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A PROTEIN KINASE C ISOZYME, nPKCε, IS INVOLVED IN THE ACTIVATION OF NF-κB BY 12-*O*-TETRADECANOYLPHORBOL-13-ACETATE (TPA) IN RAT 3Y1 FIBROBLASTS

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SUMMARY: In order to examine whether PKC is involved in the activation of NF-κB by TPA, we overexpressed a variety of PKC isozymes in rat 3Y1 fibroblasts and monitored the expression of the co-transfected reporter NF-κB gene. In contrast to TPA response element (TRE), where overexpression of a variety of PKC isozymes results in enhanced activation by TPA, activation of NF-κB by TPA is not enhanced by overexpression of PKC isozymes such as cPKCα, nPKCδ, or nPKCθ. However, the overexpression of nPKCε does result in enhancement. A kinase-negative point mutant of nPKCε, where Lys at the ATP binding site is altered to Arg, does not cause this enhancement of NF-κB activation. Further, the kinase-negative nPKCε partially suppresses endogenous NF-κB activity. These results suggest that nPKCε is specifically involved in the activation of NF-κB when cells are treated with TPA.

Protein Kinase C (PKC) has been implicated as a major component of the cellular signaling network that regulates a variety of cell functions such as cell growth and differentiation (1). PKC comprises more than ten isozymes that can be classified into three groups based on their structural and biochemical properties: TPA-sensitive and Ca^{2+}-dependent PKC, cPKC (α, βI, βII and γ); TPA-sensitive and Ca^{2+}-independent PKC, nPKC (δ, ε, η and θ); and TPA-insensitive PKC, aPKC (ζ and λ) (2). Each PKC isozyme is expressed in a cell type-dependent manner and is expected

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The abbreviations used are: PKC, protein kinase C; cPKC, conventional PKC; nPKC, novel PKC; aPKC, atypical PKC; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; TRE, TPA-response element; SRE, serum-response element; CAT, chloramphenicol acetyl transferase.

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to play some unique roles. For example, the response to mitogenic stimulation differs between nPKCδ or ε and cPKCα *in vivo* (3). Further, overexpression of nPKCε results in increased growth properties of murine fibroblasts, while overexpression of nPKCδ results in decreased growth properties (4). However, the specificity of responsive PKC members and their mode of action remain to be clarified. Further, the actual direct substrate that mediates signals from PKC to transcriptional activation remains to be clarified.

NF-κB is a transcription factor that regulates a variety of genes including immune-related and viral genes (5). The fact that TPA induces NF-κB activity (5) and PKC inhibitors abolish this induction (6) suggests that PKC is involved in NF-κB activation. PKC directly phosphorylates and inactivates the inhibitory protein of NF-κB, IκBα, *in vitro* (7, 8); however, it is not known whether PKC regulates NF-κB in this way *in vivo*.

As a first step toward clarifying the role of PKC in κB activation mediated by TPA, we examined the effect of overexpression of PKC isozymes on κB activation.

EXPERIMENTAL PROCEDURES

Plasmids-----Plasmids κB-CAT and mutant κB-CAT were obtained from Dr. T. Taniguchi (University of Tokyo) and were originally named p-55A2 and p-55A3 (9). The κB-luciferase plasmid (p-55IFNLuc) containing the enhancer/promoter region of p-55A2 instead of the original p-55IgkLuc was provided by Dr. T. Fujita (Tokyo Metropolitan Institute of Medical Science) (10). The reporter plasmid TRE-Luc, the PKC expression plasmids, and mutant nPKCε expression plasmid have been described elsewhere (3, 11, 12, 13).

Cell culture, transfection and enzyme assay----Transfection of rat 3Y1 fibroblasts was done following a standard Ca^{2+}-phosphate procedure as previously described (11). After transfection, the cells were made quiescent by keeping them in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.5% Fetal Calf Serum (FCS) (Life Technology) for 48 hours. Cells were stimulated by incubation in the presence or absence of TPA (Sigma) for the indicated times and protein extracts were prepared by a freeze-thaw method in 100 mM potassium phosphate buffer (pH 7.8). A portion (40 μg protein) of the extract was used for CAT assay (11) and another (5 μg protein) for luciferase assay (3).

Western blotting----Cells prepared for CAT and luciferase assay were analyzed by SDS-PAGE followed by immunoblotting using specific antibodies for PKC isozymes (3, 12).

RESULTS

The effect of transiently overexpressed cPKCα or nPKCε on TPA stimulated κB-reporter expression in rat 3Y1 fibroblasts was examined by monitoring CAT activity to observe the ability

of PKC isozymes to activate NF-κB. The PKC isozyme expression vector or an empty control vector was co-transfected with κB-CAT reporter plasmid. After transfection, cells were kept in DMEM supplemented 0.5% with FCS, and then treated with or without 10 ng/ml TPA for 12 hours. Although the overexpression of cPKCα did not enhance κB-reporter expression even when its expression vector was maximally transfected, that of nPKCε produced an elevation of reporter expression with the minimum amount of expression vector and this effect was dependent on the amount of nPKCε expression vector (figure 1A). In the control empty vector transfectant, TPA stimulation resulted in a moderate increase in CAT expression compared with a non-stimulated control, probably due to the activation of potent endogenous PKC isozymes including nPKCε. As shown in figure 1B, the enhancement of κB-reporter expression by overexpression of nPKCε as well as by TPA were not observed for mutant κB that does not bind NF-κB (9). These results indicate that κB-reporter expression reflects NF-κB mediated transcription and that TPA induces NF-κB activation in rat 3Y1 fibroblasts, consistent with earlier observations that TPA induces NF-κB activation in many cell lines such as 70Z cells, Jurkat cells, and HeLa cells (5). Further, cPKCα and nPKCε differ in the potency of their transcriptional activation of κB.

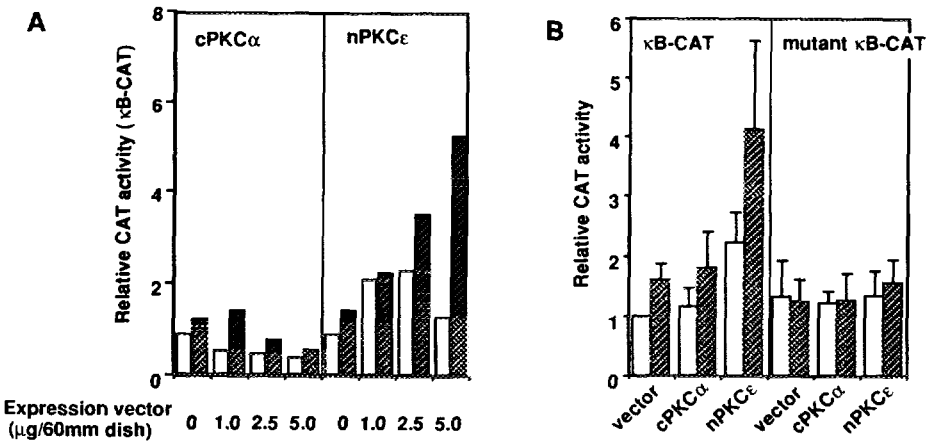

Figure 1.
(A) Effect of the overexpression of cPKCα and nPKCε. PKC expression vector (1.0, 2.5, or 5.0 μg) and κB-CAT reporter (3 μg) were transfected (60 mm dish) and total DNA was adjusted to 8 μg with empty vector. Cells were kept in DMEM supplemented with 0.5% FCS for 48 hours and were made quiescent. Then, the cells were either untreated (open bars) or treated with 10 ng/ml TPA (hatched bars) for 12 hours. A defined amount of extract (50 μg protein) was used for CAT activity assay. Each value is shown as fold activation over the value for control cells without TPA. (B) Reporter expression depends on the κB-cis element. κB-CAT or mutant κB-CAT reporter vector (3 μg) was transfected with PKC expression vector (2.5 μg) and empty vector (2.5 μg) . Cell were starved for 48 hours and either untreated (open bars) or treated with 100 ng/ml TPA (hatched bars). CAT assay was carried out as described above. Values are the means of three independent experiments.

To evaluate whether the observed enhancing effect on TPA-induced κB activation is specific to nPKCε, we next examined the effect of overexpression of other PKC isozymes on TPA-induced κB activation. PKC expression vectors were co-transfected with κB-CAT reporter genes and TRE-luciferase reporter gene. This approach made it possible to monitor simultaneously the effect of the overexpression of PKC isozymes on κB and TRE activation. As shown in figure 2A (right panel), TRE-luciferase expression with TPA stimulation was enhanced by cPKCα, cPKCβII, nPKCδ and nPKCε. This is consistent with our earlier observation that overexpression of cPKCα, cPKCβII, nPKCδ, and nPKCε all resulted in enhanced expression of TRE-CAT in a TRE-dependent manner (3, 11). nPKCθ also enhanced the expression of TRE-luciferase. These results show that the PKC isozymes are functionally expressed leading to the enhanced activation of TRE. Western blot analysis (figure 2B) confirms that 3Y1 cells express cPKCα, nPKCδ, and nPKCε, and that all

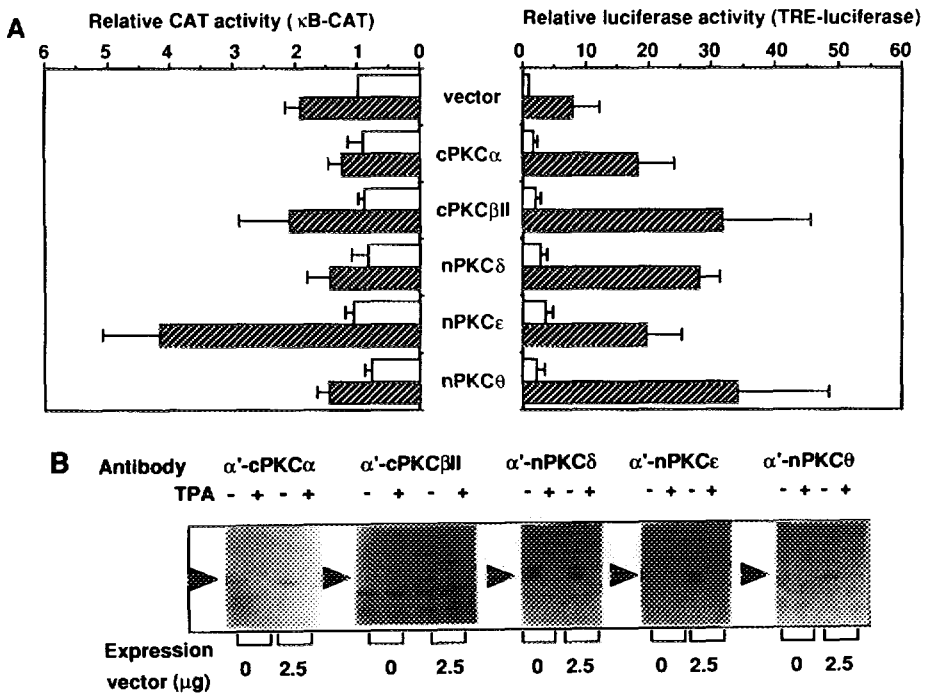

Figure 2.
(A) Effect of the overexpression of PKC isozymes on κB-CAT and TRE-luciferase reporter expression. After transfection of PKC expression vector (2.5 μg), κB-CAT reporter (3 μg) and TRE-luciferase reporter (1 μg) (60 mm dish), cells were starved and treated with (hatched bars) or without (open bars) 100 ng/ml TPA for 10 hours. CAT and luciferase activities were determined as described in "Experimental procedures". Three independent experiments were carried out. (B) Western blot analysis of endogenous and exogenous PKC isozymes. Cells were prepared as above harvested, and analyzed by SDS-PAGE followed by immunoblotting using PKC isozyme-specific antibodies. Specific bands of PKC isozymes are indicated by arrows.

the transfected PKC isozymes are expressed. It also shows the down-regulation of the PKC isozymes in TPA treated cells, that provides another evidence that the PKC isozymes are functionally expressed.

The left panel of figure 2A shows the results on κB activation. The overexpression of nPKCε resulted in a 2 to 2.5-fold enhancement of κB-CAT expression compared with the vector transfected control, while the overexpression of other PKC isozymes, cPKCα, nPKCδ, and nPKCθ, had no effect on the reporter expression. In the case of cPKCβII, a slight enhancement was sometimes observed. Similar results were obtained by monitoring κB-luciferase expression after a shorter (4 hours) stimulation by TPA (data not shown). These results with κB-reporters are in clear contrast to those with TRE reporters, indicating the specificity of nPKCε in the activation of κB.

Next we examined whether the kinase activity of nPKCε is involved in the specific activation of κB. For this purpose, we used a kinase-negative point mutant of nPKCε where Lys at the ATP binding site was substituted with Arg (13). Expression of wild type and mutant nPKCε was confirmed by Western blot analysis (figure 3A). The overexpression of mutant nPKCε did not cause enhanced κB activation; in fact, the endogenous κB activity was partially suppressed (figure 3B). This shows that the transcriptional activation of κB-reporter by nPKCε depends on kinase

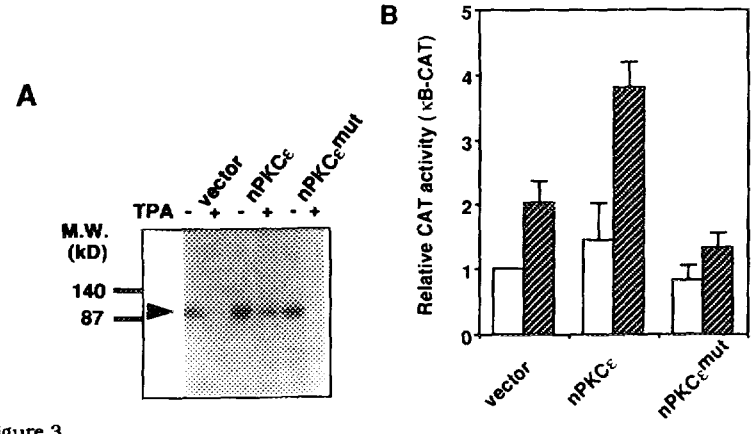

Figure 3.
(A) Western blot analysis of overexpressed nPKCε and kinase negative mutant of nPKCε. Wild type or mutant nPKCε expression vector (2.5 μg) was transfected with κB-CAT reporter (3 μg), keeping the total amounts of DNA at 8 μg with empty vector (60 mm dish). Cells were treated with or without 10 ng/ml TPA for 12 hours. The specific band representing the nPKCε isozyme is indicated by an arrow. (B) Activation of κB-CAT expression by nPKCε depends on its kinase activity. Cells were prepared as for the Western blot analysis in figure 3A. CAT activity was determined. Data from untreated cells are shown as open bar and stimulated by 10 ng/ml TPA as hatched bars. Three additional experiments gave similar results.

activity. The partial suppression of endogenous κB activity further suggests that the activation of nPKCε is necessary for the activation of NF-κB by TPA.

DISCUSSION

TPA regulates the transcription of a variety of genes and PKC is implicated as a major target of TPA in mediating such signal transduction (1). Previous studies have shown that the overexpression of several PKC isozymes caused an apparently equal enhancement of TRE or SRE activation by TPA (3, 11). As for NF-κB, there are many reports that TPA induces the activity of NF-κB, which is well known to regulate the transcription of a variety of genes (5). Further, PKC activates NF-κB DNA binding activity *in vitro* by phosphorylating and inactivating IκBα (7). However, differences among PKC isozymes in the potency of the NF-κB activator *in vivo* remain to be determined. In the present report, we have shown that the overexpression of nPKCε, but not cPKCα, nPKCδ, or nPKCθ, results in NF-κB activation by TPA in 3Y1 cells. Rat 3Y1 fibroblasts are shown to possess NF-κB activity that is inducible by TPA. In this cell line, cPKCα, nPKCδ, and nPKCε could be detected endogenously (figure 2B), therefore the effect of TPA on κB activation probably arises from the activation of endogenous nPKCε. A kinase negative nPKCε mutant did not cause enhancement, but partially suppressed reporter expression, probably due to a dominant negative effect. This also supports the idea that nPKCε is involved in NF-κB activation by TPA.

PKC has many isoforms, at least three classes, and ten isozymes, and each isozyme is expected to possess its own functional role. Some reports have demonstrated that PKCs can act as intracellular transmitters for transcriptional regulation in an isozyme-dependent manner. For example, SRE is activated by several PKC isozymes, cPKCα, βII, γ, nPKCδ and ε, while TRE is activated by all except cPKCγ (3, 11). cPKCα and cPKCβII differ in their regulation of β-myosin heavy chain gene expression in cardiac myocytes (14). Here, we provide additional evidence for the functional divergency of PKC isozymes in the regulation of transcription.

Other stimuli also activate NF-κB: cytokines, such as tumor necrosis factor α (TNFα); interleukin-1 and interleukin-2; stress, such as ultraviolet irradiation; phospholipids, such as diacylglycerol and ceramide; lipopolysaccharides; and viral infection (5, 15). Some of these stimuli have been shown, like TPA, to cause a rapid degradation of IκBα leading to the activation of NF-κB *in vivo* (16). On the other hand, PKC can increase κB site-dependent DNA binding activity *in vitro* in an ATP- and co-factor- dependent manner (7, 17), and phosphorylation by PKC renders IκBα inactive *in vitro* (7, 8). Ghosh and Baltimore (7) showed that a cPKC mixture isolated

from rat brain or partially purified cPKCα or cPKCγ activate NF-κB *in vitro*. This is not a simple agreement with the present results. The difference in cell type used or differences between *in vitro* and *in vivo* may explain the apparent discrepancy. Recently, aPKCζ, which is not activated directly by TPA, has been reported to play a significant role in the activation of NF-κB by TNFα (18, 19, 20). Interestingly, aPKCζ was demonstrated to bind a 50 kD IκB kinase which phosphorylates and inactivates IκBα *in vitro* (19). However, these observations do not explain the activation of NF-κB by TPA, since the kinase activity of aPKCζ is independent of TPA (21). How does nPKCε act as an NF-κB activator *in vivo*? If nPKCε phosphorylates IκBα directly, then nPKCε should phosphorylate more efficiently than cPKCα, nPKCδ, or nPKCθ. However if nPKCε acts on κB indirectly, then nPKCε might specifically activate another transmitter which in turn activates NF-κB. It is not known in which manner nPKCε activates NF-κB, and further study is needed.

REFERENCES

1. Nishizuka, Y. (1986) Science 233, 305-312
2. Nishizuka, Y. (1992) Science 258, 607-614
3. Ohno, S., Mizuno, k., Adachi, Y., Hata, A., Akita, Y., Akimoto, K., Osada, S.-i., Hirai, S.-i., and Suzuki, K. (1994) J. Biol. Chem. 269, 17495-17501
4. Mischak, H., Goodnight, J., Kolch, W., Martiny-Baron, G., Schaechtle, C., Kazanietz, G.K., Blumberg, P.M., Pierce, J.H., and Mushinski, J.F. (1993) J. Biol. Chem. 268, 6090-6096
5. Grill, M., Chiu, J.J.-S., and Lenardo, M.J. (1933) Int. Rev. Cytol. 143, 1-62
6. Meichle, A., Schütze, S., Hensel, G., Brunsing, D., and Krönke, M. (1990) J. Biol. Chem. 265, 8339-8343
7. Ghosh, S., and Baltimore, D. (1990) Nature 344, 678-682
8. Kerr, K.D., Inoue, J.-i., Davis, N., Link, E., Baeuerle, P.A., Bose Jr, H.R., and Verma, I.M. (1991) Genes & Dev. 5, 1464-1476
9. Fujita, T., Miyamoto, M., Kimura, Y., Hammer, J., and Taniguchi, T. (1989) Nucleic Acids Res. 17, 3335-3346
10. Fujita, T., Nolan, G.P., Liou, H.-C., Scott, M.L., and Baltimore, D. (1993) Genes & Dev. 7, 1354-1363
11. Hata, A., Akita, Y., Suzuki, K., and Ohno, S. (1993) J. Biol. Chem. 268, 9122-9129
12. Osada, S., Mizuno, K., Saido, T.C., Suzuki, K., Kuroki, T., and Ohno, S. (1992) Mol. Cell. Biol. 12, 3930-3938
13. Akita, Y., Ohno, S., Yajima, Y., Konno, Y., Saido, T.C., Mizuno, K., Chida, K., Osada, S., Kuroki, T., Kawashima, S., and Suzuki, K. (1994) J. Biol. Chem. 269, 4653-4660
14. Kariya, K.-i., Karns, L.R., and Simpson, P.C. (1991) J. Biol. Chem. 266, 10023-10026

15. Schütze, S., Potthoff, K., Machleidt, T., Berkovic, D., Wiegmann, K., and Krönke, M. (1992) Cell 71, 765-776
16. Beg, A.A., and Baldwin Jr, A.S. (1933) Genes & Dev. 7, 2064-2070
17. Shirakawa, F., and Mizel, S.B. (1989) Mol. Cell. Biol. 9, 2424-2430
18. Diaz-Meco, M. T., Berra, E., Municio, M.M., Sanz, L., Lozano, J., Dominguez, I., Diaz-Golpe , V., Lain de Lera, M. T., Alcami, J., Arenzana, F., Payá, C.V., Virelizier, J., and Moscat, J. (1993) Mol. Cell. Biol. 13, 4770-4775
19. Diaz-Meco, M. T., Dominguez, I., Sanz, L., Dent, P., Lozano, J., Municio, M.M., Berra, E., Hay, R.T., Sturgill, T.W., and Moscat, J. (1994) EMBO J. 13, 2842-2848
20. Lozano, J., Berra, E., Municio, M.M., Diaz-Meco, M.T., Dominguez, I., Sanz, L., and Moscat, J. (1994) J. Biol. Chem. 269, 19200-19202
21. Nakanishi, H., and Exton, J.H. (1992) J. Biol. Chem. 267, 16347-16354