The phorbol derivatives thymeleatoxin and 12-deoxyphorbol-13-*O*-phenylacetate-10-acetate cause translocation and down-regulation of multiple protein kinase C isozymes

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Phorbol esters such as phorbol 12-myristate,13-acetate (PMA) are potent activators of protein kinase C (PKC), and activate all PKC isozymes except ζ and λ. Recently, 12-deoxyphorbol-13-*O*-phenylacetate-20-acetate (dPPA) and thymeleatoxin (Tx) were reported to selectively activate PKCβ_1 (dPPA) and PKCα, -β, and -γ (Tx), but not PKCδ or PKCϵ in vitro. We examined the ability of these phorbol derivatives to translocate and down-regulate PKC isozymes in intact cells. Our findings demonstrate that dPPA and Tx cause translocation and down-regulation of multiple PKC isozymes, including δ and ϵ.

1. INTRODUCTION

Phosphorylation by protein kinase C (PKC) has an important role in signal transduction [1]. PKC modulates diverse cellular processes, such as secretion of hormones and neurotransmitters, gene expression, cell proliferation, and differentiation [2]. At least ten PKC isozymes have been identified, and can be divided into three, structurally related groups: conventional (cPKC; α, β_1, β_2 and γ); novel (nPKC; δ, ϵ, η/L, and θ); and atypical (aPKC; ζ and λ) [1]. cPKCs require phosphatidylserine, Ca^{2+} and diacylglycerol for activation, whereas nPKCs are independent of Ca^{2+} and aPKCs require neither Ca^{2+} nor diacylglycerol. These isozymes exhibit subtle differences in substrate specificity and kinetic properties, and differ in their tissue distribution and subcellular localization [1,3,4]. Specific functions have been suggested for the different isozymes on the basis of their heterogeneous characteristics and distribution. However, relatively little is actually known about their individual roles, since no isozyme-selective activators or inhibitors are available.

cPKC and nPKC isozymes respond to phorbol ester tumor promoters [1]. Phorbol esters mimic diacylglycerol and cause membrane association and activation of

PKC [5,6]. Phorbol esters also increase the susceptibility of PKC to proteolytic degradation, and prolonged exposure to phorbol esters causes loss of PKC activity [7,8]. Thus, phorbol esters such as phorbol 12-myristate,13-acetate (PMA) have been widely used as both acute activators and, with prolonged treatment, as inhibitors of PKC.

In addition to phorbol diesters, such as PMA, several chemically-related phorbol compounds have been isolated [9,10]. Major differences exist in the tumor-promoting capacity and other biological effects of these compounds [11–13]. Several bind and activate PKC with varying efficiency [14]. These compounds include 12-deoxyphorbol-13-*O*-phenylacetate-20-acetate (dPPA) and a mezerein analog, thymeleatoxin (Tx) [9,10]. Recently, Ryves et al. described selective activation of PKCβ_1 by dPPA and of PKCα, -β_1 and -γ by Tx, using an in vitro assay with purified PKC isozymes [15]. In this study, we investigated whether Tx and dPPA selectively activate these PKC isozymes in intact cells by assaying their ability to stimulate PKC translocation from cytosol to particulate fractions. In addition, we treated cells with Tx and dPPA for several hours to determine whether these agents could be used to selectively down-regulate specific PKC isozymes.

2. MATERIALS AND METHODS

2.1. *Materials*

PMA, Tx and dPPA were obtained from LC Services Corp. (Woburn, MA) and were stored at $-20°C$ in dimethylsulfoxide at 10^{-2} M. Final dilutions were made immediately before use. Rabbit polyclonal anti-PKCα, -δ, -ϵ, and -ζ were from Life Technologies

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Abbreviations: PMA, phorbol 12-myristate,13-acetate; dPPA, 12-deoxyphorbol-13-*O*-phenylacetate-20-acetate; Tx, thymeleatoxin; PKC, protein kinase C; PBS, phosphate-buffered saline.

(Gaithersburg, MD) and mouse monoclonal anti-PKCβ was from Seikagaku America (Rockville, MD). Peroxidase-conjugated anti-rabbit and anti-mouse antibodies were from Boehringer Mannheim (Indianapolis, IN). Other chemicals were from Sigma (St. Louis, MO).

Since dPPA can degrade in aqueous solutions, the stability of dPPA in culture medium was tested by thin-layer chromatography. dPPA was diluted to 100 μM in culture medium and was added to 35 mm culture dishes containing 2–4 × 10^6 PC12 cells per dish. After incubation for 2–24 h at 37°C, the medium was frozen, lyophilized, reconstituted in ethyl acetate, and developed in hexane/ethyl acetate (1:1) on fluorescent silica gel plates. Fresh dPPA was used as reference standard. dPPA and degradation products were visualized under UV light.

2.2. PKC translocation

PC12 cells were cultured in a humidified atmosphere of 10% CO_2 and 90% air in Dulbecco's modified Eagle medium containing 10% heat-inactivated horse serum, 5% fetal calf serum, 2 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were seeded into 175 cm² flasks (5–10 × 10^6 cells/flask) and grown to 70–80% confluence. Flasks were rinsed with buffer A containing 120 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 10 mM glucose and 25 mM HEPES, pH 7.4. PMA, dPPA or Tx were added at 100 nM in buffer A and cells were incubated at 37°C for 2 min. Cells were then dislodged from the flasks, centrifuged at 250 × g for 5 min, and resuspended in 2 ml of extraction buffer, containing 20 mM Tris-HCl (pH 7.5 at 4°C), 2 mM EDTA, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml of leupeptin and 25 μg/ml of soybean trypsin inhibitor. Cells were immediately homogenized with a Teflon–glass homogenizer, and centrifuged at 100,000 × g for 1 h at 4°C. The supernatants were stored on ice and the pellets were resuspended in 2 ml of extraction buffer containing 1% Triton X-100. The samples were mixed for 1 h at 4°C and centrifuged again at 100,000 × g for 1 h. Supernatants were diluted 4:1 (v/v) with 5× sample buffer (1× = 2% SDS, 10% glycerol, 0.025% Bromphenol blue, 62.5 mM Tris-HCl [pH 6.8]). Samples were heated at 90°C for 5 min and equal volumes of membrane and cytosol fractions (60–95 μg) were separated on 9% acrylamide gels.

2.3. Down-regulation of PKC isozymes

Cells were treated with either dPPA (10–1,000 nM) for 3 h, or Tx (10–1,000 nM) for 24 h. Cells were then collected in buffer A, centrifuged at 250 × g for 5 min and resuspended in buffer A. Cells were centrifuged at 250 × g for 5 min and the pellet was resuspended at 3 mg/ml in 1× sample buffer containing 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml of leupeptin and 25 μg/ml of soybean trypsin inhibitor. Samples were heated at 90°C for 5 min, and passed ten times each through a 21-gauge needle. After centrifugation at 10,000 × g for 10 min, 75 μg samples were subjected to SDS-PAGE on 9% acrylamide gels.

2.4. Immunoblotting

Proteins were electrophoretically transferred from gels to nitrocellulose membranes [16]. Membranes were blocked in 2% bovine serum albumin in PBS for 1 h at room temperature, before incubation with PKC isozyme-specific antibodies for 16–20 h at 4°C. Protein G-purified rabbit polyclonal anti-PKCα, -δ, -ε, and -ζ (0.5 mg/ml) were used at a dilution of 1:300 and mouse monoclonal anti-PKCβ (100 μg/ml) at 1:200. As demonstrated previously, these antibodies recognize proteins of appropriate molecular weight on Western blots, and immunoreactivity against these bands is blocked by preincubation of each antibody with its corresponding PKC isozyme-specific antigen peptide or purified PKC isozyme [16]. Membranes were washed three times for 10 min in PBS/0.2% Tween-20 and then were incubated for 1 h with peroxidase-conjugated anti-rabbit or anti-mouse antibodies (1:1,000 dilution). Membranes were then washed four times in PBS/0.2% Tween-20 and processed for detection of bands by chemiluminescence with the ECL-chemiluminescence system (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. Blots were exposed to Kodak X-AR film and the autoradiograms were scanned

with a Microscan 1000 Gel Scanner (Technology Resources Inc., Nashville, TN). Values were normalized against Coomassie stained gels as described [16]. Differences between mean density values from several experiments were compared by one way ANOVA and where significant differences were found ($P < 0.05$), the two-tailed Dunnett post-hoc test was used to identify significant differences between means of control and treated groups.

3. RESULTS

Nearly all PKCα and -β immunoreactivity in unstimulated cells was found in the cytosol, whereas a considerable portion of PKCδ, -ε, and -ζ immunoreactivity was localized in the particulate fraction (Figs. 1 and 2). As previously reported, no PKCγ-immunoreactivity could be detected in our clone of PC12 cells [16]. The antibody to PKCε recognized a doublet of 90 and 92 kDa (Figs. 1 and 3) as reported previously [16]. The different molecular masses of these two forms appear to be due to differences in phosphorylation [17]. Treatment of cells with 100 nM of either PMA, Tx, or dPPA for 2 min resulted in a shift of PKCα, -β, -δ and -ε immunoreactivity from the cytosol to the particulate fraction (Figs. 1 and 2), although translocation induced by PMA was greater than by dPPA or Tx. The distribution of PKCζ between cytosol and particulate fractions was not affected by any of the phorbol derivatives.

When stored in dilute solutions, dPPA may degrade to 12-deoxyphorbol 13-phenylacetate, which activates PKCα, -β, -γ, -δ and -ε in vitro [15]. To investigate whether dPPA degrades in cultures of PC12 cells, we analyzed the stability of dPPA in media by thin-layer chromatography. dPPA added to cultures for 2–4 h remained intact ($R_f = 0.35$–0.5), but after 12 h, another
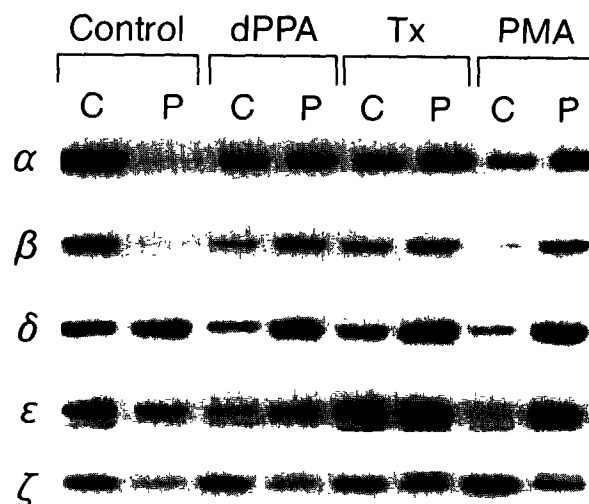

Fig. 1. Translocation of PKC-isozymes by dPPA, Tx, and PMA. Cells were treated with 100 nM dPPA, Tx, or PMA for 2 min and fractionated into cytosol (C) and particulate (P) fractions. Samples of each fraction derived from equal numbers of cells were separated by SDS-PAGE, transferred to nitrocellulose and immunostained with antibodies against PKC isozymes.

spot (R_f = 0.1–0.2) appeared, indicating degradation of dPPA. Therefore, we exposed cells to dPPA for only 3 h to examine its ability to down-regulate PKC isozymes. dPPA (1 μM) almost completely eliminated PKCα, -β, -δ and -ε immunoreactivity (Fig. 3). A lower concentration (100 nM) caused partial down-regulation of PKCβ, -δ and -ε immunoreactivity, but did not reduce levels of PKCα. Although 10 nM dPPA did not reduce PKC-immunoreactivity, it caused the appearance of a second species of PKCα, -β, -δ and -ε with decreased mobility (Fig. 3). Tx (100 nM–1 μM for 24 h) almost completely abolished PKCα, -β, -δ and -ε-immunoreactivity, and a slight reduction was already detected with 10 nM Tx

Fig. 3. Down-regulation of PKC by Tx and dPPA. Cells were incubated with 10 nM–1 μM dPPA for 3 h or with 10 nM–1 μM Tx for 24 h and subjected to SDS-PAGE, transferred to nitrocellulose, and immunostained with isozyme-specific anti-PKC antibodies. Data are from a representative experiment repeated twice with similar results. c = control lane.

(Fig. 3). PKCζ-immunoreactivity was not affected by 10 nM–1 μM dPPA or Tx.

4. DISCUSSION

In order to study the role of different PKC isozymes in various physiological functions, it would be very useful to have selective activators and inhibitors of individual PKC-isozymes or isozyme groups. In intact cells, activation of PKC isozymes is commonly assayed by measuring translocation of PKC activity and immunoreactivity to particulate fractions [18]. Translocation is thought to reflect PKC activity, since it enables the interaction of PKC with membrane phospholipids, which all PKC subspecies appear to require for activation [1]. The recent work of Ryves et al. [15] using an in vitro PKC assay suggested that dPPA might be used to selectively activate PKCβ_1, and Tx to activate PKCα, -β_1, and -γ in intact cells. However, we found that Tx and dPPA induced translocation of all PKC isozymes except PKCζ in PC12 cells. Thus, our results indicate that in intact cells, Tx and dPPA induce translocation of several PKC isozymes and are, therefore, no more isozyme-selective than PMA.

Our conclusions may contrast with those of Ryves et al. [15] because we used a translocation assay to measure PKC activation. Although association of PKC isozymes with lipid-containing particulate fractions is thought to be necessary for activation [19] membrane binding of PKC may occur without activation. For example, in inside-out erythrocyte vesicles, membrane binding of PKC is maximal at 500 nM Ca^{2+}, whereas PKC is activated by 5–50 μM Ca^{2+} when diacylglycerol
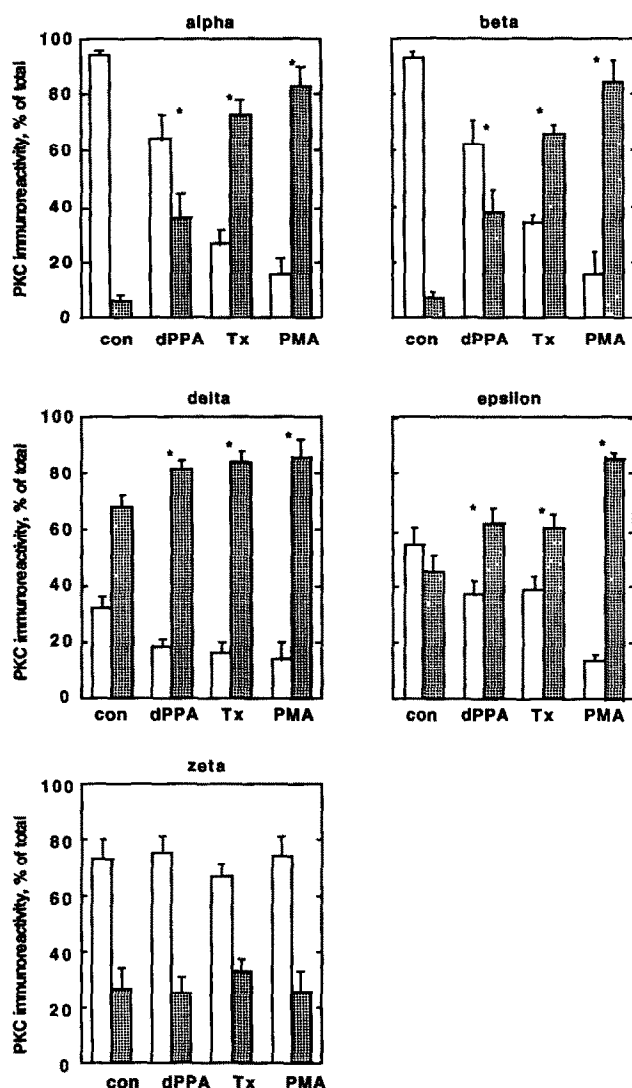

Fig. 2. The percentage of total isozyme-specific immunoreactivity found in cytosol (open bars) and particulate (filled bars) fractions in control cells (con) and after treatment with 100 nM dPPA, Tx or PMA for 2 min. Data are mean ± S.E.M. values from 4–6 experiments. P = 0.0001 (alpha), 0.0001 (beta), 0.03 (delta), 0.0004 (epsilon), 0.762 (zeta). * Significantly different from control (α = 0.05) by two-tailed Dunnett post-hoc test.
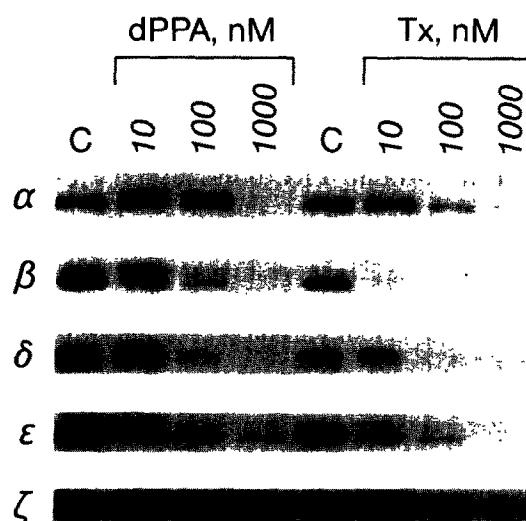
or phorbol esters are absent [19]. Moreover, in vitro, PKC activity is inhibited by salt concentrations that fail to dissociate PKC from lipid vesicles [20]. In addition, staurosporine causes PKC translocation but inhibits PKC activation [21]. However, we are unaware of any studies demonstrating PKC translocation without activation in intact cells in the absence of PKC inhibitors. On the other hand, PKC activation may occur without translocation [22,23], suggesting that, in some cases, translocation is an insensitive indicator of activation. In such cases, the inability to detect significant translocation might be due to activation of nPKCs, since a major fraction of nPKCs, especially δ, is already present in the particulate fraction of unstimulated cells (Figs. 1 and 2, and [24–27]). Despite the relative insensitivity of the translocation assay, we were able to detect translocation of nPKCs after treatment with dPPA and Tx. Thus, our results strongly suggest that dPPA and Tx activate nPKCs in intact cells.

Our results also demonstrate that dPPA and Tx can not be used to selectively down-regulate cPKCs. The down-regulation of multiple PKC-isozymes by Tx and dPPA, as well as the appearance of isozyme subspecies with decreased mobility on SDS-acrylamide gels after treatment with dPPA (Fig. 3) demonstrates that these phorbol derivatives, like PMA, have major effects on multiple PKC isozymes after prolonged exposure. Taken together with the findings of our translocation studies, these results indicate that dPPA and Tx regulate multiple cPKC and nPKC isozymes in intact cells, and are no more isozyme-selective than PMA for studies of PKC translocation or down-regulation.

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