depends on the stimulus and cell type examined. In T lymphocytes stimulated through the T-cell receptor PKC regulates the Erk/MAPK pathway at a step upstream of Ras (55, 56), whereas PKC-mediated activation of Erk by the M1 receptor or the EGFr receptor appears to act at a point downstream of Ras that is required for activation of Raf-1 (14, 57). A recent report suggests that in.
platelet-derived growth factor-stimulated cells, PKC can act downstream of MEK1, possibly by inhibiting an Erk phosphatase (58). Our results indicate that maximal activation of Erk2 in Cos7 cells following stimulation with fibronectin requires PKC activity at a step upstream of Ras because Ras-GTP binding was reduced in PKC down-regulated cells.

Activation of Ras by many receptors involves tyrosine phosphorylation of the adaptor protein Shc, which recruits the Ras exchange factor SOS to the membrane. Recruitment of SOS to Shc is mediated by the SOS-associated adaptor protein Grb2, which binds to tyrosine phosphorylated Shc through the Grb2 SH2 domain. Integrin receptor activation leads to tyrosine phosphorylation of Shc, and this event is required for integrin-induced Erk2 activation (47, 49). In this report, we show that integrin-induced tyrosine phosphorylation of Shc and association with Grb2 in Cos7 cells is dependent on PKC activity, indicating that PKC regulates a protein tyrosine kinase or protein tyrosine phosphatase (PTP) that regulates tyrosine phosphorylation of Shc. The evidence that acute TPA treatment also induces Shc tyrosine phosphorylation in some cells (59–62) supports the possibility that PKC can modulate other signaling molecules that affect tyrosine phosphorylation in Shc.

Several integrin-activated tyrosine kinases have been implicated in Shc phosphorylation following engagement of integrin receptors. Giancotti and co-workers (47, 63) have shown that integrin-induced Shc phosphorylation is mediated by caveolin-associated Fyn, a Src family tyrosine kinase. Other studies have implicated Src or Fyn kinase and FAK in integrin-regulated Shc phosphorylation (49, 64, 65). In NIH-3T3 cells attached to fibronectin, PKC inhibition reduced Src kinase activity and Erk2 activation, possibly implicating Src kinases in

Fig. 7. Quantitation of Erk2, MEK1, and Raf-1 kinase assays. Kinase assays were quantitated using a Fuji phosphoimager, and the fold activation was calculated. Results presented are a direct quantitation of autoradiographs shown in Figs. 5A (A), 5B (B), 6A (C), and 6B (D) and are representative of at least three different experiments. A, C, and D. □, FN; ●, TPA down-regulation on FN. B, □, cells transfected with vector; ○, kinase inactive PKC.

![Quantitation of Erk2, MEK1, and Raf-1 kinase assays](image-url)
some aspect of PKC regulation of Shc phosphorylation (49). The effect of PKC on Fyn activation has not been reported. However, Fyn, like Src, may be inhibited by inhibition of PKC. Tyrosine phosphorylation of Shc has also been shown to be dependent on PKC in TCR stimulated T-lymphocytes where Fyn and Lck are critical for Shc activation (66). Thus PKC may be required for Shc phosphorylation mediated by Src family kinases, either through caveolin-dependent or FAK-dependent pathways.

What are the possible mechanisms whereby PKC could activate Src family kinases? PKC and Src can physically interact with each other and directly affect each other’s activity. Association of PKCδ with Src increases the level of serine phosphorylation on Src and increases Src activity (67, 68), but higher levels of Src activity can feed back and inhibit PKCδ (69). Additionally PKC and Src can both bind to the PKCα binding protein RACK1. PKCα binding to RACK elevates its kinase activity, whereas Src activity is inhibited by RACK binding (70). Potentially, activation of PKCα could displace Src and relieve this inhibitory interaction. This model is made even more attractive by the recent finding that Rack1 can associate with β1 and β2 integrins in a PKC-dependent manner (71).

Alternatively, PKC could be required for activation of a PTP(s) that dephosphorylates the C-terminal phosphotyrosine that negatively regulates Src protein kinases, thus leading to activation of this family of kinases.

Alternatively or in addition, PKC might regulate Shc tyrosine phosphorylation by inhibiting a PTP(s) that dephosphorylates Shc. Treatment of HeLa cells with TPA or stimulation of neutrophils induces serine phosphorylation of PTP-PEST or SHP1, respectively, and inhibits their activity (15, 72). Furthermore, PTP-PEST can bind directly to Shc, an event that is up-regulated by TPA or carbachol (73), and PTP-PEST localizes to focal adhesions. Thus PKC could target tyrosine phosphatases and inhibit their activity and thereby indirectly promote Shc tyrosine phosphorylation.

PKC may also regulate Ras activation by modulating other regulatory factors, like p120 RasGAP. TPA stimulation causes an increase in GAP phosphorylation and inhibition of GAP activity toward Ras, thus allowing Ras to maintain higher levels of GTP and to be more active (55). Overexpression of GAP blocks the ability of TPA to stimulate Erk, indicating that the ability of PKC to regulate Erk activation may require inhibition of GAP (74, 75). It is also possible that PKC isoforms may regulate events downstream of Ras (e.g. Raf-1 and MEK1) as in other cell types. However, the additional contribution of these down-regulatory mechanisms remain to be determined.
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stream steps to the Erk/MAPK pathway would be masked by inhibition at earlier stages of the pathways.

In our studies overexpression of constitutively active PKC was not sufficient to activate Erk2 in suspension cells, nor did it enhance Erk2 activation even after engagement of integrin fibronectin receptors. The inability of activated PKC to enhance Erk2 activation even under conditions of integrin engagement suggests that a downstream target(s) of PKC may be limiting. It is interesting to note that we have also found that expression of a constitutively active p110 subunit of PI 3-K is unable to induce or enhance Erk2 activity in suspension cells or in cells stimulated by fibronectin, although its activity is required for integrin-induced Erk2 activation.2

The inability of constitutively active PKCs to stimulate Erk2 activation in suspension cells suggests that PKC is not sufficient to activate a pathway leading to Erk2 activation and indicates that signaling events in addition to PKC are required for Erk2 activation. Alternatively, it is possible that PKC-induced activation of Erk2 is blocked in suspension. It has been shown that EGF-induced Erk2 activation is significantly inhibited in suspended cells due to a requirement for an integrin-mediated event, possibly actin polymerization, downstream of Ras that is necessary for Raf-1 or MEK1 activation (76, 77). Integrin engagement could be required for organization of the cytoskeleton or for relieving a negative inhibitor of a step in the Erk/MAPK pathway. Inhibition of actin polymerization or actin-myosin contractility of adherent cells also blocks Erk2 activation, suggesting that actin polymerization is required for some step in Erk2 regulation (49, 78). It is possible that signals that are dependent on actin polymerization act in concert with PKC to regulate Erk2 activation.

Other regulatory enzymes also modulate the level of activation of Erk2 in integrin pathways. We previously demonstrated that fibronectin-induced Erk2 activation in Cos7 cells is also regulated by p85-PI 3-K (34). Unlike PKC, P85-PI 3-K is required for optimal activation of Raf-1, downstream of Ras. Therefore, it appears that PKC and p85-PI 3-K are activated by different integrin-regulated events that converge on the Erk/MAPK pathway at different points, but both are required for maximal Erk2 activation. This possibility is supported by the observation that inhibition of both PKC and PI 3-K reduces Erk2 activation more than either alone.3 The ability of fibronectin to induce PI 3-K activation, like Erk2, also requires actin polymerization.2 Thus cooperation between PI 3-K and PKC may require integrin-induced regulation of the actin cytoskeleton.

We also found that PKC is important for cell spreading. A role for PKC in regulating integrin-mediated cell spreading has been observed in other cell systems. For example, inhibition of PKC activity blocks spreading of HeLa cells on collagen-coated surfaces (30), and down-regulation of PKCs and PKCe with antisense oligonucleotides in vascular smooth muscle cells blocked cell spreading on fibronectin (29). Additionally, overexpression of a dominant inhibitory mutant of MARCKS, a well characterized PKC substrate, completely blocks cell spreading of fibroblasts on fibronectin (79). Recent studies have linked the ability of cells to migrate to the Erk/MAPK pathway (80–82). However, a role for Erk in regulating cell spreading has not been demonstrated, and in fact, Erk2 can be activated in the absence of cell spreading, and inhibition of the Erk/MAPK signaling pathway does not block cell spreading (83, 84). Thus PKC could be playing a role in several integrin-mediated signaling events, only some of which are dependent on Erk.

Our ability to delineate exactly which PKC isoform is involved in integrin-induced Erk2 activation has been hampered by the inability of any PKC isoforms to enhance integrin-induced Erk2 activation and by what appears to be a lack of specificity of the dominant inhibitory mutants of PKC. We did observe integrin-induced membrane translocation of PKCa, PKCβ1, and PKCe. Integrin-induced membrane translocation was not seen for PKCδ. However, we did detect increased tyrosine phosphorylation of PKCδ following adhesion to fibronectin. Thus, of the five TPA-regulated PKCs expressed in Cos7 cells, we have detected adhesion-induced regulation of four PKC isoforms. Inhibition of Erk2 activation was observed with dominant inhibitory mutants of two PKC isoforms, and three constitutively active PKC isoforms were able to rescue TPA-mediated down-regulated Erk2 activation. The cross-inhibitory effects of dominant-negative mutants of different PKC isoforms has been observed by others (35). Stimulation of MEK1 activity by TPA and Raf-1 was inhibited by three different dominant mutants of PKC, PKCa, δ, or ε, a situation analogous to fibronectin-induced Erk2 activation. Additionally, several different PKC isoforms can activate the Erk/MAPK pathway in different or in the same cell types (14, 35, 85–88). One possible interpretation of these findings is that several different PKC isoforms share some redundant functions in cells, such as Erk activation, but also perform isoform-specific functions as well. Another possibility is that specificity is lost under conditions of overexpression employed in the approaches utilized in this study.

In Cos7 cells, TPA-mediated down-regulation of PKC did not block FAK or paxillin tyrosine phosphorylation, whereas calphostin C caused a significant inhibition as reported by others (22, 25). In those same experiments, calphostin C significantly inhibited cell attachment and completely blocked cell spreading (25). These effects contrast with the inhibitory activity seen with TPA-mediated down-regulation and suggest that calphostin C has broader inhibitory activities. Alternatively, TPA-induced PKC down-regulation may not be completely effective. Prolonged TPA treatment did not completely eliminate PKC, and it is possible that the remaining PKCe activity is responsible for mediating some of these events. However, dominant-negative PKCe did not block FAK phosphorylation under conditions where it blocked Erk2 activation. It is clear that these two agents may be acting in slightly different ways, and further investigations into the role of PKC in integrin-mediated events need to be addressed by using several different approaches.

In summary, we have characterized the role of PKC in regulating integrin-mediated signaling events in Cos7 cells. These studies show that integrin modulation of PKC isoforms belonging to the classical and/or novel subclasses are required for efficient activation of Ras, Raf-1, MEK1, and Erk2 by fibronectin. PKC is involved in regulating integrin-induced Erk/MAPK pathway signaling by modulating events upstream of Ras, most likely through regulation of Shc phosphorylation. In addition, PKC is important for efficient cell spreading on fibronectin. PKC may be important for integrin-induced tyrosine phosphorylation of FAK and paxillin, but these events do not appear to be regulated by classical or novel PKC isoforms.

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