PKCε, Via its Regulatory Domain and Independently of its Catalytic Domain, Induces Neurite-like Processes in Neuroblastoma Cells

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Abstract. To investigate the role of protein kinase C (PKC) isoforms in regulation of neurite outgrowth, PKCα, βII, δ, and ε fused to enhanced green fluorescent protein (EGFP) were transiently overexpressed in neuroblastoma cells. Overexpression of PKCε-EGFP induced cell processes whereas the other isoforms did not. The effect of PKCε-EGFP was not suppressed by the PKC inhibitor GF109203X. Instead, process formation was more pronounced when the regulatory domain was introduced. Overexpression of various fragments from PKCε regulatory domain revealed that a region encompassing the pseudosubstrate, the two C1 domains, and parts of the V3 region were necessary and sufficient for induction of processes. By deleting the second C1 domain from this construct, a dominant-negative protein was generated which suppressed processes induced by full-length PKCε and neurites induced during retinoic acid- and growth factor-induced differentiation. As with neurites in differentiated neuroblastoma cells, processes induced by the PKCε-PSC1V3 protein contained α-tubulin, neurofilament-160, and F-actin, but the PKCε-PSC1V3-induced processes lacked the synaptic markers synaptophysin and neuropeptide Y. These data suggest that PKCε, through its regulatory domain, can induce immature neurite-like processes via a mechanism that appears to be of importance for neurite outgrowth during neuronal differentiation.

Key words: C1 domains • neuroblastoma cells • neuronal differentiation • neurite outgrowth • protein kinase C

The regulation of neurite outgrowth during neuronal differentiation is complex and likely to involve multiple signal transduction components. One group of enzymes that has been suggested to be involved in this process is the protein kinase C (PKC) ε isoform family. Several PKC isoforms have been shown to be present in growing axons both in vivo and in vitro (Ide, 1996). There is also experimental evidence for a function of PKCε in the regulation of neurite outgrowth in neuronal differentiation model systems, such as PC12 cells (Hundle et al., 1995, 1997) and neuroblastoma cells (Parrow et al., 1992, 1995; Fagerstrom et al., 1996).

PKCε comprises a family of serine/threonine protein kinases, consisting of at least 11 different isoforms, divided into subgroups depending on structural similarities and requirements for activators. The classical PKC isoforms (α, βI, βII, δ, and ε) are Ca2+-dependent and activated by diacylglycerol and phorbol esters. Novel PKC isoforms (γ, η, and θ) are activated by diacylglycerol and phorbol esters, but are Ca2+-independent. The atypical PKC isoforms ε and δ are insensitive to diacylglycerol and phorbol ester and are also Ca2+-independent. Finally, PKCη is structurally unique, but is activated by phorbol esters (Nishizuka, 1992; Newton, 1995; Liu, 1996).

The PKC molecule consists of one NH2-terminal regulatory domain (RD) and one COOH-terminal catalytic domain. In the resting state the enzyme is kept inactive by a pseudosubstrate motif in the RD bound to the catalytic site. To become active this locked conformation has to be changed and this is assumed to be caused by the binding of activators to the RD (Newton, 1997). The RD from classical and novel PKC isoforms contains two classes of domains, C1 and C2, which are targets for PKC activators. Diacylglycerol and phorbol ester bind C1 domains and in classical isoforms the C2 domain binds Ca2+. On the other hand, the C2 domain in novel isoforms does not bind Ca2+, putatively explaining the Ca2+-independence of these iso-
forms (Stabel and Parker, 1991; Nishizuka, 1992; Ponting and Parker, 1996).

Besides being the target for PKC activators, the RD also has been shown to be responsible for protein–protein interaction, which may direct each isoform to unique intracellular sites. RACKs (receptors for activated C-kinase) constitute one class of PKC-binding proteins that interact with activated PKC and this binding is to a large extent mediated via the C2 domain (Mochly-Rosen and Gordon, 1998). RACKs have been identified for PKCβ (Ron et al., 1994) and ε (Csukai et al., 1997), and overexpression of either the entire C2 domain or peptides derived thereof has been shown to block isoform-specific translocation and/or activation of individual isoforms (Ron et al., 1995; Johnson et al., 1996; Hundle et al., 1997). There are also several reports demonstrating protein interaction sites in the region comprising the two C1 domains (Prekeris et al., 1996; Mato-Yelin et al., 1997; Y ao et al., 1997), suggesting that depending on the interaction partner, different PKC domains may be of importance. Since there is little evidence for a substrate of PKC that is preferentially phosphorylated by one or several isoforms, but not by others, at least some of the isoform-specific effects observed have been attributed to the fact that different isoforms will localize to different intracellular sites. Due to these assumptions, and since RDs in several cases determine interaction partners and subcellular localization sites, RDs have been considered to be acting isoform specifically in a dominant-negative manner (Jaken, 1996). Studies have shown specific effects of overexpression of RDs from individual isoforms (Liao et al., 1994; Cai et al., 1997). However, there are also reports demonstrating that the effect of the full-length PKC can be mimicked by parts of, or the entire, RD indicating that some PKC effects may actually be mediated via this domain (Lehel et al., 1995a; Singer et al., 1996). Furthermore, studies with chimeras consisting of PKC molecules with the regulatory and catalytic domain derived from different isoforms have shown that isoform specificity may be mediated via either domain (Acs et al., 1997; Wang et al., 1998). Thus, to understand the molecular mechanisms for a PKC effect there is a need to identify which isoforms exert the effect and which domain(s) is/are involved in mediating it.

Neuroblastoma cell lines have been used extensively as in vitro model systems to study mechanisms regulating neuronal differentiation. In this study, two neuroblastoma cell lines, SH-SY5Y and SK-N-BE (2), were used. Both of these cell lines can be induced to differentiate with a plethora of factors (Pålman et al., 1981; Melino et al., 1993; Lavenius et al., 1994, 1995; Rossino et al., 1995). In several of these differentiation protocols there is evidence for the involvement of PKC. In particular, neurite outgrowth appears to involve PKC, and a number of PKC isoforms are present in growth cones of the differentiating cells (Parr et al., 1995; Fagerström et al., 1996). Experiments with high phorbol ester concentrations, which cause selective down regulation of PKC isoforms, have suggested that novel isoforms may be of importance in neurite outgrowth (Fagerström et al., 1996).

The aim of this study was to investigate whether increased levels of a particular PKC isoform would be sufficient to induce growth of neurites in neuroblastoma cells. To accomplish this, cDNA coding for the classical and novel PKC isoforms that are consistently expressed in neuroblastoma cell lines and tumor specimens, PKCα, βI, βII, and ε (Zeidman et al., 1999), was introduced into an expression vector and human neuroblastoma cells were transfected with these plasmids. To identify cells overexpressing the proteins, the COOH-terminal ends of the isoforms were fused to enhanced green fluorescent protein (EGFP). The results demonstrate that PKCε is the only isoform that can induce processes in neuroblastoma cells and that this effect is independent of the catalytic activity of the enzyme. By making a series of constructs expressing isolated domains of PKCε, this study demonstrates that the effect is mediated by a region from PKCε encompassing the pseudosubstrate, the two C1 domains, and parts of the V3 region. The data also indicate that this effect is of importance for neurite outgrowth during neuronal differentiation.

**Materials and Methods**

**Plasmids**

cDNA coding for full-length human PKC isoforms α, βI, βII, and ε, the RD from PKCα, βI, βII, ε, η, and ζ; or smaller fragments of PKCε were generated by PCR with introduction of appropriate restriction enzyme sites in the primers. The DNA fragments were introduced into the pEGFP-N1 vector (Clontech Laboratories, Inc.), thereby fusing the PKC cDNA with EGFP cDNA. The schematic structures of the protein products coded for by the different expression vectors are shown in Fig. 1A. Templates for the PCR reactions were for PKCα, ε, and ζ cDNA from SH-SY5Y cells; for PKCβI ATCC plasmid 80047 (Hoevear et al., 1993); for PKCβII ATCC plasmid 80049 (Aris et al., 1993); and for PKCε cDNA generated from human placenta mRNA (Clontech Laboratories, Inc.). The PKCε plasmids pPSC1aV3E and pPSC1bV3E (pPSC1V3E with DNA coding for either the second or the first C1 domain deleted) were generated with prim-

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**Figure 1.** PKC-EGFP fusion constructs used in this study. (A) List of PKC constructs produced for this study. The left column shows schematic composition of PKCε fragments coded for by the expression vectors used in this study: PS (pseudosubstrate), C1, C2, and V3 domains. The primers used for amplification of the cDNA fragments are listed in Table I. The predicted molecular weights of the fusion proteins are included. (B–D) COS cells were transfected with the plasmids listed in A and the formation of the protein products were analyzed with Western blot technique. (B) Analysis of full-length PKC isoforms α, βI, βII, and ε fused to EGFP (+) or expressed without any tags (−). Immunoblots were performed with isoform-specific antibodies. A rows indicate reactivity corresponding to PKC-EGFP fusion proteins and arrowheads point to the untagged PKC isoforms. The presence of full-length PKCα, βI, and βII and ε immunoreactivity in cell lysates from cells overexpressing EGFP fusions indicates endogenous levels of respective isoform in COS cells. The positions of two weight markers, 97 and 66 kD, are included to the left of the blot. (C) Analysis of RDs from PKCα, βI, βII, ε, η, and ζ fused to EGFP. Cell lysates from COS cells transfected with respective expression vectors were analyzed with immunoblot using an anti-GFP antibody as primary antibody. The positions of three weight markers, 97, 66, and 46 kD, are included to the left of the blot. (D) Analysis of PKCε subdomains fused to EGFP. Cell lysates from COS cells transfected with respective expression vectors were ana-

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A

Constructs with classical isoforms (PKCα and βII)

| Plasmid | Protein | PKC fragment | Primers | MW |
|---------|---------|--------------|---------|----|
| αFL    | αFL    | PKCα aa 1-671 | AFI/AR1 | 77 |
| βIIFL  | βIIFL  | PKCβIIa 1-677 | B2F1/B2R1 | 78 |
| αFLE   | αFLE   | PKCα aa 1-671 | AFI/AR2 | 106 |
| βIIFLE | βIIFLE | PKCβIIa 1-677 | B2F1/B2R2 | 106 |
| αRDE   | αRDE   | PKCα aa 1-312 | AFI/AR3 | 64 |
| βRDE   | βRDE   | PKCβIIa 1-312 | B2F1/B2R3 | 65 |

Constructs with novel isoforms (PKCζ, η, ι, and δ)

| Plasmid | Protein | PKC fragment | Primers | MW |
|---------|---------|--------------|---------|----|
| δFL    | δFL    | PKCδ aa 1-737 | EF1/ER1 | 84 |
| εFL    | εFL    | PKCε aa 1-737 | EF1/ER2 | 113 |
| δFLE   | δFLE   | PKCδ aa 1-737 | EF1/ER2 | 107 |
| εFLE   | εFLE   | PKCε aa 1-737 | EF1/ER2 | 107 |
| δRDE   | δRDE   | PKCδ aa 1-346 | EF1/ER1 | 69 |
| εRDE   | εRDE   | PKCε aa 1-346 | EF1/ER1 | 69 |
| γRDE   | γRDE   | PKCζ aa 1-342 | EF1/ER1 | 69 |
| δRDE   | δRDE   | PKCζ aa 1-342 | EF1/ER1 | 69 |
| c2PSC1E | c2PSC1E | PKCζ aa 1-298 | EF1/ER4 | 63 |
| c2PSE  | c2PSE  | PKCζ aa 1-298 | EF1/ER5 | 60 |
| c2E    | c2E    | PKCζ aa 1-146 | EF1/ER6 | 45 |
| cPSE   | cPSE   | PKCζ aa 136-178 | EF2/ER5 | 35 |
| cPSC1eE | cPSC1eE | PKCζ aa 156-232 | EF2/ER7 | 41 |
| cPSC1E  | cPSC1E  | PKCζ aa 136-298 | EF2/ER4 | 48 |
| cPSC1V3E | cPSC1V3E | PKCζ aa 136-373 | EF2/ER3 | 51 |
| c1V3E  | c1V3E  | PKCζ aa 136-373 | EF3/ER3 | 51 |
| c1E    | c1E    | PKCζ aa 136-373 | EF3/ER3 | 51 |
| cPSC1V3E | cPSC1V3E | PKCζ aa 136-373 | EF5/ER9 | 50 |
| (del 248-269) |
| cPSC1V3E | cPSC1V3E | PKCζ aa 136-373 | EF5/ER9 | 50 |

B

C

lyzed with immunoblot using an anti-GFP antibody as primary antibody. The positions of three weight markers, 66, 46, and 30 kD, are included to the left of the blots. U denotes an unspecific band at ~55 kD, which appeared in some immunoblots in C and D, when using the GFP antibody on COS cell lysates.
ers designed to amplify the entire pSC11V3E plasmid, excluding the DNA coding for the domain that should be deleted. A M13I labeled primer (Table I) was introduced in each primer, the PCR product was cleaved with MluI, and ligated. Table I lists the primers used to generate the PKC fragments. All PCR reactions were performed with Fu polymerase (Stratagene) to mini-mize introduction of mutations and all PCR-generated fragments used in this study were sequenced. The generation of the protein products of anticipated sizes were confirmed by transfecting the expression vectors into COS cells with the calcium phosphate method (Sambrook et al., 1989) and subjecting the cell lysate to Western blot analysis (Fig. 1, A–D). In addition, the Neo/SalI fragments from the plasmid, excluding the DNA (full-length PKC) were inserted into the CMS-EGFP vector (Clontech Laboratories, Inc.) to obtain expression of PKC and EGFP as two separate proteins.

**Western Blot Analysis**

COS cells were transfected with different expression vectors, washed with PBS, and lysed in buffer (10 mM Tris, pH 7.2, 160 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 1 mM EGTA, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged for 10 min at 15,000 g and 25 μg of protein was electrophoretically separated on an SDS polyacrylamide gel and thereafter transferred to Hybond-C extra nitrocellulose membranes. The transfer was done between all steps. The membranes were blocked with 3% BSA in PBS and incubated with monoclonal mouse anti–NF-160 (Sigma Chemical Co.) diluted to 1:300 for NF-160 detection; or polyclonal rabbit anti–mouse IgG-TRITC [Jackson ImmunoResearch Laboratories, Inc.] diluted to 1:300 for NPY staining; or monoclonal rat anti–NPY [Biogenesis] diluted to 1:40, respectively. After a 1-h incubation, the membranes were washed with PBS, and incubated with a goat anti-rabbit IgG-TRITC [Jackson ImmunoResearch Laboratories, Inc.] diluted to 1:300 for NPY and 1:20 for synaptophysin detection; or donkey anti–rabbit IgG-TRITC [Jackson ImmunoResearch Laboratories, Inc.] diluted to 1:300 for NPY staining) was incubated for 1 h in blocking/permeabilization solution. The secondary antibody (rabbit anti–mouse IgG-TRITC [Jackson ImmunoResearch Laboratories, Inc.] diluted to 1:100 for α-tubulin and 1:20 for synaptophysin detection; or donkey anti–rabbit IgG-TRITC [Jackson ImmunoResearch Laboratories, Inc.] diluted to 1:300 for NPY staining) was incubated for 1 h in blocking solution. EGF staining with PBS and blocking/permeabilization solution was done between all steps. For detection of neurofilament-160 (NF-160), cells were blocked for 30 min with 3% BSA in PBS and incubation with monoclonal mouse anti–α-tubulin (Sigma Chemical Co.) diluted to 1:2,000; monoclonal mouse anti-synaptophysin [clone 38, DakoPatts] diluted to 1:10; or polyclonal rabbit anti-NPY (Biogenesis) diluted to 1:40, respectively. For staining of F-actin, cells were fixed with 4% paraformaldehyde in PBS. The primary antibody (monoclonal mouse anti–α-tubulin [Sigma Chemical Co.]) diluted to 1:2,000; monoclonal mouse anti-synaptophysin [clone 38, DakoPatts] diluted to 1:10; or polyclonal rabbit anti–NPY [Biogenesis] diluted to 1:40, respectively) was incubated for 1 h in blocking/permeabilization solution. The secondary antibody (donkey anti–mouse IgG-TRITC [Jackson ImmunoResearch Laboratories, Inc.] diluted to 1:100 for α-tubulin and 1:20 for synaptophysin detection; or donkey anti–rabbit IgG-TRITC [Jackson ImmunoResearch Laboratories, Inc.] diluted to 1:300 for NPY staining) was incubated for 1 h in blocking solution. EGF staining with PBS and blocking/permeabilization solution was done between all steps. For detection of neurofilament-160 (NF-160), cells were blocked for 30 min with 3% BSA in PBS and incubation with monoclonal mouse anti–α-tubulin (Santa Cruz) and detected with an HRP-labeled secondary antibody using the SuperSignal system (Pierce Chemical Co.) as substrate. The chemiluminescence was detected with a CCD camera (Fuji Photo Film Co.).

**Cell Culture**

Human neuroblastoma SH-SY5Y, SH-SY5Y/TrkA, and SK-N-BE(2) cells were maintained in MEM supplemented with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin (GIBCO BRL). For transfection experiments, SH-SY5Y and SH-SY5Y/TrkA cells were trypsinized and seeded at a density of 350,000 cells/35-mm cell culture dish on glass cover slips in serum free medium. A five 20 min the medium was changed to medium containing serum and antibiotics, and incubated for 24 h before start of the transfections. SK-N-BE(2) cells were seeded on glass coverslips in regular growth medium (300,000 cells per dish) and transfections were initiated 24 h after seeding. In experiments where cells were treated with 12-0-tetradecanoylphorbol-13-acetate (TPA; Sigma Chemical Co.) for 4 d, or with growth factors for 40 h, the density at cell seeding was 250,000 cells/35-mm dish.

SH-SY5Y cells were transfected using 3.5 μg Lipofectin (GIBCO BRL) and 1.8 μg of DNA/ml serum free medium and SK-N-BE(2) cells were transfected with 4 μg Lipofectamine (GIBCO BRL) and 2 μg DNA, respectively, according to the supplier’s protocol.

For differentiation studies, SH-SY5Y/TrkA cells were treated for 40 h with 100 ng/ml NGF (Promega Corp.), and SK-N-BE(2) cells with 10 μM retinoic acid (RA; Sigma Chemical Co.) or 25 ng/ml ciliary neurotrophic factor (CNTF; Promega Corp.).

**Morphology Studies**

16 h after the end of transfections (unless otherwise stated) cells were fixed in 4% paraformaldehyde in PBS for 4 min, mounted on microscopy slides using a PVA-DABCO solution (9.6% polyvinyl alcohol, 24% glycerol, and 2.5% 1,4-diazabicyclo[2.2.2]octane in 67 mM Tris-HCl, pH 8.0), and used for morphological studies. Digital images were captured with a Sony D.K.C 5000 camera system. The transfected cells were considered to have long processes if the length of the process exceeded that of two cell bodies. A total of 200 transfected cells per experiment were counted.

**Confocal Microscopy**

Cells were transfected, fixed, and mounted as for morphology studies. Cells expressing various PKC–EGFP constructs and Texas red–phalloidin-stained F-actin were examined using a Bio-Rad MRC 1024 confocal system fitted with a Nikon Diaphot 300 microscope using a Nikon plan-apo 60 × 1.2 NA water immersion lens.

**Immunofluorescence and Staining of F-Actin**

Cells grown on glass coverslips were fixed with 4% paraformaldehyde as above. For detection of α-tubulin, synaptophysin, and neuropeptide Y (NPY), cells were permeabilized and blocked with 1% BSA/0.02% saponin in PBS. The primary antibody (monoclonal mouse anti–α-tubulin [Sigma Chemical Co.]) diluted to 1:2,000; monoclonal mouse anti-synaptophysin [clone 38, DakoPatts] diluted to 1:10; or polyclonal rabbit anti–NPY [Biogenesis] diluted to 1:40, respectively) was incubated for 1 h in blocking/permeabilization solution. The secondary antibody (donkey anti–mouse IgG-TRITC [Jackson ImmunoResearch Laboratories, Inc.] diluted to 1:100 for α-tubulin and 1:20 for synaptophysin detection; or donkey anti–rabbit IgG-TRITC [Jackson ImmunoResearch Laboratories, Inc.] diluted to 1:300 for NPY staining) was incubated for 1 h in blocking solution. EGF staining with PBS and blocking/permeabilization solution was done between all steps. For detection of neurofilament-160 (NF-160), cells were blocked for 30 min with 3% BSA in PBS and incubation with monoclonal mouse anti–NF-160 (Sigma Chemical Co.) diluted to 1:50 was performed for 3 h. Secondary antibody donkey anti–mouse IgG-TRITC [Jackson ImmunoResearch Laboratories, Inc.] diluted to 1:300 and incubated for 1 h after extensive washing with PBS. For staining of F-actin, cells were fixed with 4% paraformaldehyde. Cells were treated for 5 min with 0.1% Triton X-100 in PBS and incubated for 10 min with 2 μg/ml TRITC–conjugated phalloidin (Sigma Chemical Co.) in PBS. For confocal studies, fixed cells were blocked and permeabilized with 5% donkey serum and 0.3% Triton X-100 in TBS, and stained for 20 min with Texas red–conjugated phalloidin (Molecular Probes, Inc.; 25 μM/ml blocking/permeabilization solution). Coverslips were mounted on object slides with 20 μl PVA-DABCO.

**Results**

**PKCε Induces Processes in Neuroblastoma Cells**

To investigate whether increased levels of a specific PKC isoform are sufficient to induce neurites, expression vectors coding for four different PKC isoforms were trans-
ected into neuroblastoma cells. The classical and novel isoforms consistently expressed in neuroblastoma cells, PKCa, PKCbII, PKCc, and PKCe (Zeidman et al., 1999), were selected for this approach. The cDNAs coding for these isoforms were fused to cDNA coding for EGFP, generating a PKC-EGFP fusion protein when expressed.

To confirm the generation of fusion proteins, COS cells were transiently transfected with these plasmids, and cell lysates were subjected to Western blot analysis using isoform-specific antibodies (Fig. 1 B), which demonstrated the formation of proteins of the anticipated sizes.

SH-SY5Y and SK-N-BE(2) neuroblastoma cells were transfected with the vectors and the morphology of transfected cells was visualized with fluorescence microscopy (Fig. 2 A). When EGFP alone was expressed in SH-SY5Y and SK-N-BE(2) cells, the fluorescence was distributed throughout the cell. αFLE and βIIFLE (full-length PKC bound to EGFP) were mainly localized in the cytoplasm and were absent from the nucleus. δFLE localized throughout the entire cell, whereas eFLE localized mainly to the cell periphery and, in some cells, to perinuclear structures (Fig. 2 A). All fusion proteins gave rise to fluorescence of similar intensity in the transfected cells, indicating that there were no major differences in the expression levels of fusion proteins in individual cells.

The morphological effects of the overexpression of PKC isoforms were quantified by counting the number of transfected cells with cell processes longer than the length of two cell bodies. In SK-N-BE(2) cells, overexpression of eFLE induced long processes in 41% of the transfected cells, a substantially higher number than cells expressing EGFP only, where 6% of transfected cells had long processes. This effect was specific for PKCe, as overexpression of neither αFLE, βIIFLE, nor δFLE resulted in an increased number of cells with long processes (Fig. 2 B). A similar, but less pronounced pattern was observed in SH-SY5Y cells where overexpression of eFLE lead to 23% transfectants with long processes compared with 12% for cells expressing EGFP only. As in the case of the SK-N-BE(2) cells, overexpression of other PKC isoforms did not induce processes (Fig. 2 C). To exclude a potential role of EGFP in the PKCe effect, cDNA for PKCa and e were transferred from αFL and eFL, respectively, to the CMS-EGFP vector as a control. In these constructs PKC and

Figure 2. Induction of processes by PKC-e-EGFP overexpression. SH-SY5Y and SK-N-BE(2) cells were transfected with CMV-driven expression vectors containing cDNA for PKCa (α), PKCbII (β), PKCc (δ), or PKCe (ε) fused to EGFP or vector coding for EGFP only (−). Cells were fixed 16 h after transfection and mounted on object slides. (A) Fluorescence images of cells expressing PKC-EGFP fusion proteins. Comparison with bright-field microscopy images showed that the fluorescence from the PKC-EGFP fusion protein made the entire cell visible. The cells shown are typical for each treatment. For PKCe cells with processes are shown. (B and C) Quantification of the morphological effects shown in A. The number of transfected SK-N-BE(2) (B) and SH-SY5Y (C) cells with processes longer than the length of two cell bodies were counted. Data (mean ± SEM, n = 3) are presented as percent transfected cells with long processes. (D) Following transfection of SH-SY5Y and SK-N-BE(2) cells with EGFP or eFLE, 2 μM GF109203X was added to the medium. Cells were fixed 16 h after transfection and the number of cells with long processes were counted. Data (mean ± SEM, n = 2) are presented as transfected cells with long processes.
EGFP are expressed as separate proteins. SK-N-BE (2) cells were transfected with these vectors, and 5% of PKCα and 31% of PKCε overexpressing cells had processes. This demonstrates that the process induction of PKCε-EGFP is not dependent on EGFP.

To investigate whether the changes in cell morphology provoked by overexpression of PKCε-EGFP can be blocked by inhibition of PKC, the transfectants were treated with GF109203X (Fig. 2 D). This inhibitor did not cause a decrease in the percentage of transfected cells with long processes. The concentration used (2 μM) is in the range that inhibits the catalytic activity of classical and novel PKC isoforms in vitro (Martiny-Baron et al., 1993) and blocks TPA-induced expression of fos and jun genes in neuroblastoma cells (Ding et al., 1998). Thus, the induction of processes by PKCε appears to be independent of the catalytic activity of the enzyme.

The Regulatory Domain of PKCε Is Sufficient to Induce Processes

The fact that overexpression of full-length PKCε induced processes in the presence of GF109203X suggested an independence of the kinase activity. To analyze whether the PKC RD is sufficient for the effect, vectors coding for the RDs of PKCα, β, δ, and ε fused to EGFP, were created. The RDs of the remaining novel isoforms PKCγ and PKCζ, which are not expressed in neuroblastoma cells, were also included as a comparison (Fig. 1 A). All constructs were sequenced and found free of mutations. The constructs were expressed in COS cells (Fig. 1 C) where Western blot analysis confirmed formation of proteins of the anticipated sizes.

SH-SY5Y and SK-N-BE (2) cells were transfected with the vectors and all fusion proteins gave rise to fluorescence of similar intensity in transfected cells (Fig. 3), with the exception of εRDE. εRDE caused a weaker fluorescence suggesting lower levels of this protein. As in the case for full-length PKCζ and PKCβII, their corresponding RD-EGFP fusion proteins localized mainly outside the nucleus with a tendency to perinuclear enrichment (Fig. 3 A). Neither of these RDs induced a major increase in the percentage of transfected cells with long processes, suggesting no major difference in intracellular concentration.

All fusion proteins containing the two C1 domains (εRDE, c2PSC1E, PSC1E, c1V3E, and c1E; see Fig. 1 A for structural description) were not detected in the nucleus, and displayed a tendency to enrich in perinuclear structures (Fig. 4 A). Some fusion proteins, particularly εRDE and εPSC1V3, also seemed to localize to the plasma membrane. C2-containing proteins without the C1 domains (cC2E and cC2PSE; see Fig. 1 A) localized throughout the cell, and the smaller proteins (εPSE and εPSC1aE) were primarily present in the nucleus.

When cell morphology was examined, it was evident that the fragment from PKCε containing the pseudosubstrate, the C1 domains, and the V3 region (εPSC1V3E) was necessary and sufficient to induce processes (Fig. 4, A–C). 48% of the SH-SY5Y cells expressing this protein exhibited long processes. In SK-N-BE (2), the corresponding number was 59%. When the pseudosubstrate (c1V3E) or the V3 (εPSC1E) was removed from the PSC1V3 fragment, no substantial induction of processes could be observed in either cell line. It is notable that in SH-SY5Y cells more εPSC1V3- than εRDE-expressing cells had processes (48% versus 36%), suggesting that removal of the C2 domain enhances the process-inducing capacity (Fig. 4 B). It was also evident that the other constructs did not have a major effect on process induction.

Intracellular Distribution of PKCε Subdomain Fragments

Fluorescence microscopy suggested that the PKCε fragments localized to different intracellular sites. To investigate a possible correlation between the localization and process-inducing ability of the fragments, transfected SH-SY5Y cells were analyzed with confocal microscopy (Fig. 5). Full-length PKCε fused to EGFP localized uniformly outside the nucleus. The smallest fragment that induced processes, εPSC1V3E, displayed a distinct plasma membrane localization. Removal of the pseudosubstrate led to the complete loss of plasma membrane localization, as εC1V3E could only be seen in the perinuclear area of the cell. This suggests that the pseudosubstrate might be necessary for targeting of PKCε to the plasma membrane (Fig. 5). Removal of the hinge region from the PSC1V3 fragment generating εPSC1E, which is incapable of inducing processes, did not cause a loss of plasma membrane localization (Fig. 5). In conclusion, these data suggest that localization to the plasma membrane, for which the pseudosubstrate and the C1 domains are required, is necessary, but not sufficient for the process induction. The V3 region needs to be present for optimal function of the fragment.

Inhibition of Process Outgrowth by the Use of Inhibitory PKCε Constructs

The previous results demonstrate that PKCε through the
PSC1V3 fragment has the capacity to induce processes in neuroblastoma cells. To address the question of whether this capacity is a part of the molecular events driving neurite outgrowth in neuroblastoma cells differentiating in response to growth factors and RA, an attempt was made to find an εPSC1V3E-derived construct that could inhibit the process formation, putatively by acting in a dominant-negative manner. The two constructs that were most similar to εPSC1V3E, i.e., εPSC1E and εC1V3E, and did not display a process-inducing capacity, were initially evaluated for this purpose. Neither construct had a major effect on neurite outgrowth in RA-differentiated SK-N-BE(2) cells (data not shown). Thereafter, cDNA coding for either the first (C1a) or the second (C1b) C1 domain was deleted in the εPSC1V3E construct, forming εPSC1bV3E and εPSC1aV3E, respectively (Fig. 1, A and D). SK-N-BE(2) cells were transfected with these vectors, and vector coding for EGFP only (Fig. 6 A). Neither protein induced processes in untreated cells, demonstrating that both C1 domains are required for this effect. In fact, there was a slight suppression of the number of cells with processes in εPSC1aV3E-expressing cells (Fig. 6 A). After treatment with RA, 57% of EGFP-expressing cells and 52% of εPSC1bV3E-transfected cells had neurites. In contrast, only 18% of εPSC1aV3E-expressing cells had processes, demonstrating a neurite suppressing effect of this protein. Treatment with CNTF gave results that followed the same pattern as in RA, albeit with generally fewer neurite extending cells (Fig. 6 A). The constructs were also evaluated for NGF-driven neurite outgrowth of SH-SY5Y cells stably expressing the high affinity NGF receptor, TrkA (Fig. 6 B). Also in this differentiation protocol, expression of εPSC1aV3E, but not εPSC1bV3E, caused a substantial decrease in the...
number of neurite-bearing cells, both in control and NGF-exposed cells. These results demonstrate that the protein lacking the second C1 domain (ePSC1aV3) inhibits neurite outgrowth in several neuronal differentiation protocols, whereas the protein with the first C1 domain deleted (ePSC1bV3E) has no such effect.

To test whether the C1-deleted constructs have similar effects on processes induced by overexpression of PKC\(\epsilon\) or ePSC1V3E, cells were cotransfected with ePSC1aV3E or ePSC1bV3E at a 1:3 ratio into SK-N-BE(2) cells (Fig. 6, C and D). Cotransfection with ePSC1bV3E gave rise to fewer cells with processes than when either eFLE or ePSC1V3E alone was transfected, but substantially more process-bearing cells than when ePSC1bV3E alone was transfected into the cells. It is likely that the lower number of cells with processes in this cotransfection protocol could be due to a significant proportion of cells expressing only ePSC1bV3E, cells that will fluoresce, but will not have processes. On the other hand, cotransfection with ePSC1aV3E gave a lower number of cells with processes than did cotransfection with ePSC1bV3E. Thus, the ePSC1V3 fragment with the second C1 domain deleted (ePSC1aV3E) acts in a dominant-negative manner suppressing processes induced by overexpression of PKC\(\epsilon\) and inhibiting neurite outgrowth in several neuronal differentiation protocols. This suggests that the effect of the PSC1V3 region from PKC\(\epsilon\) may be a common mechanism for these processes.

### Characteristics of ePSC1V3E-induced Processes

All PKC\(\epsilon\)-derived, process-inducing constructs caused similar morphological changes of transfected cells. The outgrowth of processes was accompanied by a shrinkage of the cytoplasm and a rounding up of the cell body, which was most apparent in SK-N-BE(2) cells. Untreated SH-SY5Y cells generally have smaller cell bodies than SK-N-BE(2) cells, but a tendency towards rounding up of the cell body was observed in the SH-SY5Y cells also. The overall morphology of the processes differed slightly between the two cell lines. In SH-SY5Y cells, generally one process per cell was observed, but this process frequently carried several branches of various lengths (Figs. 2 A, C, 3 A, 8, and 4 A, PSC1V3), but in some cells two or more processes extending from the same cell were seen (Fig. 3 A, C and D). The SK-N-BE(2) cells generally had more than one process per cell, and these processes were frequently branched.

To address whether the ePSC1V3E-induced processes have characteristics associated with neurites, expression of cytoskeletal components and synaptic markers were analyzed. The ePSC1V3E-induced processes in SH-SY5Y...
cells were compared with neurites obtained after 4 d of treatment with 16 nM TPA, a protocol that causes SH-SY5Y cells to differentiate neuronally (Fig. 7). The experiments show that both ePSC1V3E-induced processes and the neurites of differentiated SH-SY5Y cells were composed of α-tubulin (Fig. 7, A–D) and NF-160 (Fig. 7, E–H). The cells were also stained for F-actin (Fig. 7, I–L), which besides staining of the main branches of the processes, also revealed an intense staining either at the tip of the processes (Fig. 7 L) or at sites where the processes have sharp bends (not shown). These actin-rich structures resemble the growth cones in TPA-differentiated cells (Fig. 7 J), suggesting that ePSC1V3E-induced processes express growth cones. Staining for the presence of synaptic proteins NPY (Fig. 7, M–P) and synaptophysin (Fig. 7, Q–T) in TPA-differentiated SH-SY5Y cells (Fig. 7, M, N, Q, and R) was positive, while the processes of cells transfected with ePSC1V3E were negative (Fig. 7, O, P, S, and T). This shows that ePSC1V3E-induced processes are neurite-like, but lack important properties of functional neurites. Furthermore, no overall increase in the expression of NPY or synaptophysin could be detected in the ePSC1V3E-transfected cells, suggesting that this PKCe fragment does not induce complete differentiation of neuroblastoma cells. The characteristics of processes induced by eFLE were similar to ePSC1V3E-induced processes (not shown).

**Colocalization of PKCe-EGFP and F-Actin**

An interesting issue is why overexpression of the RDs of both PKCe and PKCd (εRDE and δRDE) induced processes, whereas for full-length isoforms the same effect only was obtained with PKCe (εFLE) and not with PKCd (δFLE). A unique feature of PKCe, compared with other isoforms, is the presence of an actin-binding site between the C1 domains (Prekeris et al., 1996). Binding to F-actin via this site in vitro has been shown to maintain PKCe in an open conformation (Prekeris et al., 1998), which may result in exposure of structures in the RD essential for the process-inducing capacity of this isoform. If this interaction is important for the process induction of eFLE, it would be expected to detect colocalization of F-actin and eFLE. F-actin in eFLE-transfected SH-SY5Y cells was stained with Texas red-conjugated phalloidin and the
Figure 7. Phenotypic characterization of processes induced by εPSC1V3. SH-SY5Y cells overexpressing εPSC1V3E were compared with TPA-differentiated SH-SY5Y cells for cytoskeletal composition and expression of synaptic proteins. 16 h after transfection with εPSC1V3E, or after 4 d of treatment with 16 nM TPA of SH-SY5Y cells, the cells were fixed, and immunofluorescence with TRITC-conjugated secondary antibodies or TRITC-phalloidin staining of F-actin was performed. TPA-differentiated (A, B, E, F, I, J, M, N, Q, and R) and εPSC1V3E-expressing cells (C, D, G, H, K, L, O, P, S, and T) were analyzed for expression of α-tubulin (B and D), NF-160 (F and H), F-actin (J and L), NPY (N and P), and synaptophysin (R and T). εPSC1V3-transfected cells were visualized using the fluorescence of EGFP (C, G, K, O, and S). The weak staining seen in P and T was similar to the background staining seen when only secondary antibody was used.
colocalization of F-actin and eFLE was analyzed with confocal microscopy. Several processes were analyzed and it was evident that the proteins were colocalized in some parts of the processes (Fig. 8). This result thus indicates that an interaction between eFLE and F-actin may take place in the processes.

Discussion

This study was designed to examine the role of PKC isoforms in neurite outgrowth regulation and identify structures in the PKC molecule of importance for its function in this process. For this purpose, we used neuroblastoma cell lines which have been extensively used to study neuronal differentiation. Of the classical and novel PKC isoforms that are consistently expressed in neuroblastoma cells (PKCα, βII, δ and ε; Zeidman et al., 1999), only overexpression of PKCε induced processes in these cells. PKCε has been suggested to be of importance for neurite outgrowth in PC12 cells where overexpression of PKCε, but not PKCδ, potentiated NGF-induced neurite outgrowth (Hindle et al., 1995). The effect of PKCε in PC12 cells was suppressed by PKC inhibitors, which contrasts the results in the present study which demonstrates that the effect of PKCε was independent of its kinase activity. Furthermore, in PC12 cells, overexpression of PKCε did not by itself induce processes. It is thus likely that PKCε may regulate neurite outgrowth by a number of mechanisms. In neuroblastoma cells, several PKC isoforms are enriched in growth cones, but studies with phorbol ester treatment, which downregulates the classical isoforms, have suggested a role for PKCε or another novel isoform in supporting the growth cone (Fagerström et al., 1996). These facts, together with the results from the present study, highlight PKCε as one PKC isoform of importance in neurite outgrowth regulation.

In this study, the PKC isoforms were fused to EGFP to visualize transfected cells and to facilitate an examination of the intracellular distribution of the expressed proteins. EGFP, in its native fluorescent form, is a highly condensed molecule (Ormö et al., 1996). Approaches to fuse PKC isoforms with GFP variants have been successfully used to follow the translocation of PKCζ (Feng et al., 1998), PKCγ (Sakai et al., 1997), PKCδ (Ohmori et al., 1998; Shirai et al., 1998), and PKCε (Shirai et al., 1998). When examined, this fusion has been shown not to influence the catalytic activity of the enzyme. GFP variants have also been fused to smaller proteins or isolated domains, such as histone 2B (Kanda et al., 1998), pleckstrin homology domains (Stauffer et al., 1998), and PKC C1 domains (Oancea et al., 1998) without any obvious loss of function. Furthermore, as shown in this study, regardless of the position of EGFP was at the COOH terminus of intact PKCε or if it was placed COOH-terminally of the RD in constructs where the catalytic domain was deleted, processes were induced in neuroblastoma cells. This suggests that the effect on the process induction is independent of the position of EGFP. Processes were also induced when PKCε, without being fused to EGFP, was overexpressed. Several subdomains of PKCε that were fused to EGFP did not induce processes at all, further indicating that the effects observed in this study are not mediated by EGFP.

As previously mentioned, the effect of PKCε was independent of enzymatic activity and of the presence of the catalytic domain, since expression of the RD was sufficient to induce processes. In fact, the RD could induce processes more potently than the full-length PKCε, suggesting that the catalytic domain may inhibit this function of the RD. The RD from PKCδ and ζ also induced processes in the transfected cells, despite the inability of full-length PKCδ to do so. Cells transfected with RDE displayed less fluorescence than the other RD transfectants, probably indicating a lower level of expression of fusion protein in these transfectants. It is possible that the RD from PKCδ...
would have had the same effect if the protein levels in each individual cell had been higher. These results may suggest that the novel isoforms PK Cδ and PK Cγ, and perhaps PK Cα, could have the capacity to induce processes under proper conditions. An interesting feature possibly explaining the selective effect of full-length PK Cε, is the actin binding site which is present only in this isoform (Prekeris et al., 1996). When PK Cε binds actin it is maintained in an open active confirmation exposing the catalytic domains and the RDs (Prekeris et al., 1998), which thereby can exert its activity. There was a colocalization of εFLE and F-actin in processes, a finding which may indicate that this interaction might be important for the selective effect of PK Cε, although further experimentation is necessary to establish this interaction as crucial for process induction.

The finding that the PK Cε effect is insensitive to PK C inhibitors and could be mimicked by the RD is somewhat surprising. Since RDs of PK C isoforms have been suggested to act in a dominant-negative manner, the effects obtained in this study may be due to a dominant-negative effect of PK Cε and its RD on another endogenous PK C isoform. If this were the case, it would be expected to see an induction of processes upon inhibition of this isoform with PK C inhibitors. However, treatment of the neuroblastoma cells with GF109203X did not cause an elevated number of processes. It could be argued that this lack of process induction is due to the fact that GF109203X also inhibits other kinases that are critical for the induction of processes. If so, it would be expected that GF109203X should suppress the processes also in PK Cε-overexpressing cells, since the kinase of importance for processes also would be inhibited under these conditions. Furthermore, if the effects of the PK Cε constructs are dominant-negative, the suppression by εPSC1V3E of PK Cε-induced processes, RA-, and NGF-induced neurites implies that this construct would act in a dominant-negative manner towards a dominant-negative effect in the first case, whereas in the latter protocols it would simply act in a dominant-negative way. Therefore, we think that the most plausible explanation for the effects observed in this paper is that PK Cε RD induces processes through a mechanism that does not involve dominant-negative effects.

There are other reports where parts of, or the entire PK C RD exert the same effects as the complete enzyme. PK Cα was shown to activate phospholipase D in a PK C activator-dependent, but PK C activity-independent fashion, and phospholipase D was activated by PK Cα regulatory, but not catalytic domain in vitro (Singer et al., 1996). A further example is the inhibition of Golgi-specific sulfation of glycosaminoglycan chains in cells overexpressing PK Cε, which can be mimicked by overexpressing the PK Cε C1 domains only (Lehel et al., 1995a).

When examining the role of the different domains of PK Cε RD in process induction, it was evident that a fragment centering on the two C1 domains was sufficient and necessary for this effect. Interestingly, the C2 domain, which is of importance for RA CK binding (Csukai et al., 1997), was not of importance for the process-inducing capacity. In fact, expression in SH-SY 5Y cells suggested that removal of the C2 domain from the RD, generating PSC1V3, slightly increased the ability to induce processes.

There are several examples demonstrating that protein interaction with the RD is mediated via the C1 domains. Beside the previously mentioned actin binding site in PK Cε located between the C1 domains (Prekeris et al., 1996), a homologue of 14-3-3 has been shown to bind the Dictyostelium myosin II heavy chain-specific PK C through the PK C C1 domain (M atto-Y elin et al., 1997). In addition, binding of the pleckstrin homology domain from the tyrosine kinase Btk was shown to be dependent on the pseudosubstrate and the C1 domain from PK Cε (Y ao et al., 1997). Using an overlay assay, it was shown that the second C1 domain from PK Cγ bound several proteins from Xenopus laevis oocyte cytosol extracts (Pawelczyk et al., 1998). Taken together, these results indicate an important role for the C1 domains in PK C protein interactions. Thus, it is conceivable that the effects observed in this study are due to the C1 domains interacting with other proteins, thereby eliciting the observed morphological changes. However, there was also a dependence on the pseudosubstrate and parts of the V3 domain for the induction of processes. These structures have been shown to be of importance for localization of PK Cε C1 domains to the plasma membrane in NIH 3T3 fibroblasts (Lehel et al., 1995b). In line with that report, the process-inducing fragment, PSC1V3, localized almost exclusively to the plasma membrane, but this localization was lost when the pseudosubstrate was removed. This was accompanied with a loss of process-inducing capacity, which suggests that a plasma membrane localization is necessary for this effect. However, a plasma membrane localization per se of the C1 domains is not sufficient, since the PSC1 fragment to a large extent appeared to be present at the plasma membrane without inducing processes.

Removal of the second, but not of the first, C1 domain generated a fragment that suppressed neurite outgrowth during RA-, CNTF-, and NGF-driven neuronal differentiation. Since this same fragment also acted in a dominant-negative manner towards processes induced by PK Cε overexpression, these results suggest that the observed effects of PK Cε is not only observed upon overexpression of the protein, but may indeed be of importance for neurite outgrowth that accompanies neuronal differentiation. However, given the abundance of proteins with C1 domains (Hurley et al., 1997), it cannot be excluded that during neuronal differentiation effects reported in this study are mediated via other C1 containing proteins. The results obtained with the C1-deleted constructs also illustrate the different properties of the two C1 domains that have been described (Szallasi et al., 1996; Hunn and Quest, 1997; Bögi et al., 1998).

From the present results, it is not possible to draw definite conclusions regarding the mechanisms whereby PK Cε constructs elicit processes. To exclude the possibility that the increase in process-bearing cells is not due to a selection of cells with process-inducing capacity, the number of SH-SY 5Y cells expressing EGF P or εRD E following transfection were counted. There was a lower percentage of εRD E-expressing cells (4.1 ± 0.5% of EGF P- versus 2.8 ± 0.6% εRD E-expressing cells), but this difference is too low to account for the increase in process-bearing cells (5% in EGF P- to 32% in εRD E-expressing cells). Furthermore, the few processes that could be observed in...
EGFP-expressing cells were much shorter than processes in cells transfected with PKCε constructs. This was also true for EGFP-expressing cells that were kept in culture for up to 4 d. This suggests that transfection with PKCε constructs does not result in an enhancement of a basal rate of process generation, but rather induces some events that eventually lead to the generation of neurite-like processes. This process generation may be mediated via cytoskeletal mechanisms, effects on the interaction of the cell with the substrate, or some other mechanism. It does not seem to involve altered expression of differentiation-coupled genes, since no increase in expression of NPY or synaptophysin, in the cell bodies or the processes, could be observed in ePSC1V3E-overexpressing cells. These proteins are elevated upon neuronal differentiation of neuroblastoma cells. Thus, it is likely that PKCε overexpression induces processes in undifferentiated cells and does not elicit a complete neuronal differentiation program. Both α-tubulin and NF-160 were present at apparently similar levels in the processes in ePSC1V3E-overexpressing cells and in neurites in neurally differentiated neuroblastoma cells, indicating that the processes induced by the PKCε fragment to some extent display neuronal features. Such a dissociation between the physical induction of neurites and the accompanying increase in neuronal differentiation markers generally present in neurites has also been observed after overexpression of a constitutively active phosphatidylinositol 3 kinase in PC12 cells (Kobayashi et al., 1997).

In conclusion, this study demonstrates that PKCε, but not PKCα, βII, or δ, induces neurite-like processes in neuroblastoma cells and this effect can be ascribed to a region encompassing the pseudosubstrate, the two C1 domains, and parts of the V3 domain. Identification of a dominant-negative construct derived from this region indicates that this effect of PKCε is of relevance for neurite outgrowth during neuronal differentiation.

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