An Inhibitory Fragment Derived from Protein Kinase Cε Prevents Enhancement of Nerve Growth Factor Responses by Ethanol and Phorbol Esters*

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We have studied nerve growth factor (NGF)-induced differentiation of PC12 cells to identify PKC isozymes important for neuronal differentiation. Previous work showed that tumor-promoting phorbol esters and ethanol enhance NGF-induced mitogen-activated protein (MAP) kinase activation and neurite outgrowth by a PKC-dependent mechanism. Ethanol also increases expression of PKCδ and PKCe, suggesting that one of these isozymes regulates responses to NGF. To examine this possibility, we established PC12 cell lines that express a fragment encoding the first variable domain of PKCe (amino acids 2–144), which acts as an isozyme-specific inhibitor of PKCε in cardiac myocytes. Phorbol ester-stimulated translocation of PKCe was markedly reduced in these PC12 cell lines. In addition, phorbol ester and ethanol did not enhance NGF-induced MAP kinase activation or neurite outgrowth in these cells. In contrast, phorbol ester and ethanol increased neurite outgrowth and MAP kinase phosphorylation in cells expressing a fragment derived from the first variable domain of PKCeδ. These results demonstrate that PKCe mediates enhancement of NGF-induced signaling and neurite outgrowth by phorbol esters and ethanol in PC12 cells.

Protein kinase C (PKC) is a multigene family of phospholipid-dependent, serine-threonine kinases that plays a central role in cell growth and differentiation. Molecular cloning studies have identified 10 isozymes encoded by 9 different mRNAs (1, 2). Based on sequence homology and biochemical properties, the PKC gene family has been divided into three groups: "conventional" PKCs (α, βI, βII, and γ) regulated by calcium and diacylglycerols or phorbol esters; "novel" PKCs (ε, δ, θ, and η), which are calcium-independent but diacylglycerol- and phorbol ester-sensitive; and "atypical" PKCs (ζ, ι/λ), which are insensitive to calcium, diacylglycerol, and PMA. In addition, two related phospholipid-dependent kinases, PKCμ and protein kinase D, share sequence homology in their regulatory domains to novel PKCs and may constitute a new subgroup (3, 4).

Several studies with tumor-promoting phorbol esters suggest that PKCα modulates neural differentiation. Phorbol esters induce neural tissue from ectoderm in Xenopus embryos (5) and elicit neurite outgrowth from chick sensory ganglia (6, 7), chick ciliary ganglion neurons (8), several human neuroblastoma cell lines (9, 10), and rat PC12 cells (11, 12). Studies using purified isoforms, kinase-defective mutants, and transgenic or mutant cell lines have implicated PKCα, βI, θ, ε, and ζ in the differentiation of nonneural cells (13–17). Overexpression of PKCε or β in Xenopus embryos enhances neural induction (18), but little else is known about the identity of specific PKC isoforms that regulate neural differentiation.

Recent evidence suggests that PKCe plays a role in neural differentiation and plasticity. PKCe is expressed predominantly in the nervous system and is particularly abundant in the hippocampus, olfactory tubercle, and layers I and II of cerebral cortex (19). Within immunoreactive neurons, it is localized to the Golgi apparatus and to axons and presynaptic nerve terminals (19). PKCe is activated by growth factors that stimulate neural differentiation such as insulin (20) and NGF (21). In addition, in developing chick brain, it is the major isozyme found in nondividing, differentiating neurons (22).

Further evidence for involvement of PKCe in neural differentiation has come from studies with PC12 cells. PC12 cells are derived from neural crest and, when treated with NGF or fibroblast growth factors, undergo dramatic biochemical and morphological differentiation, developing several characteristics of mature sympathetic neurons (23). PKC-activating phorbol esters enhance NGF-induced activation of ERK1 and ERK2 mitogen-activated protein (MAP) kinases and neurite outgrowth in PC12 cells, suggesting that PKC modulates responses to NGF (11, 12, 24). Studies with ethanol-treated PC12 cells helped direct us toward the PKC isoyme responsible for this effect. Like phorbol esters, ethanol increases NGF-induced MAP kinase activation and neurite outgrowth through a PKC-dependent mechanism (11, 24). Ethanol promotes PKC-mediated phosphorylation in PC12 cells by increasing levels of messenger RNA and protein for two PKC isoforms, PKCe and PKCeδ (25, 26). Recently, we found that overexpression of PKCe, but not of PKCeδ, enhances NGF-induced MAP kinase activation and neurite outgrowth (27). These findings establish PKCe as a positive modulator of neurite growth. They also suggest
that PKCe mediates the neurite-promoting effect of ethanol and phorbol esters in PC12 cells. However, proof of this hypothesis requires studies with PKC isozyme-specific inhibitors or cells lacking specific PKC isoforms.

In the current study, we used specific inhibitors of PKCδ and PKCe to investigate whether PKCe mediates enhancement of neurite outgrowth by phorbol esters and ethanol. To achieve this goal we used dominant-negative inhibitors based on the amino acid sequences for PKCδ and PKCe. This approach is based on the observation that upon activation, PKC isoforms translocate to specific intracellular sites where they appear to bind anchoring proteins, termed RACKs (receptors for activated C-kinase) (28). One such protein that has been cloned is RACK1, which interacts with the C2 domain of conventional PKCs (29). The sites of interaction between RACK1 and the C2 domain of PKCδ have been mapped, and short peptides derived from these domains inhibit translocation and activation of PKCδ in cardiac myocytes and Xenopus oocytes (29–31). Holmology has been noted between the unique first variable region of PKCe (eV1) and the C2 domain of conventional PKCs (32), suggesting that, similar to the C2 domain of conventional PKCs, eV1 may contain a binding site for an PKCe-specific RACK. If that is the case, then expression of an eV1 fragment should inhibit PKCe translocation and function. Indeed, recent work has shown that an eV1 fragment and a peptide corresponding to amino acids 14–21 in this region prevent phorbol ester-induced PKCe translocation and function. Therefore, we decided to test whether PKCe specifically mediates enhancement of MAP kinase activation and neurite growth by phorbol esters and ethanol in PC12 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—NGF (2.5 S) was purchased from Collaborative Research (Bedford, MA). Geneticin (G418), laminin, and poly-l-ornithine (30–70 kDa) were purchased from Sigma. Phorbol 12-myristate 13-acetate (PMA) was from LC Laboratories (Woburn, MA). Antibodies were purchased from the following sources: peroxidase-conjugated goat anti-rabbit IgG from Boehringer Mannheim, fluorescein-conjugated goat anti-rabbit IgG from Cappel (Durham, NC), anti-phospho-MAP kinase antibody from New England Biolabs (Beverly, MA), and anti-Flag M2 antibody from Eastman Kodak Co.

**Cell Culture**—PC12 cells, originally obtained from Dr. John A. Wagner (Cornell University, New York, NY), were cultured in plastic tissue culture flasks at 37 °C in Dulbecco’s modified Eagle’s medium containing 5% heat-inactivated horse serum, 5% fetal calf serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin, in a humidified atmosphere of 90% air and 10% CO2. For studies of neurite outgrowth, cells were plated at a density of 30–40 × 103 cells/well on 24-well plastic culture plates pretreated for 1 h with poly-l-ornithine (100 μg/ml in 15 mM sodium borate, pH 8.4). In some experiments, cells were plated at a density of 20 × 103 cells/well on 8-well glass chamber slides (Nunc, Naperville, IL) treated first with poly-l-ornithine and then laminin (30 μg/ml) overnight. Cells were cultured in medium containing 50 ng/ml of NGF for 4 days, and neurites were measured as described previously (27). Ethanol-treated cultures were wrapped in Parafilm to prevent evaporation of ethanol, as in prior studies (24, 25).

**Immunofluorescence Microscopy**—To detect PKCe immunoreactivity, cells plated on glass chamber slides were incubated in PBS (137 mM NaCl, 2.7 mM KCl, 1.47 mM KH2PO4, 8 mM Na2HPO4, 0.5 mM MgCl2, 0.9 mM CaCl2, pH 7.2) containing 2% paraformaldehyde for 30 min and 4% paraformaldehyde for 30 min at 4 °C. Cells were washed three times in PBS and incubated for 2 h in PBS containing 1% normal goat serum and 0.1% Triton X-100. Cells were incubated 48 h at 4 °C in PBS containing 2 mg/ml of bovine serum albumin, 0.1% Triton X-100, and 2 μg/ml of rabbit anti-PKCδ antibody (24) provided by Dr. Susan C. Kiley (W. Alton Jones Cell Science Center, Lake Placid, NY). Cells were washed three times in PBS, and immunoreactivity was detected using fluorescein-conjugated goat anti-rabbit IgG as described previously (27). Images were detected using a liquid-cooled CCD camera (Photometrics Ltd., Tucson, AZ) fitted with a Thompson 7883 chip (384 × 576 pixels) attached on an Olympus IMT-2 inverted microscope equipped with a × 1.3 numerical aperture objective. Exposure times were 0.5 s, and images were stored on an Apple Macintosh Quadra 950 computer. Fluorescence intensity in growth cones and cytoplasm was measured using the program BIDS Image (Oncor Imaging Systems, Gaithersburg, MD).

To detect PKCe immunoreactivity, cells were incubated with a flag polyclonal antibody against PKCe (0.2 μg/ml) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) as described previously (27). Immunofluorescence in 0.5-μm optical sections was detected with a Bio-Rad MRC 1024 confocal laser-scanning microscope equipped with a Nikon ×60, 1.4 numerical aperture oil immersion objective. Nuclei were stained by incubating cells in PBS containing 2 mg/ml of bovine serum albumin, 0.1% TO-3 (Molecular Probes, Eugene, OR) for 2 h at 25 °C. After three washes in PBS, slides were dried, and coverslips were mounted with Vectashield (Vector, Burlingame, CA). TO-3 immunofluorescence was detected by confocal laser-scanning microscopy. Images were analyzed in BIDS Image to measure the area of each nucleus at its widest diameter.

**Generation of Cell Lines**—The plasmid pDM27 (33), containing a flag-epitope tag followed by the sequence encoding amino acids 2–144 of PKCe was used to amplify a 480-base pair fragment containing a NotI site at the 5’-end and a XbaI site at the 3’-end. This amplified fragment, containing an ATG start codon, the Flag epitope sequence, and the PKCe sequence, was subcloned into the NotI and XbaI sites of pcR/RSV (Invitrogen, San Diego, CA) to generate the plasmid pcR/VE1. Another plasmid, pDM68 (33), containing the Flag epitope followed by the sequence encoding amino acids 2–144 of PKCδ was used to amplify and subclone a homologous Flag-tagged PKCe sequence into pcR/RSV to generate the plasmid pcR/VE1. PC12 cells (105) were suspended in 0.5 ml of Ca2+- and Mg2+-free PBS containing 137 mM NaCl, 2.7 mM KCl, 1.47 mM KH2PO4, 8 mM Na2HPO4, pH 7.2, and were electroporated with 80 μg of pcR/VE1, pcR/VE1, or pcR/RSV as described previously (27). Genetically modified cells were added at 400 μg/ml to specific-activated medium and were added at 200 μg/ml to maintain cultures. For each vector, 46 clones were selected, expanded, and then examined for expression of Flag-tagged PKCe fragment mRNA using RT-PCR and for Flag immunoreactivity by Western analysis.

**RT-PCR**—Poly(A) mRNA was isolated from 105 cells using a MicroFast Track mRNA isolation kit (Invitrogen, San Diego, CA). Reverse transcription with 100 ng of mRNA was carried using a Stratagene (La Jolla, CA) RT-PCR kit according to the manufacturer’s protocol. Amplification of cDNA was achieved using the forward primer (5’-ACACTGGCCGCCGGATGACTACAAGGACGACGAT-3’) and the reverse primer 5’-AGCGGCTGATCCTATGCTTCTATCTGTTCTTTA-3’ for pcR/VE1, and 5’-ACAGACCTCTAGAGCGGTTCATAGTTGGGAA-3’ for pcR/VE1. Both primers were designed to specifically amplify the Flag-V1 sequences and not the V1 sequences of endogenous PKCe and PKCe in cells. Samples were heated to 94 °C, and amplification was started by the addition of Taq DNA polymerase. The amplification cycle was as follows: annealing for 45 s at 48 °C; elongation for 1 min at 72 °C, and denaturation for 1 min at 94 °C. Amplification was repeated for 30 cycles.

**Western Analysis for Flag and Phospho-MAP Kinase Immunoreactivity**—To detect expression of Flag-tagged peptides, cells were cultured on poly-l-ornithine-coated, 100-mm tissue culture dishes at a density of 6 × 106 cells/dish. Medium was removed, and cells were rinsed twice at 4 °C with buffer A (120 mM NaCl, 5 mM KCl, 1.4 mM CaCl2, 0.8 mM MgSO4, 1 mM Na2HPO4, 10 mM glucose, 25 mM HEPES, pH 7.4). Cells were scraped into 1 ml of buffer A containing 40 μg/ml leupeptin, 40 μg/ml aprotinin, 5 μM phenylmethylsulfonyl fluoride and then were frozen on dry ice. Concentrated 5 × sample volume was added to 400-μl frozen samples to yield a final solution containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 12.5 mM mg/m bromophenol blue. Samples were heated at 90 °C for 10 min, passed five times through a 26-gauge needle, and centrifuged at 10,000 × g for 10 min. Samples (80 μl) were separated by
SDS-polyacrylamide gel electrophoresis using 14% gels. Proteins were electrophoretically transferred for 2 h at 4 °C to Hybond-C extra membranes (Amersham Corp.). Membranes were blocked for 1 h with 3% nonfat dry milk dissolved in Tris-buffered saline (TBS; 20 mM Tris HCl, pH 7.4, 137 mM NaCl). Blots were then incubated with anti-Flag M2 antibody (10 μg/ml) for 2 h at 25 °C. Blots were washed three times with TBS containing 0.05% Tween-20 (TBS-T) for two minutes and then incubated with goat anti-mouse IgG-peroxidase-conjugated antibody (1:1000 dilution) in blocking solution overnight at 25 °C. Blots were washed three times for 15 min in TBS-T and once with TBS. Immunoreactive bands were detected with the ECL kit from Amersham.

Activation of ERK1 and ERK2 MAP kinases was assayed with a phospho-specific 42/44-kDa MAP kinase rabbit polyclonal antibody raised against a phosphotyrosine peptide corresponding to residues 196–209 of human ERK1. The antibody detects phosphorylation of ERK1 at tyrosine 204, which is required for ERK1 activation (35). The antibody also detects phosphorylation of the corresponding activating tyrosine of ERK2. Blots from 11% polyacrylamide gels were washed for 5 min with 25 ml of buffer B containing 58 mM NaH₂PO₄, 17 mM Na₂HPO₄, and 68 mM NaCl, pH 7.4. Membranes were blocked in buffer B containing 0.1% Tween 20 and 5% milk (blocking buffer) for 1 h. Blots were incubated overnight at 4 °C with 1 μg/ml of anti-phospho-MAP kinase antibody in buffer B containing 0.05% Tween 20 and 5% bovine serum albumin (incubation buffer). They were then washed three times for 5 min with 15 ml of blocking buffer and incubated with goat anti-rabbit alkaline phosphatase-conjugated antibody (1:2000 dilution) in incubation buffer for 1 h at 25 °C. Blots were finally washed three times with 15 ml of blocking buffer, and immunoreactive bands were detected using the Western-Light chemiluminescent detection system from Tropix, Inc. (Bedford, MA).

PKC Translocation—Cells (3–4 × 10⁶) were plated on 100-mm plastic tissue culture plates. After 48 h, cultures were rinsed at 37 °C twice with 10 ml of medium and incubated with or without 30 nM PMA for 2 min. Cells were rapidly rinsed twice at 4 °C with Ca²⁺- and Mg²⁺-free PBS and then were scraped into 1 ml of ice-cold buffer C containing 20 mM Tris HCl, pH 7.5, 2 mM EDTA, 10 mM EGTA, 40 μM leupeptin, 40 μM Nα-galactosidase, 20 μg/ml soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride. Cells were homogenized at 4 °C with 10 strokes of a Teflon-glass homogenizer. Sucrose was added to a final concentration of 250 mM, and the sample was homogenized with 10 additional strokes. An aliquot of 800 μl was centrifuged at 150,000 × g for 1 h, and the supernatant was frozen on dry ice. The pellet was dispersed in 800 μl of buffer C by sonication in a Branson Sonifier 450 for 2 s at a setting of six. Samples of supernatant and pellet suspension derived from 100 μg of crude homogenate were separated by SDS-polyacrylamide gel electrophoresis using 10% gels and analyzed for PKCδ and PKCe immunoreactivity by Western analysis as described previously (27).

Miscellaneous Procedures—Protein concentrations were measured by the Bradford method (36) using bovine IgG standards. Results are expressed as mean ± S.E. values, and differences between means were analyzed by ANOVA. Where p < 0.05, the significance of differences between means was evaluated by the Scheffe F-test or the Newman Keuls test.

RESULTS

Analysis of Transfected Cells—Stably transfected PC12 clones were tested for expression of the first variable domain of PKCe or PKCδ by RT-PCR (Fig. 1A) and Western analysis (Fig. 1B). Two clones, V1δ and V1δ2, expressing the V1 region of PKCe (δV1), and two clones, V1e and V1e2, expressing the V1 region of PKCe (εV1), were expanded for further studies.

To determine whether the δV1 and εV1 fragments expressed by our PC12 clones act as PKCe isozyme-selective inhibitors, we analyzed cDNA samples—

"FIG. 1. RT-PCR and Western analysis of transfected PC12 cells demonstrating expression of δV1 and εV1 fragments. PC12 cells were transfected with the parent vector pRc/RSV (C1), the vector pRcV-1 containing the cDNA sequence for δV1 (V1δ, V1δ2), or with pRcV-1 containing the cDNA sequence for εV1 (V1ε, V1ε2). A, 1.2% agarose gels of RT-PCR products showing expression of δV1 fragment mRNA in clones V1δ and V1δ2 and εV1 fragment mRNA in clones V1ε and V1ε2. No δV1 mRNA or εV1 mRNA (not shown) was detected in C1 cells. B, Western blots showing 17-kDa Flag immunoreactivity in clones V1δ and V1δ2 and in clones V1ε and V1ε2, co-migrating with purified Flag-tagged εV1 expressed in bacteria (εV1 fragment). The same molecular mass was detected for Flag-tagged δV1 purified from bacteria (not shown). Std, DNA size markers."

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fluorescence intensity of each growth cone and of the cytoplasm at the base of its neurite shaft. In PC12 (0.73 ± 0.02; n = 71) and C1 (0.76 ± 0.02; n = 55) cells, this ratio was significantly greater (p < 0.05; ANOVA and Scheffe F-test) than ratios measured in V1δ1 (0.49 ± 0.01; n = 83) and V1ε1 (0.50 ± 0.01; n = 71) cells. These results demonstrate that V1δ and V1ε fragments alter the localization of their corresponding PKC isozymes in cells undergoing NGF-induced differentiation. In addition, expression of V1ε is associated with an increase in nuclear size.

**Growth and Neurite Formation in Cell Lines Expressing V1δ or V1ε**—The growth rates of V1δ- and V1ε-expressing clones, parent PC12 cells, and C1 cells were similar before NGF treatment (data not shown). After culture in poly-l-ornithine-treated culture dishes with 50 ng/ml NGF for 4 days, the number of neurite-bearing cells and the length of neurites was similar in all cell lines (Tables I and II). However, in NGF-treated V1ε1 and V1ε2 cells, neither ethanol nor PMA (10 nM) increased neurite length or the percentage of cells that expressed neurites. In contrast, ethanol and PMA increased neurite length and the percentage of neurite-bearing cells in NGF-treated PC12, C1, V1δ1, and V1δ2 cultures. When cells were cultured on glass slides treated with poly-l-ornithine and coated with laminin, the results were qualitatively similar but more dramatic because NGF-induced neurite outgrowth was especially robust following treatment with ethanol or PMA in all but V1ε1 and V1ε2 cultures (Fig. 5, A and B). Therefore, expression of the V1ε fragment, but not of the V1δ fragment, appears to prevent enhancement of neurite extension by PMA or ethanol in NGF-treated cells.
FIG. 4. Indirect immunofluorescence of PKCε in V1-expressing cells. Parent PC12 cells (PC) and cells transfected with the empty pRc/RSV vector (C1) or pRcV1 (V1ε1 and V1ε2) were cultured for 4 days with 50 ng/ml NGF prior to fixation and immunostaining with antibody against PKCε. The arrows show prominent PKCε immunoreactivity in growth cones of PC12 and C1 cells and reduced immunoreactivity in growth cones of V1ε1 and V1ε2 cells. Bar, 30 μm.

**TABLE I**

Percentage of cells expressing neurites after treatment with NGF plus PMA or ethanol

| Cell line | NGF | NGF + PMA | NGF + ethanol |
|-----------|-----|-----------|---------------|
| PC12      | 38 ± 1 | 57 ± 1* | 60 ± 2*        |
| C1        | 38 ± 1 | 56 ± 2*  | 60 ± 2*        |
| V1ε1      | 36 ± 1 | 56 ± 2*  | 62 ± 2*        |
| V1ε2      | 40 ± 1 | 59 ± 1*  | 62 ± 1*        |
| V1ε1      | 32 ± 2 | 30 ± 1   | 33 ± 3         |
| V1ε2      | 40 ± 1 | 40 ± 1   | 43 ± 1         |

* Significantly different compared with treatment with NGF alone (ANOVA and Scheffe F-test).

**TABLE II**

Neurite length after treatment with NGF plus PMA or ethanol

| Cell line | NGF | NGF + PMA | NGF + ethanol |
|-----------|-----|-----------|---------------|
| PC12      | 24.7 ± 1.5 | 47.6 ± 2.9* | 46.4 ± 2.8* |
| C1        | 23.6 ± 1.8  | 42.6 ± 2.4*  | 43.8 ± 3.2*  |
| V1ε1      | 24.2 ± 1.6  | 39.3 ± 2.4*  | 46.5 ± 2.4*  |
| V1ε2      | 24.2 ± 1.6  | 44.8 ± 2.1*  | 46.8 ± 2.9*  |
| V1ε1      | 21.7 ± 1.4  | 27.4 ± 1.7   | 22.5 ± 1.4   |
| V1ε2      | 21.1 ± 1.7  | 29.0 ± 1.9   | 27.9 ± 1.9   |

* Significantly different compared with cells treated with NGF alone (ANOVA and Scheffe F-test).

**MAP Kinase Phosphorylation in PKCε-transfected Cells**

NGF and basic fibroblast growth factor stimulate sustained activation of ERK1 and ERK2 MAP kinases in PC12 cells, which is important for their neuronal differentiation (37). Previous work has shown that treatment with either PMA or ethanol increases NGF-induced phosphorylation and activation of ERK1 and ERK2 (24). Since overexpression of PKCε also enhances NGF-induced MAP kinase phosphorylation (27), we examined whether expression of εV1 would prevent enhancement of MAP kinase activation by PMA or ethanol.

ERK1 and ERK2 are activated by dual phosphorylation on neighboring tyrosine and threonine residues (35). We measured activation of ERK1 and ERK2 by Western analysis using an anti-phospho-MAP kinase antibody that specifically detects phosphorylation of the activating tyrosine of each enzyme. As described previously in PC12 cells (24), NGF stimulated phosphorylation of ERK1 and ERK2 in C1 cells with a biphasic time course (Fig. 6A). Phosphorylation was maximal after 5–10 min (peak phase) and then declined to a lower level (plateau phase) that was maintained for at least 2 h. A similar pattern of phosphorylation was observed in V1ε1, V1ε2, V1ε1, and V1ε2 cells (Fig. 6). As previously observed in PC12 cells (24), co-treatment with 10 nm PMA or pretreatment with 100 μM ethanol for 6 days increased NGF-induced ERK phosphorylation. This was particularly evident during the plateau phase of ERK phosphorylation, which was elevated to levels achieved during the peak phase in C1 and V1ε2 cells (Fig. 6A). A similar increase in phosphorylation was also observed in V1ε1 cells (Fig. 6, B and C). In contrast, the plateau phase of ERK phosphorylation was not increased in V1ε1 or V1ε2 cells treated with ethanol or PMA (Fig. 6, A–C). Therefore, expression of the εV1 fragment specifically inhibits PMA- or ethanol-induced ERK phosphorylation in NGF-treated cells.

**DISCUSSION**

The current results identify PKCε as the PKC isozyme responsible for enhancement of NGF responses by phorbol esters and ethanol in PC12 cells. We previously found that phorbol esters enhance NGF-induced neurite outgrowth and MAP kinase activation in PC12 cells (11, 24). Ethanol also increases these responses to NGF by a PKC-dependent mechanism (11, 24). Recently, we found that overexpression of PKCε also enhances responses to NGF (27), suggesting that PKCε mediates the neurite-promoting effect of PMA and ethanol. In this paper, we investigated this issue directly, by creating PC12 cell lines that express peptides encoding V1 domains of PKCζ or PKCε, which act as isozyme-selective translocation inhibitors (33). We found that expression of the εV1 fragment selectively inhibited PMA-induced translocation of PKCε, whereas expression of the δV1 fragment specifically inhibited translocation of PKCδ. Cells expressing these peptides showed no alterations in cell growth. However, expression of εV1 prevented enhancement of NGF-induced MAP kinase activation and neurite growth by PMA or ethanol. In contrast, expression of δV1 did not alter enhancement of NGF responses by these agents. These findings indicate that PKCε mediates enhancement of neurite outgrowth and MAP kinase activation by PMA or ethanol in NGF-treated PC12 cells.

Our data are consistent with a recent study (33) in which a δV1 fragment, an εV1 fragment, and an εV1-derived peptide were introduced into cardiac myocytes by transient permeabilization. These fragments selectively inhibited PMA-induced...
translocation of their corresponding PKC isozyme and not translocation of other isozymes concomitantly activated in these cells. Furthermore, the εV1 fragment and the short peptide derived from it inhibited phorbol ester- or hormone-induced regulation of contraction rate, whereas the δV1 fragment or translocation inhibitors of PKCb did not. Together with the data presented here, these studies reinforce the concept that translocation of PKC is required for its function (28) and indicate that isozyme-selective inhibitors of PKC translocation can be used to determine the function of individual isozymes in a variety of cells.

The ability of the PKCb and -ε fragments to act as isozyme-selective translocation inhibitors is also consistent with the hypothesis that each contains a binding site for a corresponding, isozyme-specific RACK (28). This hypothesis is further supported by the finding of structural homology between εV1 (32) and the C2 domain of conventional PKCs, which contains a RACK1 binding site (29). Indeed, an PKCe-specific RACK, RACK2, that has recently been cloned, binds the εV1 fragment in vitro.2

Prominent PKCe immunoreactivity was found in neurites and growth cones of NGF-treated parent PC12 and C1 cells. This was reduced in processes of cells expressing εV1, suggesting that εV1 displaces endogenous PKCe from binding sites in neurites and growth cones. Localization of PKCe to growth cones is consistent with a role for this isozyme in regulating neurite outgrowth (38). Immunoprecipitation-kinase assays indicate that NGF activates PKCe in PC12 cells (21), suggesting that NGF-induced localization of PKCe to neurites and growth cones may involve activation of this isozyme. This suggests that an PKCe-specific RACK may reside in growth cones and neurites.

In PC12 and C1 control cells, PKCb immunoreactivity was most prominent asymmetrically next to the nucleus. Expression of δV1 was associated with redistribution of PKCb immunoreactivity to the perinuclear region and with an increase in

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PKCe Inhibitor and NGF

FIG. 6. The eV1 fragment prevents enhancement of NGF-stimulated MAP kinase phosphorylation by PMA or ethanol. PC12 cells transfected with the empty pcR/RSV vector (C1), pR6V-1 (V181), or pR6V-1 (V1e1) were treated with 50 ng/ml of NGF alone (NGF) or with NGF and 10 mM PMA (NGF + PMA). Some cells were pretreated with 100 mM ethanol for 6 days prior to the addition of NGF in the continued presence of ethanol (NGF + EtOH). Tyrosine phosphorylation of ERK1 and ERK2 was detected by Western analysis of cell lysates using an anti-phospho-MAP kinase antibody. A, Western blots showing the time course of ERK1 (upper band) and ERK2 (lower band) tyrosine phosphorylation in clones C1, V182, and V1e1. Similar results were observed in clones V181 and V1e2 and in parent PC12 cells (data not shown). B and C, quantitation of ERK1 (B) and ERK2 (C) phosphorylation in C1 control cells and in V1 fragment-transfected cells after 60 min of treatment with 50 ng/ml NGF (open bars), NGF and PMA (black bars), or NGF and ethanol (gray bars). Data are expressed as a percentage of maximal phospho-ERK1 or phospho-ERK2 immunoreactivity observed after 5–10 min of NGF treatment and are from 2–4 experiments. Immunoreactivity was significantly increased by treatment with PMA or ethanol only in C1, V181, and V182 cells but not in V1e1 or V1e2 cells (ANOVA, Scheffe F-test).

nuclear size. It is difficult to speculate on the physiologic significance of these changes, since the function of PKC ε in these cells is not yet known. However, the findings clearly indicate that eV1 alters the localization of PKC ε and produces a unique change in cell morphology.

NGF-induced activation of ERK1 and ERK2 involves phosphorylation and binding of phosphatase C ε and the adapter protein Shc to the NGF receptor tyrosine kinase TrkA (39). Within minutes of NGF binding, phosphorylated Shc also forms a complex with another adapter protein, Grb2, and with the guanine nucleotide exchange factor mSOS (40), leading to activation of Ras (41) and sequential activation of B-Raf (42), MAP kinase kinase-1 (43, 44), and ERK1 and ERK2 (45). This pathway appears essential for NGF-induced neurite outgrowth, since dominant negative inhibitors of Ras (46) or MAP kinase kinase-1 (47) block NGF-induced neurite outgrowth in PC12 cells. PKCe could enhance ERK activation by increasing the activity of members of this pathway. For example, PKCe appears to phosphorylate and activate Raf-1 (48), suggesting that other PKC isozymes, such as PKCe, might also modulate Raf kinases. In addition, NGF stimulates binding of Shc to F-actin in PC12 cells (49). NGF also activates PKCe (51), and upon activation, PKCe binds F-actin in nerve terminals (50). This raises the intriguing possibility that Shc or proteins complexed with Shc interact with PKCe when both Shc and PKCe are anchored to actin. We do not yet know if the eV1 fragment prevents binding of PKCe to actin, since actin binds to PKCe at a site between the first and second cysteine-rich regions of the C1 domain, which is outside of the eV1 domain (50). Whether binding of PKCe to actin is important for regulation of MAP kinases and neurite outgrowth also remains to be determined.

The major effect of phorbol esters or overexpressed PKCe is to increase the late plateau phase of MAP kinase activation rather than the initial peak phase (24, 27). These kinetics suggest that PKCe may act by inhibiting dephosphorylation of ERK1 and ERK2 rather than by promoting ERK activation. MAP kinase phosphatase (MKP)-1, MKP-2, and hVH3 are dual specificity protein phosphatases that are expressed in the brain and dephosphorylate and inactivate ERKs (51, 52). MKP-1 and MKP-2 mRNAs are constitutively expressed at low levels in PC12 cells and are increased by NGF (51). Inhibition of MKP-1 expression in PC12 cells does not accelerate the early phase of MAP kinase inactivation following stimulation with growth factors (53). Instead, protein phosphatase 2A and an unidentified protein-tyrosine phosphatase appear to mediate the rapid phase of inactivation in these cells (54). The role of dual specificity phosphatases in regulating the later plateau phase of NGF-induced MAP kinase activation is not known, but NGF induction of mRNA for MKP-1 and MKP-2 peaks at 1–2 h (51), suggesting that they may regulate this phase. If this is the case, then inhibition of these phosphatases by PKCe may account for enhanced activation of ERK1 and ERK2.

ERK activity is also regulated by negative feedback inhibition. Phosphorylation of mSOS by MAP kinase promotes dissociation of mSOS from tyrosine-phosphorylated Shc and epidermal growth factor receptors (55, 56). In addition, MAP kinase kinase-dependent phosphorylation of mSOS has been reported to cause dissociation of mSOS-Grb2 complexes, interrupting mSOS activation of Ras (57). Moreover, ERK1 phosphorylates MAP kinase kinase, and this phosphorylation ap-
pears to reduce MAP kinase kinase activity (58). Therefore, inhibition of mSOS or MAP kinase retrophosphorylation may be mechanisms by which PKCe could enhance MAP kinase activation.

Together with studies in primary neurons (20, 22, 59), our findings suggest that PKCe modulates neural differentiation. This may be a mechanism for enhancement of neurite growth by neurotransmitters that activate receptors coupled to phospholipase C and could be important for activity-dependent remodeling of synapses during normal development (60). Our studies also suggest that excessive activation of PKCe may contribute to abnormal neurite growth observed in certain disease states. Chronic abuse of ethanol can damage the nervous system by disrupting the growth and remodeling of dendrites and axons. In certain brain regions, ethanol increases the growth of neural processes and terminals (61–65). This is particularly striking in the hippocampus, where expression of PKCe is high (19). There prenatal exposure to ethanol causes marked overgrowth of dentate granule cell axons (mossy fibers) into the stratum pyramidale and stratum oriens of CA3, which may contribute to cognitive dysfunction (65). In addition, abnormal growth of mossy fibers into the supragranular layer of the dentate gyrus is found in some humans with temporal lobe epilepsy and in animals following stimuli that induce epilepsy (66). Future studies with transgenic and PKCe mutant mice or with eV1-derived inhibitory peptides may allow us to examine the role of PKCe in normal development, alcohol-related neurologic disorders, and epileptogenesis.

REFERENCES

1. Selbie, L. A., Schmitz-Peiffer, C., Sheng, Y., and Biden, T. J. (1993) J. Biol. Chem. 268, 24296–24302
2. Nishizuka, Y. (1992) Science 255, 607–614
3. Johannes, F.-J., Prestle, J., Eis, S., Oberhagemann, P., and Pfizenmaier, K. (1988) Nature 334, 618–620
4. Valverde, A. M., Sinnett-Smith, J., Van Lint, J., and Rozengurt, E. (1994) Mol. Cell. Biol. 14, 267–274
5. Roivainen, R., Hundle, B., and Messing, R. O. (1994) in Toward a Molecular Basis of Alcohol Use and Abuse (Janson, B., Jovall, H., Rydberg, U., Ferenius, L., and Valve, B. L., eds) pp. 29–38, Birkhäuser Verlag, Basel
6. Hundle, B., McMahon, T., Dodgier, J., and Messing, R. O. (1995) J. Biol. Chem. 270, 3053–3059
7. Mochly-Rosen, D. (1995) Science 268, 247–251
8. Roivainen, R., McMahon, T., and Messing, R. O. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 839–843
9. Ron, D., Luo, J., and Mochly-Rosen, D. (1996) J. Biol. Chem. 271, 24180–24187
10. Zhang, X.-H., El-Sherif, N., Ron, D., Mochly-Rosen, D., and Bourtjoglou, M. (1996) J. Biol. Chem. 271, 4328–4331
11. Roivainen, R., Hundle, B., and Messing, R. O. (1994) in Toward a Molecular Basis of Alcohol Use and Abuse (Janson, B., Jovall, H., Rydberg, U., Ferenius, L., and Valve, B. L., eds) pp. 29–38, Birkhäuser Verlag, Basel
12. Okada, M., Gershon, E. M., and Nishizuka, Y. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 725–730
13. Berra, E., Diaz-Meco, M. T., Dominguez, I., Municio, M. M., Sanz, L., Lozano, A., and Martinez, J. (1993) FEBS Lett. 335, 289–293
14. Basu, T., Warne, P. H., and Downward, J. (1994) Oncogene 9, 3483–3491
15. Nakafuku, M., Saito, T., and Kaziro, Y. (1992) J. Biol. Chem. 267, 19448–19454
16. Jaiswal, R. K., Moodie, S. A., Wolfman, A., and Landreth, G. E. (1994) Mol. Cell Biol. 14, 694–6952
17. Powell, C. T., Leng, L., Dong, L., Kiyokawa, H., Busquets, X., O'Rourke, C., Marks, P. A., and Rifkind, R. A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 147–151
18. Otte, A. P., and Moon, R. T. (1992) Cell 68, 1021–1029
19. Saito, N., Itani, A., Totani, Y., Otsawa, I., Koide, H., Fujisawa, N., Ogita, K., and Tanaka, C. (1993) Brain Res. 607, 241–248
20. Heidenreich, K. A., Toled, S. P., Brunton, L. L., Watson, M. J., Daniel-Izzakian, S., and Struclovici, B. (1990) J. Biol. Chem. 265, 15076–15082
21. Ohmichi, M., Zhu, G., and Saltiel, A. R. (1993) Biochem. J. 295, 767–772
22. Mangoura, D., Sogus, V., and Dawson, G. (1993) J. Neurosci. Res. 35, 488–498
23. Greene, L. A., Sobel, M. M., and Teng, K. K. (1991) in Culturing Nerve Cells (McKerracher, L., and Gold, G., eds) pp. 207–226, MIT Press, Cambridge, MA
24. Roivainen, R., Hundle, B., and Messing, R. O. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1891–1895
25. Messing, R. O., Petersen, P. J., and Henrich, C. J. (1991) J. Biol. Chem. 266, 23428–23432
26. Roivainen, R., Hundle, B., and Messing, R. O. (1994) in Toward a Molecular Basis of Alcohol Use and Abuse (Janson, B., Jovall, H., Rydberg, U., Ferenius, L., and Valve, B. L., eds) pp. 29–38, Birkhäuser Verlag, Basel
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