Protein Kinase C-δ mRNA Is Down-regulated Transcriptionally and Post-transcriptionally by 12-O-Tetradecanoylphorbol-13-acetate*

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Activation of protein kinase C-δ (PKC-δ) by 12-O-tetradecanoylphorbol-13-acetate (TPA) is followed by a gradual decrease in detectable protein 12–24 h later in the mouse B lymphoma cell line A20. Down-regulation is associated with TPA-induced proteolysis and a 50–86% decrease in PKC-δ mRNA 0.5–24 h post-treatment which is due to both a 50% decrease in transcription and accelerated degradation of PKC-δ mRNA as determined using the pulse-chase method. Destabilization of PKC-δ mRNA is also observed when actinomycin D is added to cells pretreated with TPA for 2 h; however, addition of actinomycin D or cycloheximide prior to TPA treatment blocks destabilization. Addition of PKC inhibitors to TPA-treated cells also blocks destabilization of PKC-δ mRNA. Cells treated with TPA for 4 h contain an activity not found in control cells which destabilizes PKC-δ mRNA but not glyceraldehyde-3-phosphate dehydrogenase mRNA in vitro. Addition of TPA to control extracts fails to increase degradation of PKC-δ mRNA in vitro, suggesting that treatment of intact cells is required to induce the synthesis of a factor(s) that destabilizes PKC-δ mRNA. This factor(s) then acts along with transcriptional and post-translational regulatory mechanisms to down-regulate PKC-δ.

Protein kinase C (PKC)1,2-δ is a widely distributed member of the PKC family of serine/threonine protein kinases (for review, see Ref. 1). Unlike conventional PKC isoforms, PKC-δ is activated by diacylglycerol or the tumor promoter 12-O-tetradecanoyl-13-acetate (TPA) in the presence of phosphatidylserine without a requirement for Ca2+ (for review, see Ref. 2). Abundant expression of PKC-δ in several cell types including B lymphoma cells (3–5), myeloid cells (3), fibroblasts (6,7), neural crest-derived PC12 cells (8), and glioma cells (9) suggests that this enzyme is of central importance in intracellular signaling.

The biological functions of PKC-δ are largely unknown; however, this enzyme has been implicated as a regulator of cell growth. Overexpression or selective activation of PKC-δ by mitogens can lead to cell differentiation (8, 10, 11), cell division arrest (12), or slowed cell growth (13–15). Specific substrates phosphorylated in vitro by PKC-δ include the elongation factor eEF-1α (16) and the high affinity immunoglobulin E receptor (17). PKC-δ itself can be phosphorylated on tyrosine residues in response to platelet-derived growth factor (18), neurotransmitters (10), and expression of transfected c-Ha-ras (19), indicating a complex mode of regulation involving cross-talk between tyrosine kinases and other signaling components.

The amount of PKC-δ in cells can be regulated, thereby altering cellular responses to external stimuli. PKC-δ is increased in cultured neural cells following chronic exposure to ethanol (20), in rabbit corpora lutea by estrogen (21), and in a B lymphoblastoid cell line by antibody to the major histocompatibility complex class II antigen (22). On the other hand, PKC-δ is decreased in peritoneal macrophages by nitric oxide (25) and in B cells by nonmitogenic anti-IgM antibody (22). Down-regulation of PKC-δ following activation by phorbol esters or diacylglycerol occurs in several cell types including fibroblasts (13, 23, 24) and murine melanocytes (14). In murine melanocytes, down-regulation of PKC-δ occurs by a post-translational mechanism without a detectable change in steady-state mRNA (14).

As described in this report, PKC-δ is also down-regulated by TPA in murine A20 B lymphoma cells; however, in these cells, a decrease in PKC-δ mRNA is observed. The mechanisms involve both a decrease in the rate of transcription and an increase in the rate of mRNA degradation. The post-transcriptional regulatory mechanism can be activated in vitro and appears to require de novo protein synthesis.

**Experimental Procedures**

Materials—Calphostin C and TPA were obtained from Calbiochem-Novabiochem Corporation (La Jolla, CA). Chelerythrine chloride was from LC Laboratories (Woburn, MA). Goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase, 3,3′-tetramethylbenzidine, and the peptide corresponding to amino acids 662–673 of PKC-δ were from Life Technologies, Inc. Rabbit antiserum against the carboxy-terminal domain of PKC-δ (amino acids 662–673) was from R & D Systems (Minneapolis, MN). [5,6-3H]Uridine (35–50 Ci/mmol), [α-32P]UTP (3,000 Ci/mmol), and [α-32P]dATP (3,000 Ci/mmol), and [α-32P]UTP (3,000 Ci/mmol) were from DuPont NEN. MagnaGraphTM nylon transfer membranes and NitroBindTM nitrocellulose membranes were obtained from Micron Separations Inc. (Westboro, MA).

Cell Culture and Experimental Treatment—A20 cells were obtained from American Type Culture Collection (Rockville, MD) and maintained as described previously (5). Cells were resuspended in fresh medium at a density of 2–3 × 106 cells/ml and incubated for 2 h at 37 °C prior to the addition of either 1 mg/ml TPA in dimethyl sulfoxide to a final concentration of 100 ng/ml or an equal volume of dimethyl sulfoxide (control). Western Blot Analysis of PKC-δ—Cell extracts were prepared from 5 × 107 cells/treatment in 500 μl of ice-cold homogenization buffer (20 mM Tris-HCl, pH 7.4, 5 mM EGTA, 1 mM phenethylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin) as described by Szallasi et al. (25). The cell extracts were centrifuged for 1 h at 100,000 × g. The protein concentration of the supernatant was determined (27), and proteins from the supernatant (40 μg) and pellet were resolved on 9% SDS-polyacrylamide gels (28) and transferred to a nitrocellulose membrane at 200 mA for 2 h at 15 °C (29). The membrane was incubated for 1 h in BLOTTO (20 mM Tris-HCl, pH 7.8, 180 mM NaCl, and 3% nonfat dry milk) followed by incubation with a 1:750-fold dilution of rabbit
anti-PKC-δ antiserum overnight at 4°C in BLOTTO. The membrane was washed in BLOTTO and subsequently incubated with a 1:1,000-fold dilution of goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase in BLOTTO. Chromogenic visualization of bound antibody was done using 3,3′,5,5′-tetramethylbenzidine as a substrate (5).

RNA Extraction and Northern Blot Analysis—Total cellular RNA was purified (30, 31), resolved on 1.2% agarose gels containing formaldehyde, and transferred to nylon transfer membranes (5). Northern blots were hybridized to 32P-labeled synthetic oligonucleotide probes (32–34) complementary to PKC-δ mRNA and to mRNA encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5). The labeled RNAs were detected by autoradiography at -70°C with an intensifying screen and quantified by whole band analysis using a BioImage scanning densitometer (Millipore, Bedford, MA).

Nuclear Run-off Analysis—Full-length GAPDH, PKC-α, and PKC-δ cDNA inserts were isolated from pB7 (35), pMPK20 (36), and pL-δ (13), respectively, and 2–3 μg of each was bound to nylon transfer membranes. Nuclei were prepared from 1–2 × 10^6 cells and immediately frozen in liquid nitrogen (37). The transcription reaction mixture containing 200 μl of nuclei plus 200 μl of 2× transcription buffer (10 mM Tris-HCl, pH 8.0, 300 mM KCl, 5 mM MgCl2, 5 mM dithiothreitol, 1.0 mM ATP, 1.0 mM CTP, 1.0 mM GTP) with 100 μCi of [α-32P]UTP was incubated at 26°C for 30 min (38). Labeled transcripts were purified (39) from 30 ml aliquots for each treatment (6 × 10^6 cpm) were hybridized to cDNAs bound to a nylon transfer membrane for 3 days at 42°C and then washed as described previously (38). Transcripts hybridizing to GAPDH, PKC-α, and PKC-δ cDNA were detected by autoradiography and quantified by whole band analysis.

Pulse-Chase Analysis of mRNA Half-life—Turnover of PKC-δ and GAPDH mRNA was determined as described by Zhu et al. (40) with modifications. 2–3 × 10^6 cells at a density of 2–3 × 10^6 cells/ml were pulsed with 100 μCi/ml [5,6-3H]uridine for 48 h and chased with unlabeled 10 mCi uridine and 10 mCi cytidine for 2 h at 37°C. The cells were subsequently maintained in medium containing 1 mCi uridine and 1 mCi cytidine for 2 h prior to treatment. Total RNA (6.8–7.5 × 10^6 cpm/mg) was purified (30, 31), and 150 μg of each sample (5 × 10^6 cpm/ml) was hybridized to 1–2 μg of PKC-δ cDNA insert or pLTR (13) immobilized on a nylon transfer membrane. Membranes were washed (38) and the hybridized RNA eluted in 1 N NaOH at 95°C for 2–3 min. Each sample was neutralized with 1 N HCl and counted in a liquid scintillation analyzer.

Assays for mRNA Stability in Vitro—The postnuclear extract and nuclei were prepared from 5–6 × 10³ cells/treatment as described by Wager and Assoian (41). Nuclei were then washed once in 1 ml of Nonidet P-40 buffer (10 mM Tris–HCl, pH 7.9, 150 mM NaCl, 0.5% (v/v) Nonidet P-40) and resuspended in 1 ml of ice-cold nuclear extraction buffer (5 mM HEPES, pH 7.9, 300 mM NaCl, 15 mM MgCl2, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonfyl fluoride, 0.2 mM EDTA, 26% (v/v) glycerol). Nuclear proteins were prepared as described by Dertinger and Latchman (42). Reaction mixtures (100 μl) containing 20 μg of purified RNA, 0.1–12% postnuclear or nuclear extract in Nonidet P-40 buffer were incubated at 4°C for 30 min. The reactions were terminated by the addition of 100 μl of phenol/chloroform, and the remaining RNA was purified and analyzed on Northern blots.

RESULTS

PKC-δ was found recently to be the most abundant isozyme in A20 cells (5). To determine if PKC-δ was activated and then down-regulated by TPA, anti-PKC-α antibody was used to detect PKC-δ at various times following exposure to TPA (Fig. 1). At zero time, two forms of PKC-δ, which are likely to correspond to two major phosphorylation variants (7), were detected primarily in the cytosol. The addition of a competitor peptide (amino acids 662–673 of PKC-δ) specifically blocked binding of the antibody to both forms. Following TPA treatment, about 50% of the total PKC-δ translocated to the membrane fraction within 10 min and was retained there for at least 18 h (Fig. 1A). An increase in the proportion of PKC-δ exhibiting decreased electrophoretic mobility on SDS-polyacrylamide gels was also seen in the cytosol. PKC was activated as indicated by increased phosphorylation of the 80-kDa myristoylated alanine-rich protein kinase C substrate and induction of c-fos (see Ref. 5).

Down-regulation of PKC-δ was seen in both the cytosol and membrane fractions 6–24 h post-treatment. Complete down-regulation was not, however, seen in the cytosol even after a 48-h treatment (data not shown). Since several PKC isozymes were found previously to be down-regulated rapidly by TPA-induced proteolysis (14, 44–46), the amount of PKC-δ in control and TPA-treated cells was determined in the presence or absence of the translational inhibitor, cycloheximide (Fig. 1B). In the presence of cycloheximide, PKC-δ decreased by 30% for control cells, whereas a 65% decrease was seen for TPA-treated cells at 24 h, indicating that protein turnover was greater following TPA treatment. A 90% decrease in PKC-δ over 24 h was, however, seen in the absence of cycloheximide, which suggested that TPA was reducing production of PKC-δ as well as increasing its degradation.

To determine if PKC-δ mRNA levels were down-regulated, Northern blots of RNA from control and TPA-treated cells were analyzed (Fig. 2). PKC-δ mRNA was decreased within 15 min following treatment and was reduced to 14% of the control within 4 h. Steady-state PKC-δ mRNA remained low for 12 h, then gradually increased 12–48 h post-treatment to the control level.

The TPA-induced decrease in PKC-δ mRNA was due, in part,
to a decrease in the rate of transcription (Fig. 3). No change was observed in the transcription rate for PKC-α, which was expressed at a low level. When compared with the constitutively expressed GAPDH, a 50% decrease in the transcription rate for PKC-δ was seen 2–24 h post-treatment (Fig. 3B). The transcription rate returned to pretreatment levels after 48 h (data not shown).

TPA treatment also destabilized PKC-δ mRNA; however, in contrast to transcriptional down-regulation, this mechanism required ongoing mRNA and protein synthesis (Fig. 4). When actinomycin D was added 2 h following TPA treatment, the half-life for PKC-δ mRNA was more than 180 min in control cells and 50 min in TPA-treated cells (Fig. 4B). Accelerated degradation of PKC-δ mRNA was also seen when 10 μg/ml cycloheximide and actinomycin D were simultaneously added 2 h after TPA treatment (data not shown). Accelerated degradation of PKC-δ mRNA could not, however, be detected if actinomycin D was added 20 min prior to TPA treatment (Fig. 4A). Likewise, the stability of PKC-δ mRNA was unaffected by TPA in cells pretreated with cycloheximide and assayed in the presence of actinomycin D 2–6 h after TPA or control treatments (Fig. 4D).

The stability of PKC-δ mRNA was also assayed using the pulse-chase method (Fig. 5). In this assay, TPA treatment decreased the half-life of PKC-δ mRNA from >8 h to 160 min. The longer half-lives calculated using the pulse-chase method were due in part to destabilization of PKC-δ mRNA by actinomycin D. When actinomycin D was added to cells, 50.8% of the [3H]uridine-labeled PKC-δ mRNA was degraded within 6 h compared with 24.6% in the absence of actinomycin D (data not shown). The measured half-lives of other mRNAs have also been found to be longer using the pulse-chase method compared with Northern blot analysis following the addition of actinomycin D (47). In any case, both assays reveal destabilization of PKC-δ mRNA by TPA.

TPA-treated cells were found to contain an activity that destabilized PKC-δ mRNA in vitro but did not affect the stability of GAPDH mRNA (Figs. 6 and 7). The addition of extracts from control cells to reaction mixtures containing deproteinated RNA did not destabilize PKC-δ mRNA, whereas postnuclear or nuclear extracts from TPA-treated cells increased PKC-δ mRNA degradation. A 50% decrease in the ratio of PKC-δ to GAPDH mRNA was seen when RNA was incubated in reaction mixtures containing 2.7% postnuclear extract (3.5 mg/ml protein) from TPA-treated cells, whereas no decrease was seen when incubated with 2.7% postnuclear extract (3.4 mg/ml) from control cells. Likewise, incubation of RNA with 12% nuclear extract (1.8 mg/ml protein) from control cells did not alter the ratio of PKC-δ to GAPDH mRNA, whereas 12% nuclear extract (1.7 mg/ml protein) from TPA-treated cells resulted in a 40% decrease in the ratio of PKC-δ to GAPDH mRNA. Divalent cations were required, since addition of 50 mM EDTA blocked degradation of PKC-δ mRNA in TPA-treated extracts (data not shown). These data suggest that component(s) required for
selective degradation of PKC-δ mRNA were found primarily in the cytosol.

PKC-δ mRNA destabilization required treatment of intact cells with TPA. The addition 1–100 ng/ml TPA to control lysates failed to increase degradation of PKC-δ mRNA, and addition of the PKC inhibitor chelerythrine chloride (48) had no effect in the in vitro assay (Fig. 7A). TPA-induced degradation of PKC-δ mRNA appeared to be mediated through activation of PKC since addition of calphostin C, a highly specific PKC inhibitor (49), to TPA-treated cells partially blocked degradation of PKC-δ mRNA in a dose-dependent manner (Fig. 7B). Taken together, these data show that down-regulation of PKC-δ mRNA occurs as a result of several mechanisms that include increased protein turnover, decreased mRNA stability, and transcriptional repression.

DISCUSSION

Activation of PKC is generally correlated with phosphorylation and translocation to cell membranes (for review, see Refs. 1 and 2). In A20 cells, some PKC-δ is translocated to the membrane fraction; however, most remains in the cytosol following activation by TPA. This is in contrast to the rapid and almost complete translocation of PKC-δ in NIH 3T3 cells (13). Two forms of PKC-δ are detected by Western blot analysis, and a relative increase in one form of PKC-δ, which has a slower electrophoretic mobility on SDS-polyacrylamide gels, is seen after TPA treatment. These data are consistent with previous reports showing phosphorylation of PKC-δ upon activation (7, 18, 23, 43).

Down-regulation of PKC-δ by TPA occurs in several cell types (13, 14, 26); however, the time course and mechanisms appear to be variable. In melanocytic cells, down-regulation is primarily due to proteolysis with little effect on PKC-δ synthesis (14), whereas in A20 cells increased proteolysis and decreased synthesis of PKC-δ both occur. TPA-activated mechanisms that decrease transcription and PKC-δ mRNA half-life contribute significantly to down-regulation. Down-regulation of PKC mRNA by TPA has not been seen by others who have reported only post-translational down-regulation of conven-
Protein Kinase C-δ mRNA Down-regulation

Fig. 5. PKC-δ mRNA stability as determined by the pulse-chase method. Cells were prelabeled with [5,6-3H]uridine and then incubated with 100 ng/ml TPA (●) or without TPA (○). Total cellular RNA was purified, and radioactive PKC-δ mRNA was quantified as described under "Experimental Procedures." Each value is expressed as the relative percent of mRNA and is the mean of two independent experiments.

Fig. 6. Degradation of PKC-δ mRNA in vitro. Cells were incubated without (Control) or with 100 ng/ml of TPA for 4 h. In vitro RNA degradation assays containing the indicated percentage of either post-nuclear (panel A) or nuclear (panel B) extracts were performed as described under "Experimental Procedures." Incubation with 10 mM vanadyl ribonucleoside complex (V) or without 20 μg of RNA (−R) is indicated. PKC-δ and GAPDH mRNAs were detected by Northern blot analysis and autoradiography.

Fig. 7. Effects of TPA and PKC inhibitors on PKC-δ mRNA degradation in vitro and in vivo. Panel A, postnuclear extract from control cells was incubated with 10 mM vanadyl ribonucleoside complex (V), with 20 mM chelerythrine chloride (Ch), and the indicated concentration of added TPA at 4 °C for 30 min. PKC-δ and GAPDH mRNAs were detected by Northern blot analysis and autoradiography. Panel B, cells were treated for 2 or 4 h with TPA as indicated or alternatively treated for 2 h with TPA followed by 2 h with the indicated concentration of calphostin C (CC). Densitometry was used to quantify PKC-δ and GAPDH mRNA. The relative percentage of mRNA detected at the 2-h TPA treatment is shown.

TPA has been well characterized as an agent that modulates transcription through activation of AP-1 (for review, see Ref. 50) and/or NF-κB (for review, see Ref. 51) transcription factors. Although generally regarded as positive regulators, AP-1 (52-54) and NF-κB (55) can function as negative regulators of transcription. Another PKC isoform, PKC-β, has been found to have AP-1 binding sites in a region that negatively regulates cell type-specific transcription in transient transfection assays (56). Rapid transcriptional down-regulation of PKC-δ, which is not inhibited by cycloheximide, is consistent with activation of a preexisting transcription factor by TPA.

In recent years the importance of post-transcriptional regulatory mechanisms has become recognized widely (for review, see Refs. 59 and 60). TPA selectively stabilizes some mRNAs (57, 58) and destabilizes others (57) including estrogen receptor mRNA (59), α1B-adrenergic receptor mRNA (60), and muscle-specific mRNAs in avian skeletal muscle (57) without altering stability of GAPDH mRNA (57, 61). Destabilization of PKC-δ mRNA contributes significantly to the overall decrease in PKC-δ mRNA 2-24 h following TPA treatment in A20 cells. In control cells, PKC-δ mRNA is relatively stable with a half-life of more than 8 h which is reduced to 160 min in TPA-treated cells as determined using the [3H]uridine pulse-chase method. TPA-induced degradation of PKC-δ mRNA is also observed when transcription is blocked by actinomycin D; however, the stability of PKC-δ mRNA is generally decreased by actinomycin D. Others have also observed destabilization of mRNAs by actinomycin D (47) and increased stability of mRNAs using the [3H]uridine pulse-chase method (61), which suggests that the [3H]uridine pulse-chase method may reflect mRNA turnover rates more accurately.

TPA treatment appears to induce activation of cytoplasmic components that destabilize PKC-δ mRNA since deproteinated...
PKC-δ mRNA is degraded upon addition of TPA-treated extracts but not control extracts in an in vitro assay. The addition of EDTA to the TPA-treated lysate blocks PKC-δ mRNA degradation, indicating that divalent cations are required. Others have observed that divalent cations are required for a diversity of regulated ribonuclease systems (41, 57). In U937 cells, TPA treatment leads to a post-transcriptionally controlled increase in TGF-β1 mRNA which can be detected in vitro (41). This observed increase in TGF-β1 mRNA is due to inhibition of a ribonuclease system present in cytoplasmic extracts from control U937 cells (41). Cytoplasmic nucleases systems activated or inactivated by TPA must apparently be capable of targeting specific mRNAs.

Activation of PKC is required for increased degradation of PKC-δ mRNA since addition of a specific PKC inhibitor, calphostin C, prevents PKC-δ mRNA degradation. Because TPA exerts a dual effect on PKCs, activating initially and then down-regulating subsequently, some of the effects of TPA can be attributed to activation, whereas others may be due to down-regulation. Zhu et al. (40) attributed TPA-induced destabilization of muscle-specific mRNAs to down-regulation of PKC since the time course for PKC down-regulation precedes destabilization of muscle-specific mRNAs. On the other hand,Saceda et al. (59) attributed TPA-induced destabilization of estrogen receptor mRNA to PKC activation since a protein kinase inhibitor blocks destabilization. Both the time course and experiments utilizing PKC inhibitors are consistent with a mechanism requiring activation.

The induction of PKC-δ mRNA-specific nucleolytic activity requires transcription and translation. Actinomycin D does not interfere directly with the TPA-induced nucleolytic activity since addition of actinomycin D 2 h following treatment does not block the increased PKC-δ mRNA degradation, although PKC-δ mRNA from both control and TPA-treated cells turns over more rapidly in the presence of actinomycin D. TPA treatment of extracts from control cells fails to induce an activity directly, which increases degradation of PKC-δ mRNA in vitro; this is consistent with a requirement for intact cells and new transcription. The time course for down-regulation of PKC-δ mRNA is also consistent with rapid transcriptional down-regulation followed by a decrease in PKC-δ mRNA stability 2–4 h later. These results are in agreement with Zhu et al. (40), who found that actinomycin D and cycloheximide blocked accelerated turnover of muscle-specific mRNAs in TPA-treated avian muscle cells. This requirement for transcription/translation suggests that a TPA-inducible component is also necessary for accelerated turnover of PKC-δ mRNA; however, actinomycin D and cycloheximide could, in some other manner, alter the ability of cells to respond to TPA. Rapid turnover of unstable mRNAs has long been hypothesized to require a labile endonuclease or mRNA-binding protein since inhibitors of translation often stabilize these mRNAs (57). Direct evidence for the existence of a labile nuclease activity is, however, generally lacking (57). A soluble destabilizing factor does, however, appear to be present in cytoplasmic extracts from TPA-treated A20 cells, which specifically accelerates degradation of PKC-δ mRNA.

PKC-δ mRNA is relatively stable and does not have the extensive AUUUArepeats (3) seen in unstable mRNAs such as c-fos (57). The stop codon is imbedded in a region that contains one AUUAA element; however, single elements do not usually increase mRNA instability. The 3′-untranslated region also contains an AU repeat that could be a target for endonuclease attack (3). At this time, regulatory elements within PKC-δ mRNA have not been identified, and further studies would be required to define regions of the PKC-δ mRNA which determine stability.

Since PKC-δ has been implicated in control of cell differentiation and cell growth arrest (8, 10–15), the decreased availability of this signal transducer following activation could significantly alter cellular responses to mitogenic stimuli. Control over the stability of PKC-δ mRNA amplifies transcriptional regulation contributing to the observed shift in protein levels. Negative feedback regulation of PKC-δ therefore involves coordinated mechanisms that both increase proteolysis and decrease production of this key regulatory enzyme.
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