Different Protein Kinase C Isoforms Determine Growth Factor Specificity in Neuronal Cells

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Although mitogenic and differentiating factors often activate a number of common signaling pathways, the mechanisms leading to their distinct cellular outcomes have not been elucidated. In a previous report, we demonstrated that mitogen-activated protein (MAP) kinase (ERK) activation by the neurogenic agents fibroblast growth factor (FGF) and nerve growth factor is dependent on protein kinase Cζ (PKCζ), whereas MAP kinase activation in response to the mitogen epidermal growth factor (EGF) is independent of PKCζ in rat hippocampal (H19-7) and pheochromocytoma (PC12) cells. We now show that EGF activates MAP kinase through a PKCζ-dependent pathway involving phosphatidylinositol 3-kinase and PKD1 in H19-7 cells. PKCζ, like PKCδ, acts upstream of MEK, and PKCζ can potentiate Raf-1 activation by EGF. Inhibition of PKCζ also blocks EGF-induced DNA synthesis as monitored by bromodeoxyuridine incorporation in H19-7 cells. Finally, in embryonic rat brain hippocampal cell cultures, inhibitors of PKCζ or PKCδ suppress MAP kinase activation by EGF or FGF, respectively, indicating that these factors activate distinct signaling pathways in primary as well as immortalized neural cells. Taken together, these results implicate different PKC isoforms as determinants of growth factor signaling specificity within the same cell. Furthermore, these data provide a mechanism whereby different growth factors can differentially activate a common signaling intermediate and thereby generate biological diversity.

Treatment of cells with different growth factors such as epidermal growth factor (EGF) or fibroblast-derived growth factor (FGF) often leads to distinct biological outcomes such as mitogenesis, neurogenesis, or apoptosis. Since these factors stimulate tyrosine kinase receptors that in turn activate common signaling cascades, the explanation for these differences in specificity has not been obvious. In general, two types of models have been proposed. First, it is possible that the same intermediates are utilized by both receptors, but the variations in activation kinetics, signal amplitude, or cellular localization result in different outputs. Second, it is possible that distinct intermediates are responsible for the differences in specificity. These two models can be reconciled if the same general families of signaling molecules are utilized by both receptor systems, but differences in the specific isoforms generate diversity in kinetics, amplitude, localization, or substrate selectivity.

One of the major targets of growth factor stimulation that can lead to diverse endpoints dependent on the degree of activation is the Ras Raf/MEK/mitogen-activated protein (MAPK) kinase signaling cascade. Although activated Ras and Raf were originally identified as mediators of neoplastic transformation, recent studies have suggested that these proteins can promote cell cycle arrest, differentiation, and even apoptosis in normal cells (49, 71). For example, in NIH 3T3 cells, moderate Raf activation elicits cell proliferation, but high activation leads to reversible p21-mediated cell cycle arrest (71). Expression of activated Raf in nonimmortalized human lung fibroblasts results in rapid and irreversible cell cycle arrest and senescence mediated by the cdk4 inhibitor p16 (75). In these examples, regulation of inhibitors of the cell cycle-dependent kinases by Raf can lead to a feedback inhibition of cellular growth. Expression of other proteins such as Fos and Jun during the G1 phase of the cell cycle can also vary dependent on the duration of the MAPK (ERK) signal (12). Thus, the extent of MAPK activation may influence the outcome of growth factor signaling cascades.

Recently, we have shown that activation of ERK by FGF or nerve growth factor but not EGF requires selective activation of a specific protein kinase C (PKC) isoform, PKCζ, in neuronal cells (13). More than 10 PKC isoforms have been cloned and can be categorized according to endogenous and exogenous activators (reviewed by Dekker and Parker [15]). Phorbol esters and diacylglycerol activate classical (α, βI, βII, γ) and novel (δ, ε, η, θ, and ι) PKC isoforms, with the activation of the former also requiring calcium. Activation of the atypical isoforms (αλ and ζ) is independent of both calcium and phospholipids. Many of the PKCs, including the atypical isoform PKCζ, have been shown to be involved in ERK activation (4, 6, 7, 16, 41, 55, 64, 66).

PKCζ has been implicated in several cellular processes including apoptosis, protein synthesis, and differentiation (3, 18, 42, 46, 69, 72). Recently, this isoform was shown to be involved in the activation of P70S6K by EGF in a phosphatidylinositol-3’ kinase (PI-3 kinase) and 3’-phosphoinositide-dependent kinase 1 (PDK1)-dependent manner (51). Similarly, PKCζ cooperates with P1-3 kinase-γ to mediate Ras-independent ERK activation by a Gi protein-coupled receptor (63). PKCζ mediates platelet-derived growth factor-induced ERK activation by a Raf-1 and phosphatidylinositol-specific phospholipase C (PC-PLC)-dependent cascade in Rat-1 cells (6, 66). PC-PLC and PKCζ are also required for lipopolysaccharide (LPS)-induced ERK activation in macrophages (45). Most recently,
PKCζ was implicated in the activation of ERK by insulin in adipocytes (52). Finally, a dominant-negative mutant of PKCζ severely impairs activation of MAP/ERK kinase (MEK) and ERK by serum and tumor necrosis factor alpha (4). Thus, PKCζ seems to play a role in growth factor-induced ERK activation in a variety of cell types.

In this study, we investigated the role of PKCζ as a mediator of EGF-induced ERK activation in a conditionally immortalized rat hippocampal cell line (H19-7) and in primary rat embryonic hippocampal cells. The H19-7 cell line was generated from a temperature-sensitive rat E17 hippocampal cell line with a recombinant vector expressing a temperature-sensitive simian virus 40 large T antigen (21). At the permissive temperature (33°C) when the large T antigen is expressed, cells proliferate in response to EGF. When shifted to the nonpermissive temperature (39°C) where the large T antigen is inactivated, H19-7 cells express neuronal differentiation markers upon stimulation by EGF but not EGF (21, 36, 37). Furthermore, like other conditionally immortalized neuronal cell lines (57), H19-7 cells are progenitor cells capable of migration and neuronal differentiation when grafted into the hippocampi of postnatal rats (U. England, R. A. Fricker, E. M. Eves, M. R. Rosner, and K. Wiclorin, unpublished data). In a previous study, we demonstrated that PKCζ can mediate MEK/ERK activation and neuritogenesis in both H19-7 and PC12 cells in response to neurogenic factors (13).

We now demonstrate that a parallel but distinct cascade involving PKCζ is required for EGF-induced ERK activation and mitogenesis in H19-7 cells. PKCζ is activated by EGF in a PI-3 kinase- and PDK1-dependent manner and, like PKCδ, activates ERK upstream of MEK. Furthermore, studies with selective PKC inhibitors indicate that ERK activation by EGF or FGF requires PKCζ or PKCδ, respectively, in primary embryonic hippocampal cells. Taken together, these results demonstrate that PKCζ mediates EGF-induced ERK activation by MEK in neuronal cells and provide evidence that different PKC isoforms play a role in mediating the specific effects of various growth factors in the same cell type.

MATERIALS AND METHODS

Materials. Receptor-grade EGF was purchased from Biomedical Technologies Inc. (Stoughton, Mass.). Basic FGF (bFGF) was purchased from Research Diagnostics Inc. (Flanders, N.J.). Normal goat serum (NGS) was purchased from Vector Laboratories, Inc. (Burlingame, Calif.). 9-Bromo-2-deoxyuridine (BrdU), 5-fluoro-2-deoxyuridine (FurdU), phorbol 12-myristate 13-acetate (PMA), Wortmannin, myelin basic protein (MBP), peroxidase-conjugated goat anti-rabbit immunoglobulin (IgG), antisera against phosphotyrosine, and antibodies against PKCζ were purchased from Transduction Labs (Lexington, Ky.). The purified, kinase-dead MEK (MEK[K97A]) was a gift from Angus MacNichol (University of Chicago). Antisense oligonucleotides (ECL) were purchased from Pharmacia Biotech AB (Uppsala, Sweden). Enhanced chemiluminescence (ECL) reagents and [γ 32P]ATP (6000 Ci/mmol) were purchased from DuPont/NEN Research Products (Boston, Mass.). The ECL-Plus Western blotting detection system was from Amersham Pharmacia Biotech (Piscataway, N.J.). The purified, kinase-dead MEK (MEK[K97A]) was a gift from Angus MacNichol (University of Chicago).

Plasmids. The activated MEK2E and HA-tagged mouse ERK2 constructs were described previously (36). The FLAG-Raf construct was a gift from Andrey Shaw (Washington University). HA-tagged PKCζ constructs were described previously (59). Myc-tagged p101 was a gift from J. Downward (Imperial Cancer Research Fund, London, England). Myc-tagged PDK1 constructs were a gift from A. Toker. HA-MEK1 was a gift from M. Marshall (Indiana University). Plasmid DNAs were prepared by CsCl-ethidium bromide gradient centrifugation as previously described (36) or by purification through columns as instructed by the manufacturer (Qiagen, Chatsworth, Calif.).

Cell culture. The immortalized H19-7 cells were generated from embryonic rat hippocampal cells as previously described (21). Cells were maintained in 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 400 µg of G418 at 33°C. Cells were serum starved in N2 medium overnight prior to treatment.

Dissection and culture of primary rat hippocampal cells. Hippocampi were dissected from E16 Sprague-Dawley (Harlan Sprague Dawley, Indianapolis, Ind.) rat embryos and placed in cold 25 mM HEPES buffer plus additives as described elsewhere (47). The hippocampal pieces were triturated 15 times with a P-1000 Pipetten pipette and allowed to settle for 5 min. The cell suspension was centrifuged at 250 × g for 5 min at 4°C. The cell pellet was then resuspended and plated as described previously (24). Briefly, the cells were resuspended in Dulbecco’s modified Eagle medium–Ham’s F-12 (Life Technologies, Gaithersburg, Md.) with insulin (25 µg/ml), transferrin (100 µg/ml), 60 µM putrescine, 30 mM sodium selenite, 20 mM progestrone, and sodium pyruvate (0.11 mg/ml). The cells were plated onto polyornithine- and fibronectin-coated 12- and 6-well tissue culture dishes at 2.5 × 104 cells/well, respectively. Cells were grown in 5% CO2 at 37°C, and fresh medium was added every other day. bFGF (10 ng/ml) was added to the medium for the first 4 days in culture to enhance proliferation, and then the cells were allowed to differentiate without bFGF for 4 days.

Immunocytochemistry. Cells were fixed with 4% paraformaldehyde and washed with phosphate-buffered saline (PBS). The cells were incubated with primary antibodies diluted in 0.5% NGS-0.01% Triton X-100-PBS for 2 h, washed with PBS for 30 min, incubated with Texas red-conjugated secondary antibodies (Cappel, Durham, N.C.), and washed with PBS for 30 min. The cells were characterized with antibodies to nestin (Rat-401; Developmental Hybridoma Bank, Iowa City, Iowa), microtubule-associated protein 2, (MAP-2) (HM-2; Sigma), and glial fibrillary acidic protein (GFAP; DAKO, Carpinteria, Calif.). Cells were examined at 400× with an inverted fluorescence Leica microscope supplied with rhodamine filters.

Transient transfections. Cells (2 × 106) were seeded on 100-mm diameter plates and incubated overnight. The medium was changed to serum-free Opti-Mem (Gibco/BRL), and cells were transfected with a total of 20 µg of plasmid DNA and 80 µl of TransIT LT-1 as specified by the manufacturer (Pan Vera Corp., Madison, Wis.). Ten percent of the total plasmid DNA consisted of pGreen Lantern-1 (Gibco/BRL), and the percentage of green protein-expressing cells was scored to normalize transfection efficiency between groups. Cells were split (1:2) 24 h posttransfection and kept quiescent for 16 h prior to treatment and harvesting. All experiments were done 48 h posttransfection.

Treatment of cells with phosphorothioate-modified oligonucleotides. H19-7 cells were seeded at 2 × 106/well in six-well polystyrene-coated plates and transfected with 10 µg of oligonucleotide, using 60 µl of TransIT-LT-1 according to the manufacturer’s protocol. Cells were left to incubate for 4 h and then switched to N2 medium at 39°C, and a further 30 µM (final concentration) oligonucleotide was added for 48 h prior to treatment. The antisense sequences used were 5’ GAAAGGAGATGCCTCAA 3’ for PKCζ and 5’ GTGCGTCC TGTCGGCCAT 3’ for PKCζ (22). The antisense sequence for PKCζ on nucleotides 10 to 27 of the murine coding sequence, while the sequence for PKCζ is based on the start codon plus the next 15 downstream nucleotides. The appropriate sense sequence was used as control.

In vitro FLAG-Raf, HA-MEK1, HA-PKζ, and HA-ERK2 kinase assays. FLAG-Raf, HA-MEK1, HA-PKζ, and HA-ERK2 were overexpressed in H19-7 cells as described above. Cells were then treated with or without the appropriate growth factor followed by two washings with ice-cold PBS. Cells were lysed with 1% Triton X-100-PBS containing 1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 40 mM β-glycerophosphate, 100 mM NaCl, 50 mM NaF, 2 mM EDTA, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, aprotinin (1 µg/ml), leupeptin (1 µg/ml), and 20 µM N-acetylphenylalanine and incubated on ice for 30 min. The cell debris was removed by centrifugation (15,000 rpm for 10 min at 4°C), and protein concentrations were determined by the Bio-Rad (Hercules, Calif.) protein assay using bovine serum albumin as the standard. Monoclonal antibodies M5 and HA.11, against the FLAG and HA epitope, respectively, were used together with a protein G-Sepharose 4B (Pharmacia) slurry of 50 µg of M5 or HA.11 to 1 ml of a 50:50 slurry of protein G-Sepharose in TLEB overnight at 4°C. Lysates were precleared with 50 µl of protein G-Sepharose for 30 min at 4°C. 40 µl of the antibody-protein G-Sepharose complex was added to 300 µl of TLEB for 2 h at 4°C. Extracts were then washed three times with TLEB and two times in kinase buffer (1× kinase buffer is 25 mM HEPES [pH 7.4], 10 mM MgCl2, 1 mM MnCl2, 1 mM EDTA, 10 mM DTT, 1 µg/ml each of aprotinin, leupeptin, and pepstatin A, 0.1% NP-40, 0.1% SDS) and 200 µl of 1× kinase buffer.
dithiothreitol, and 0.2 mM sodium vanadate). The final pellet was resuspended in 30 μl of kinase buffer, and reactions were started by addition of 50 μM ATP, 5 μCi of [γ-32P]ATP, and either 100 ng of purified MEK(K97A) or 1 μg of MEK-FL (for Raf kinase assay), 1 μg of ERK2 (K25R) (for MEK kinase assays), or 5 μg MBP (for ERK and PKC kinase assays) and carried out for 20 min at 30°C. Reactions were stopped by addition of 10 μl of 6× concentrated sample buffer and boiling for 5 min at 100°C. Beads were pelleted by centrifugation (14,000 rpm for 5 min), and supernatants were loaded onto a 10 or 13% acrylamide separating gel. Proteins were transferred to nitrocellulose and subjected to autoradiography.

**Western analysis.** Cell extracts (10 to 20 μg) were resolved on a 10% acrylamide separating gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a nitrocellulose membrane. Membrane blocking, washing, antibody incubation, and detection by ECL were performed as previously described (37). When antibodies against phosphospecific peptides were used, blots were stripped by washing six times with 5 min each with TBS-T (10 mM Tris [pH 7.5], 100 mM NaCl, 0.1% Tween 20) (0.1%) at room temperature (RT). 30 min at 55°C with stripping buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 100 mM 2-mercaptoethanol), and finally six times for 5 min each with TBS-T at RT. The stripped blots were then reprobed with the corresponding non-phosphospecific antibody to ensure equal protein loading.

**Detection of proliferating cells by BrdU staining and immunofluorescence.** H19-7 cells (10^5) were plated at about 30% confluency in either 6- or 12-well each with TBS-T at RT. The stripped blots were then reprobed with the corresponding non-phosphospecific antibody to ensure equal protein loading.

**RESULTS**

PI-3 kinase mediates EGF- but not FGF-induced ERK activation in H19-7 cells. Our previous work demonstrated that the activation of ERK1 and -2 by EGF in H19-7 cells in N2 medium is inhibited by wortmannin, an inhibitor of PI-3 kinase. Since wortmannin is not a specific inhibitor of PI-3 kinase at the concentration used (200 nM) (14), a more specific inhibitor, LY294002 (68), was tested. As shown in Fig. 1, both wortmannin and LY294002 blocked EGF-induced ERK activation. In contrast, neither PI-3 kinase inhibitor suppressed ERK activation by FGF. This result confirms our previous findings that PI-3 kinase mediates EGF- but not FGF-induced ERK activation in H19-7 cells.

**EGF but not FGF activates PKCζ in H19-7 cells.** Since FGF selectively activates ERK in H19-7 cells via a PKCζ-dependent mechanism (13), we investigated the possibility that EGF activation of ERK occurs by a parallel but distinct PKC cascade. On the basis of expression and PKC inhibitor studies in H19-7 cells (13), only the atypical PKCs, ζ and η, are possible candidates. Given that EGF activates ERK in a PI-3 kinase-dependent manner and PKCζ has been shown to be a downstream effector of PI-3 kinase (28, 39, 62), we initially focused on PKCζ. To determine whether EGF stimulates PKCζ, the effect of EGF treatment on PKCζ activity was directly measured. H19-7 cells were transfected with an expression vector for HA-PKCζ and then either left untreated or stimulated with EGF or FGF. As shown in Fig. 2A, the in vitro kinase activity of HA-PKCζ, after normalization for protein expression, was higher when the enzyme was immunoprecipitated from EGF-treated cells. Addition of a PKCζ peptide inhibitor (58) to the kinase assay abrogated EGF-induced PKCζ kinase activity, indicating that the kinase reaction was specific for PKCζ. In contrast, FGF treatment of cells did not stimulate PKCζ. These results indicate that EGF but not FGF selectively activates PKCζ.

Recent studies have shown that Thr410 is the site within the activation loop that must be phosphorylated for PKCζ activity (10, 39). PKD1, a downstream effector of PI-3 kinase, can phosphorylate Thr410 and activate the enzyme. To determine whether PKCζ isolated from EGF-stimulated cells is phosphorylated at this residue, H19-7 cells were transfected with HA-PKCζ and the cells were either left untreated or stimulated with EGF or FGF. HA-PKCζ was immunoprecipitated from the cell lysates, resolved by SDS-PAGE, and then analyzed by immunoblotting with an antibody directed against the phosphorylated Thr410 residue. As shown in Fig. 2B, only the PKCζ expressed in the EGF-treated cells was phosphorylated at this site within the activation loop. These results support the previous finding that EGF but not FGF activates PKCζ in these cells.

**PKCζ antisense oligonucleotides are specific and do not block expression of other PKC isoforms.** To determine directly whether PKCζ mediates the activation of ERKs by EGF, we used antisense oligonucleotides (19, 33, 56). Initially, we determined whether the antisense oligonucleotides acted specifically to suppress PKCζ and not other PKC isoforms. H19-7 cells were transfected with phosphorothioate-modified antisense oligonucleotides directed against PKCζ or -6 (see Materials and Methods), and lysates were collected and analyzed for PKC expression by immunoblotting. As shown in Fig. 3, treatment of cells with PKCζ antisense oligonucleotides decreased the amount of immunoreactive PKCζ by 76%, whereas other PKC isoforms were unaffected. In contrast, treatment of cells with PKCζ sense oligonucleotides had no effect on expression of any of the PKC isoforms. As a control, cells were also chronically treated with PMA, which selectively down regulates classical and novel PKC isoforms but not the atypical PKCζ or PKCζ (Fig. 3). Treatment of cells with antisense PKCζ oligonucleotides showed similar selectivity, decreasing expression of PKCζ by over 90% but leaving the...
other PKC isoforms unaffected. Taken together, these data show that treatment of H19-7 cells with antisense PKCζ oligonucleotides is an effective method for investigating the function of the PKCζ isoform.

**PKCζ mediates EGF-induced ERK activation in H19-7 cells.** To determine whether PKCζ plays a role in ERK induction by EGF, two approaches were used. First, H19-7 cells were transfected with PKCζ antisense or sense oligonucleotides, and ERK activation following EGF stimulation was determined by immunoblotting the cell extracts with anti-active MAPK phosphospecific antibody. This antibody recognizes the phospho-ylated form of the conserved TGY motif within the activation loop in ERKs (11). Addition of the PKCζ antisense oligonucleotides decreased the levels of PKCζ expression and abrogated EGF-induced ERK activation, while control sense oligonucleotides had no effect (Fig. 4A). These results indicate that PKCζ expression is required for ERK activation by EGF.

As a complementary approach, cells were transfected with a kinase-inactive mutant of PKCζ (PKCζKR) to determine whether it acts as a dominant-negative inhibitor of ERK activation by EGF. Thus, HA-tagged PKCζ constructs (59) were cotransfected with HA-ERK2 into H19-7 cells, and the cells were either untreated or stimulated with EGF. Following stimulation, cell lysates were immunoprecipitated with anti-HA antibody, and the immunoprecipitates were assayed for ERK kinase activity using MBP as a substrate. Since HA-PKCζ is also immunoprecipitated under these conditions, the PKCζ inhibitor peptide was also included in the kinase assay to suppress any MBP phosphorylation by PKCζ. Finally, the results of this experiment were confirmed using FLAG-tagged PKCζ (data not shown). As shown in Fig. 4B and C, EGF stimulation caused a threefold increase in normalized HA-ERK2 kinase activity relative to nonstimulated cells. Cotransfection with wild-type or the catalytic portion of PKCζ (PKCζwt or PKCζCAT) resulted in an approximately fourfold increase in EGF-induced HA-ERK2 activation compared to nonstimulated cells. Conversely, the kinase-dead PKCζKR mutant decreased the level of EGF-stimulated HA-ERK2 activation to 1.3-fold, suggesting that the kinase activity of PKCζ is required. Analysis of HA-ERK2 activation by Western blotting with anti-phospho-ERK yielded similar results (data not shown). These data confirm that activated PKCζ is an intermediate in the EGF-induced ERK activation pathway in H19-7 cells.

**PKCζ is required for EGF-induced MEK activity.** Since PKCζ mediates ERK activation, we initially determined whether PKCζ acts upstream of MEK. H19-7 cells were transfected with PKCζ antisense oligonucleotides, the cells were stimulated with EGF, and the cell extracts were immunoblotted with antibodies specific for phosphothreonine 410 (pT410) PKCζ. Membranes were then stripped and reprobed for HA-tagged proteins using rat antibody 3F10. Data are representative of two independent experiments.

**FIG. 2.** EGF selectively activates HA-PKCζ in H19-7 cells. (A) H19-7 cells were transfected with 8 μg of pcDNA3 (CTRL), 2 μg of HA-ERK2 plus 6 μg of pcDNA3, or 8 μg of HA-PKCζ. Cells were then switched to N2 medium at 39°C and left untreated or stimulated with 10 ng of FGF or EGF per ml for 10 min. Following treatment, cells were lysed, and HA-tagged constructs were immunoprecipitated with HA.11 antibody. The samples were resolved by SDS-PAGE (13% gel) and assayed for ERK or PKCζ activity using MBP as a substrate as described in Materials and Methods. In one sample, 10 μM PKC peptide inhibitor (HA-PKCζ + pep) was added to the kinase mixture. Membranes were then probed for HA-tagged proteins using rat antibody 3F10. The amount of 32P incorporated into MBP was measured by PhosphorImager analysis and normalized to the amount of HA protein in each sample. The mock (CTRL) lane was arbitrarily set to 1. (B) H19-7 cells were transfected with 10 μg of HA-PKCζ. Cells were then switched to N2 medium at 39°C and left untreated or stimulated with 10 ng of FGF or EGF per ml for 10 min. Following treatment, cells were lysed, and HA-tagged constructs were immunoprecipitated with HA.11 antibody. The samples were resolved by SDS-PAGE (10% gel) and immunoblotted with antibodies specific for phosphothreonine 410 (pT410) PKCζ. Membranes were then stripped and reprobed for HA-tagged proteins using rat antibody 3F10. Data are representative of two independent experiments.

**FIG. 3.** Antisense oligonucleotides selectively suppress isoform-specific PKCs. H19-7 cells were pretreated with either sense (S) or antisense (AS) PKCζ, or δ phosphorothioate-modified oligonucleotides as described in Materials and Methods and then either untreated (CTRL) or stimulated with 800 nM PMA for 18 h. Cells were then lysed; the lysates were resolved by SDS-PAGE (10% gel) and assayed for PKCζ expression by immunoblotting with anti-PKCζ antibodies.
MEK as determined by phosphorylation of its activation loop. As shown in Fig. 5A, treatment of H19-7 cells with antisense but not sense PKCζ inhibited EGF-induced MEK activation, indicating that PKCζ acts upstream of MEK.

To confirm this result, MEK activity was also assayed directly. An HA-tagged MEK1 construct was cotransfected into H19-7 cells with HA-PKCζwt, PKCζKR, or PKCζCAT, and the cells were stimulated with EGF. Following immunoprecipitation with anti-HA antibody, HA-MEK1 activity was measured by in vitro kinase assay using MBP as a substrate as described in Materials and Methods; 10 μM PKC peptide inhibitor was included in the kinase mixture. Membranes were then probed for HA-tagged proteins using rat 3F10 antibody. (C) Quantification of ERK activation. The amount of 32P incorporated into MBP was measured by PhosphorImager analysis and normalized to the amount of HA protein in each sample. The mock lane was arbitrarily set to 1. Data are the means ± standard deviations from three independent experiments.

PKCζ potentiates EGF-induced Raf-1 activation. To narrow down the potential targets of PKCζ action, we determined whether PKCζ activity potentiates or is required for stimulation of Raf-1 by EGF. A FLAG-tagged c-Raf-1 construct was cotransfected into H19-7 cells along with HA-PKCζwt, HA-
PKCζ, or HA-PKCζKR, and the cells were stimulated with EGF. Following immunoprecipitation of FLAG-Raf-1 with anti-FLAG antibody, Raf-1 activity was assessed by in vitro kinase assay using inactive MEK as a substrate. As shown in Fig. 6, EGF is a weak activator of Raf-1 kinase activity in H19-7 cells. However, in EGF-treated cells cotransfected with HA-PKCζwt in addition to FLAG-Raf-1, Raf-1 kinase activity was enhanced 13-fold. Cotransfection of cells with HA-PKCζCAT in addition to FLAG-Raf-1 augmented EGF-induced Raf-1 kinase activity only fourfold. Consistent with the ERK and MEK1 results, expression of HA-PKCζKR completely abrogated FLAG-Raf-1 kinase activity. These data demonstrate that although EGF alone is a poor activator of Raf-1 kinase activity, the activation can be significantly enhanced by coexpression of PKCζ.

**PDK1 is required for EGF-induced ERK activation.** Since EGF-induced ERK activation is PI-3 kinase and PKCζ dependent in H19-7 cells, we were interested in identifying other
ERK activation in H19-7 cells involves PI-3 kinase, PDK1, and PKCζ.

**PKCζ is required for EGF-induced mitogenesis in H19-7 cells.** The ERK family of MAPK regulates cellular growth in a variety of tissues. At the permissive temperature (33°C), EGF induces a mitogenic response in H19-7 cells. Since PKCζ is a mediator of EGF-induced ERK activation, we determined whether EGF stimulation of DNA synthesis in these cells is dependent on PKCζ. To monitor DNA synthesis, we assayed for incorporation of the nucleoside analog BrdU into DNA by immunostaining with an anti-BrdU antibody. H19-7 cells were serum starved for 3 days at 33°C and then stimulated with EGF or FGF in the presence of BrdU for 24 h. As shown in Fig. 8, only 8% of the untreated control cells could be immunostained with an antibody to BrdU. FGF induced a fourfold increase (32%) in BrdU incorporation, while EGF caused 80% of the cells to incorporate BrdU, indicating that EGF is a more potent mitogen than FGF in H19-7 cells. To determine whether PKCζ mediates growth factor-stimulated DNA synthesis, almost 80% of the H19-7 cells were depleted of PKCζ by preincubation with PKCζ antisense oligonucleotides (Fig. 3). Under these conditions, only 25% of the cells incorporated BrdU following EGF stimulation. This number is probably an underestimate of the inhibition, since not all of the cells have taken up the antisense PKCζ oligonucleotides. No effect on DNA synthesis was observed when EGF-treated cells were preincubated with sense PKCζ or when FGF-treated cells were preincubated with antisense PKCζ oligonucleotides. These results indicate that PKCζ selectively mediates EGF-induced DNA synthesis.

**Different PKC isoforms regulate ERK activation by EGF versus FGF in primary hippocampal neural cultures.** To determine whether the PKC-dependent growth factor signaling pathways identified in H19-7 cells are similarly activated in primary cells, we examined the response of E16 rat hippocampal cells to EGF versus FGF. Initially, the hippocampal cells were treated with FGF (10 ng/ml) in N2 medium for 4 days to expand the cultures and then incubated in N2 medium alone for 4 days to suppress mitogenesis. Immunostaining of the cells with antibodies for progenitor (nestin), neuronal (anti-MAP-2), or glial (anti-GFAP) markers indicated that 77% of the cells expressed nestin, 17% ± 3.2% of the cells expressed MAP-2, and 2.7% ± 0.1% of the cells expressed GFAP (Fig. 9A). These results indicate that the hippocampal cells were primarily neurally differentiated progenitor cells. Thus, these cells are similar to the H19-7 cells that express nestin and function as progenitors in vivo. Treatment with 10 ng of either FGF or EGF per ml for 10 min stimulated ERK activation, as shown by immunoblotting cell extracts with anti-phospho-ERK antibodies. Pretreatment with chelerythrine chloride, a selective inhibitor of the classical and novel PKCs, specifically inhibited FGF-induced ERK activation (Fig. 9B). However, pretreatment of cells with a myristoylated peptide inhibitor of PKCζ that is cell permeable (62) specifically suppressed ERK activation by EGF but had no effect on FGF-induced ERK activity. Conversely, pretreatment of cells with rottlerin, an inhibitor of PKCζ, decreased ERK activation by FGF but not EGF. Similarly, rottlerin selectively blocked ERK activation by FGF, and the PI-3 kinase inhibitor wortmannin specifically inhibited ERK activation by EGF in embryonic day 14.5 mouse cortical cultures (data not shown). These results indicate that EGF and FGF activate similar signaling cascades in primary as well as immortalized hippocampal neural cells.

intermediates in this pathway. Although PKCζ can be activated by the products of PI-3 kinase (62), recent studies have shown that PKCζ is directly regulated by PDK1, which phosphorylates the Thr410 site within the activation loop (10, 39). As shown above, EGF-stimulated PKCζ is phosphorylated at this key site (Fig. 2B). Therefore, we determined whether PKD1 may be required for EGF-induced ERK activation in H19-7 cells. As shown in Fig. 7A, expression of a membrane-targeted, constitutively active PI-3 kinase (p110CAAXA) was sufficient to activate ERK, and this activation was blocked by PKCζKR suggesting that PKCζ is a downstream effector of PI-3 kinase in H19-7 cells. Consistent with this observation, the cells expressing Le Good et al. (39), a kinase-dead mutant of PKD1 (PKD K110N) blocked EGF-induced ERK activation (Fig. 7B). Together, these results suggest that the pathway controlling EGF-induced
The results described here demonstrate that PKC\(z\) is required for EGF-induced ERK activation and DNA synthesis in hippocampal H19-7 cells. The activation of PKC\(z\) occurs by a PI-3 kinase and PDK-dependent pathway and is downstream of Ras (5, 8, 17). Furthermore, in primary E16 hippocampal cells, EGF-induced ERK activation also requires PKC\(z\), and FGF activation of ERK is dependent on PKC\(z\). These data complement our previous findings that PKC\(z\) is required for FGF-induced ERK activation and can mediate neuritogenesis in H19-7 cells and PC12 cells. Taken together, these results demonstrate that different isoforms of PKC mediate growth factor-specific activation of ERK and lead to different biological outcomes within the same cell (Fig. 10).

PKC\(z\) is often associated with a differentiation- or growth-suppressive function, whereas PKC\(\delta\) has been implicated in cell metabolism and proliferation. For example, PKC\(z\) promotes differentiation of myeloid progenitors into macrophages (44) and, when overexpressed, blocks growth in vascular smooth muscle, capillary endothelial, NIH 3T3, and CHO cells (26, 30, 43, 70). Src-mediated transformation in rat fibroblasts is blocked by PKC\(z\) (40), and overexpression of PKC\(z\) in the skin of transgenic mice prevents tetradecanoyl phorbol acetate-induced tumor promotion (50). On the other hand, PKC\(z\) mediates Ras-independent activation of ERK by the Gi protein-coupled LPS receptor (63) as well as EGF-induced p70S6K activation (51). Sajan et al. (52) recently showed a requirement for PKC\(z\) in growth factor signaling. While the dominant-negative mutant of PKC\(z\) gave consistent results and has been used previously, this approach is not sufficient to implicate a particular isoform since various PKC dominant-negative mutants inhibit other members of the PKC family (reference 27 and data not shown). Peptides derived from the pseudosubstrate regions of particular PKC isoforms and chemical inhibitors that have 10- to 100-fold selectivity for a particular isoform such as rottlerin are very powerful tools. Perhaps the most convincing approach involves the use of antisense oligonucleotides (33, 56). Taken together, these methods provide strong evidence for a selective mobilization of different PKC isoforms by growth factors.

The mechanism by which these differential cascades are initiated is not entirely clear. Both the EGF and FGF receptors are associated through their juxtamembrane domains with distinct docking proteins, termed Gab1 and FRS2, respectively, and it is likely that the signaling molecules recruited to the receptors via these adapter proteins play an important role in initiating the specific cascades. In the case of FGF, receptor stimulation leads to complex formation between FRS2 and a variety of signaling molecules including the adapter Grb2, the Ras activator Sos, and the Shp2 tyrosine phosphatase (35). This complex has been implicated in the subsequent activation of MAPK and differentiation of PC12 cells (29). Gab1 has been found to similarly mediate EGF signaling (31). Although PKC\(z\) (39) and PKC\(\zeta\) (10, 39) have both been shown to be activated by a PI-3 kinase- and PDK1-dependent pathway in some cells, our results suggest that this pathway is selectively activated by EGF but not FGF in neuronal cells. The primary target of PKC\(z\) activation could be either Raf or MEK. Surprisingly, although overexpression of PKC\(z\) can potentiate Raf activation by EGF, EGF barely stimulates Raf-1 in cells expressing only endogenous PKC\(z\). This result suggests that under physiological conditions, EGF activates the MEK/ERK cascade by a Raf-independent mechanism, and the potentiation of Raf is an artifact of PKC\(z\) overexpression. Thus, the primary site of PKC\(z\) action, like that of PKC\(\zeta\) (13), could be MEK rather than Raf. This possibility is consistent with previous studies indicating that the only PKC shown to activate MEK directly is PKC\(\zeta\), and this signaling pathway is Raf independent (55). Thus, PKCs may modulate the ERK
pathway by Raf-1-independent as well as -dependent pathways.

The results that we obtained suggest that the activation of ERKs by PKC\(\text{z}\) is subject to a number of regulatory mechanisms. In several of our experiments, expression of exogenous PKC\(\text{z}\text{wt}\) was significantly more effective at activating ERK than the catalytically active form of PKC\(\text{z}\). One explanation for these results is that the N-terminal regulatory domain of PKC\(\text{z}\) is required for maximal stimulation of Raf. This possibility is supported by a recent finding that the 14-3-3 site in the regulatory domain of PKC\(\text{z}\) potentiates binding of PKC\(\text{z}\) to Raf-1 (65). In other experiments, differences were observed in the ability of coexpressed PKC\(\text{z}\text{wt}\) to activate exogenous Raf versus exogenous ERK. There are several possible explanations for these results. Perhaps only the endogenous PKC\(\text{z}\) is able to activate ERK. However, it is more likely that the lack of stimulation of the downstream exogenously expressed ERK is due to a limitation in the amount of endogenous upstream activators of ERK. It has recently been shown that synergistic activation of exogenously expressed JNK requires coexpression of the upstream activators MEK kinase 2 and JNK kinase 2 (9). Thus, formation of a similar complex between Raf, MEK, ERK, and PKC\(\text{z}\text{wt}\) may be required to maximally activate ERK.

The specific mechanisms by which PKC isoforms mediate growth factor activation of ERKs appear to be tissue specific. Phorbol ester-sensitive PKCs are required for EGF stimulation of Raf in NIH 3T3 and COS cells (7), and FGF stimulation of ERKs is independent of PKC\(d\) in NIH 3T3 cells (13). In contrast, FGF requires PKC\(d\) downstream of Raf but upstream of MEK in both H19-7 and PC12 cells (13). Similar cascades were observed in primary neuronal cells, although in these cells FGF can act as both a mitogen and a neurogenic factor (67). Thus, studies with embryonal cortical and hippocampal cultures from both mice and rats indicated that EGF activation of ERKs was suppressed by PI-3 kinase and PKC\(\text{z}\) inhibitors, and FGF activation of ERKs and MEKs was suppressed by rottlerin and inhibitors of the novel PKCs (see Results; data not shown). Presumably, in different cell types, other modulating factors reflecting different intracellular environments act in conjunction with the specific PKC isoforms to determine the final biological outcome of growth factor stimulation.

The mechanism by which PKCs regulate MEK activation is the subject of current investigation. In one possible mechanism by which PKC\(d\) might activate MEK, at PKC acts as a scaffold to bring a multiprotein complex together in order to mediate efficient transduction of the signal down the cascade. A number of scaffolding proteins in the MAPK cascade have now been identified, including MEK partner 1 (54), JNK-interacting protein 1 (73), and JNK/stress-activated protein kinase-associated protein 1 (32). An alternative mechanism is inactivation of an inhibitor acting on one of the intermediates in the ERK cascade. A primary candidate is the newly identified Raf kinase inhibitor protein (74). In either case, PKC kinase activity appears to be required, since the kinase-inactive mutant blocked PKC function. Finally, similar to the action of PAK1, PKC might directly phosphorylate and potentiate the activity of MEK (25). At least one group showed that LPS-stimu-
lated PKC\(\zeta\) can phosphorylate MEK in vitro (45). In contrast, several groups have commented that PKC\(\zeta\) phosphorylates MEK weakly if at all compared to its ability to phosphorylate Raf (34, 55, 66). Further studies should resolve these possibilities.

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REFERENCES

becomes neurototoxic via the N-methyl-D-aspartate receptor when intracellular energy levels are reduced. Brain Res. 451:205–212.


