0026-895X/95/020258-08$03.00/0

MOLECULAR PHARMACOLOGY, 47:258–265, 1995.

Differential Regulation by Anti-Tumor-Promoting 12-Deoxyphorbol-13-Phenylacetate Reveals Distinct Roles of the Classical and Novel Protein Kinase C Isozymes in Biological Responses of Primary Mouse Keratinocytes

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Received June 30, 1994; Accepted November 14, 1994

SUMMARY

12-Deoxyphorbol-13-phenylacetate (dPP) is the prototype for a new class of phorbol derivatives that function as protein kinase C (PKC) activators with potent anti-tumor-promoting activity. To explore the mechanism of action of dPP, we have conducted detailed analyses of the translocation and down-regulation patterns of individual PKC isozymes in mouse primary keratinocytes upon dPP treatment. PKC-α, -δ, and -ϵ were very quickly (within 2–5 min) translocated from the soluble fraction to the Triton X-100-soluble particulate fraction. PKC-δ and -ϵ were translocated with 2 orders of magnitude higher potency than was PKC-α. After translocation, PKC-α, -δ, -η, and -ϵ were down-regulated; the down-regulation of PKC-ϵ contrasts with its retention after phorbol-12-myristate-13-acetate or bryostatin treatment. As was the case with translocation, dPP down-regulated the novel PKC isozymes (δ, ϵ, and η) with 2 orders of magnitude higher potency (ED_{50}, about 1–2 nM), compared with PKC-α (ED_{50}, about 100 nM). dPP induced transglutaminase activity, ornithine decarboxylase activity, and cornification with potencies similar to that for PKC-α translocation. On the other hand, dPP caused inhibition of EGF binding with a potency similar to that for the translocation of the novel PKC isozymes. Although the generality of its selectivity in different cell types remains to be determined, at least in keratinocytes dPP is a powerful tool for dissecting the involvement of the classical and novel PKC isozymes in biological responses. The unique regulatory pattern of PKC-ϵ could contribute to the anti-tumor-promoting activity of dPP.

Activation of PKC has been widely assumed to lead to tumor promotion, based on the discovery that the receptor for the potent tumor promoter PMA is PKC (for reviews, see Refs. 1 and 2). This simple model of PKC activation leading to tumor promotion faces several complications. First, it has been shown in the past decade that PKC is a family of at least 11 isozymes with distinct properties (3). Second, potent PKC activators can either be inactive as tumor promoters or, indeed, function as inhibitors of tumor promotion. For example, bryostatin 1 proved to be inactive as a first-stage promoter (4) and very weak as a second-stage promoter or complete promoter (5), and it acted as a strong inhibitor of first-stage promotion (4) and a modest inhibitor of complete promotion (5). Even more dramatically, some 12-deoxyphorbol-13-monoesters are completely inactive as tumor promoters and very potent inhibitors of complete promotion (6). They also inhibit PMA-induced inflammation and hyperplasia (7). Third, the bryostatins not only induce a distinct pattern of biological responses but also show distinctly different regulation of individual PKC isozymes, compared with typical phorbol esters. For example, in fibroblasts and mouse primary keratinocytes bryostatin 1 induces biphasic down-regulation of PKC-δ, in good correlation with its biphasic biological responses (8, 9), and bryostatin 1 inhibits PMA-induced down-regulation of PKC-δ with dose-response curves identical to those for its inhibition of PMA-induced biological responses (8). These observations and others suggest the working hypothesis that some PKC isozymes might be involved in tumor formation and others in the inhibition of tumor promotion.

In this paper we examined the regulation of the individual PKC isozymes α, δ, ϵ, η, and ζ by a 12-deoxyphorbol-13-monoester, dPP, that shows potent anti-tumor-promoting activity. The PKC isozymes examined comprise all of the PKC isozymes that have been detected so far in cultured primary mouse keratinocytes (10) and represent all three classes of PKC isozymes (α, classical; δ, ϵ, and η, novel; ζ, atypical).

ABBREVIATIONS: PKC, protein kinase C; dPP, 12-deoxyphorbol-13-phenylacetate; PMA, phorbol-12-myristate-13-acetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGF, epidermal growth factor.

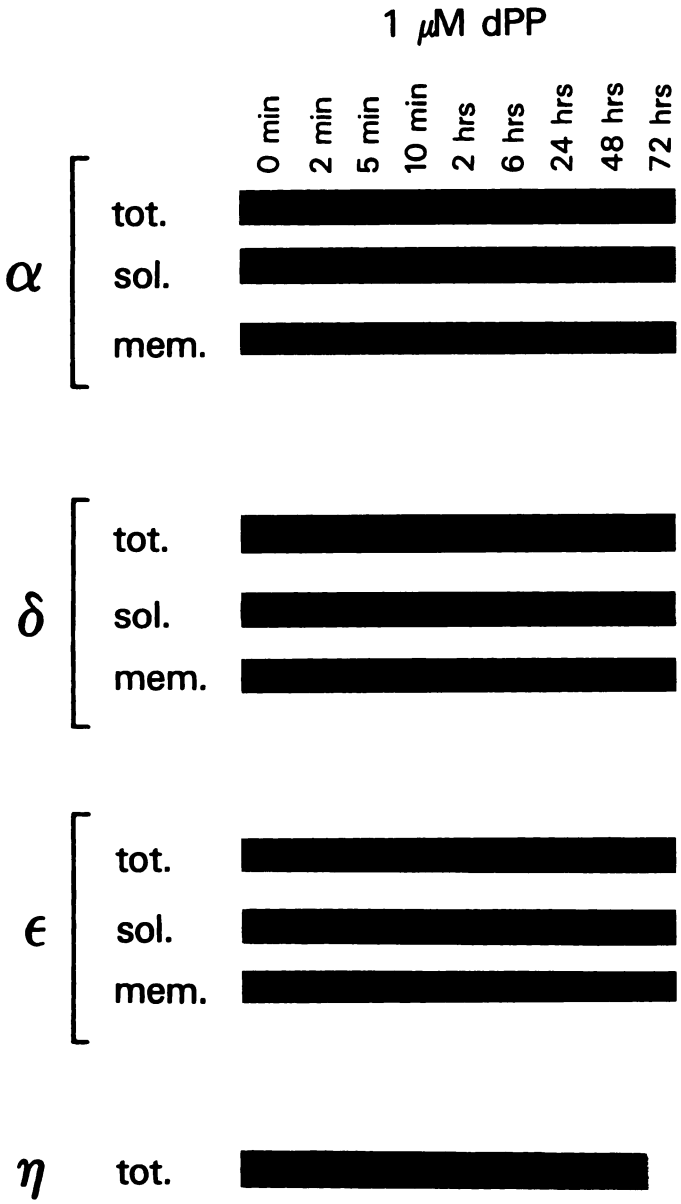

Fig. 1. Translocation and down-regulation of PKC isozymes induced by 1 μM dPP. Primary mouse keratinocytes were treated as indicated for 2, 5, or 10 min or 2, 6, 24, 48, or 72 hr. Samples for SDS-PAGE were prepared and Western immunoblotting was performed as described in Experimental Procedures. Equal amounts of protein from the total (10 μg of protein/lane), soluble (10 μg of protein/lane), and Triton X-100-soluble (5 μg of protein/lane) fractions were loaded in each lane. *tot.*, total fraction; *sol.*, soluble fraction; *mem.*, Triton X-100-soluble fraction. Identical results were obtained in two more independent sets of experiments.

Although two classes of high affinity receptors for the phorbol esters distinct from PKC are known (the chimaerin and unc-13 classes), the former is limited to brain and testes and the latter has been reported only in nematodes (for review, see Ref. 11). We report that dPP shows a unique pattern of isozyme regulation and that the regulation of individual PKC isozymes in mouse keratinocytes correlates with different biological responses induced by dPP.

Experimental Procedures

Primary keratinocytes were prepared from newborn BALB/c mice by the trypsin flotation technique and were cultured in low-calcium (0.05 mM) Eagle's minimal essential medium containing 8% fetal calf serum and antibiotics, as described previously (12). dPP was purchased from LC Services (Woburn, MA).

For Western blot analysis of the PKC isozymes, treatments were started 3 days after the cells were plated. At that time they had attained 80–90% confluency. All compounds were applied in ethanol (0.1% final concentration). Western blot analysis was performed as described previously (9). The cells were harvested in 20 mM Tris·HCl, pH 7.4, containing 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 20 μM leupeptin and were lysed by sonication. Total cell lysates were fractionated by ultracentrifugation. The cytosolic fraction represents the supernatant after centrifugation at 100,000 × *g* for 1 hr at 4°. The Triton X-100-soluble particulate fraction was prepared by 1-hr extraction of the pellet with the same buffer containing 1% Triton X-100 and subsequent centrifugation for 1 hr at 100,000 × *g*. The remaining pellet is the Triton X-100-insoluble fraction. The protein samples were subjected to SDS-PAGE according to the method of Laemmli (13) and were transferred to nitrocellulose membranes. Western blots were stained with 0.1% Ponceau S solution in 5% acetic acid (Sigma Chemical Co., St. Louis, MO) to determine the protein content of individual lanes. The protein staining was found to be linear up to 30 μg of protein/lane. Monoclonal antibody against the catalytic domain of PKC-α was purchased from Upstate Biotechnology (Lake Placid, NY) and used at a 2 μg/ml concentration. Affinity-purified polyclonal antibody against the carboxyl terminus (amino acids 662–673) of PKC-δ was purchased from Research and Diagnostics Antibodies (Berkeley, CA) and applied at a 1/50,000 dilution. Polyclonal antibody against the carboxyl terminus (amino acids 480–492) of PKC-ζ was purchased from Research and Diagnostics Antibodies and applied at a dilution of 1/5000. Monoclonal antibody raised against a 19.9-kDa amino-terminal fragment corresponding to residues 1–175 of PKC-ϵ was purchased from Transduction Laboratories (Lexington, KY) and used at a dilution of 1/250. Polyclonal antibody against the carboxyl terminus (amino acids 669–683) of PKC-η was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and applied at a dilution of 1/250. The demonstration of specificity and lack of cross-reactivity of the primary antibodies for the PKC isozymes was described in a previous paper (8). The blots were incubated overnight at 4° with the indicated amounts of primary antibody dissolved in 4% milk in phosphate-buffered saline. The PKC isozymes were detected by horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories, Richmond, CA) and quantitated as described previously (9). For densitometric quantitation, we used the National Institutes of Health Image 1.52 program (written by Dr. Wayne Raspband, National Institutes of Health).[1]

Cornification was measured by the quantitation of cornified envelopes induced as described previously (14). Ornithine decarboxylase activity was quantitated as the release of CO_2 from L-[^{14}C]ornithine, as described previously (15). Transglutaminase activity was determined by measuring cross-linking of [^{3}H]putrescine to dimethylcasein as described (14). ^{125}I-EGF binding was assessed by incubating the previously treated keratinocytes with ^{125}I-EGF for 5 hr on ice and then measuring the specifically bound radioactive ligand with a scintillation counter, as described elsewhere (16).

The metabolism of dPP was assessed by using radioactively labeled ligand. [20-^{3}H]dPP (9.5 Ci/mmol) was prepared by DuPont-NEN from dPP-20-aldehyde (LC Services) and purified in our laboratory by high pressure liquid chromatography. For the analysis of metabolism, [20-^{3}H]dPP was diluted with nonradioactive dPP to achieve the desired final specific activity. [^{3}H]dPP (1 μM, 0.9 Ci/mmol) was added to the keratinocytes (cultured in standard 12-well plates) in standard keratinocyte medium for 5 min, 2 hr, 6 hr, 1 day, 2 days, or 3 days. The medium was collected, lyophilized, and extracted with methanol. The methanol extracts were dried down, redissolved in a small volume of

[1] The program is available electronically via Internet by anonymous ftp from zippy.nimh.nih.gov or from Library 9 of the MacApp forum on CompuServe and is available on floppy disk from NTIS, 5285 Port Royal Rd., Springfield, VA 22161.

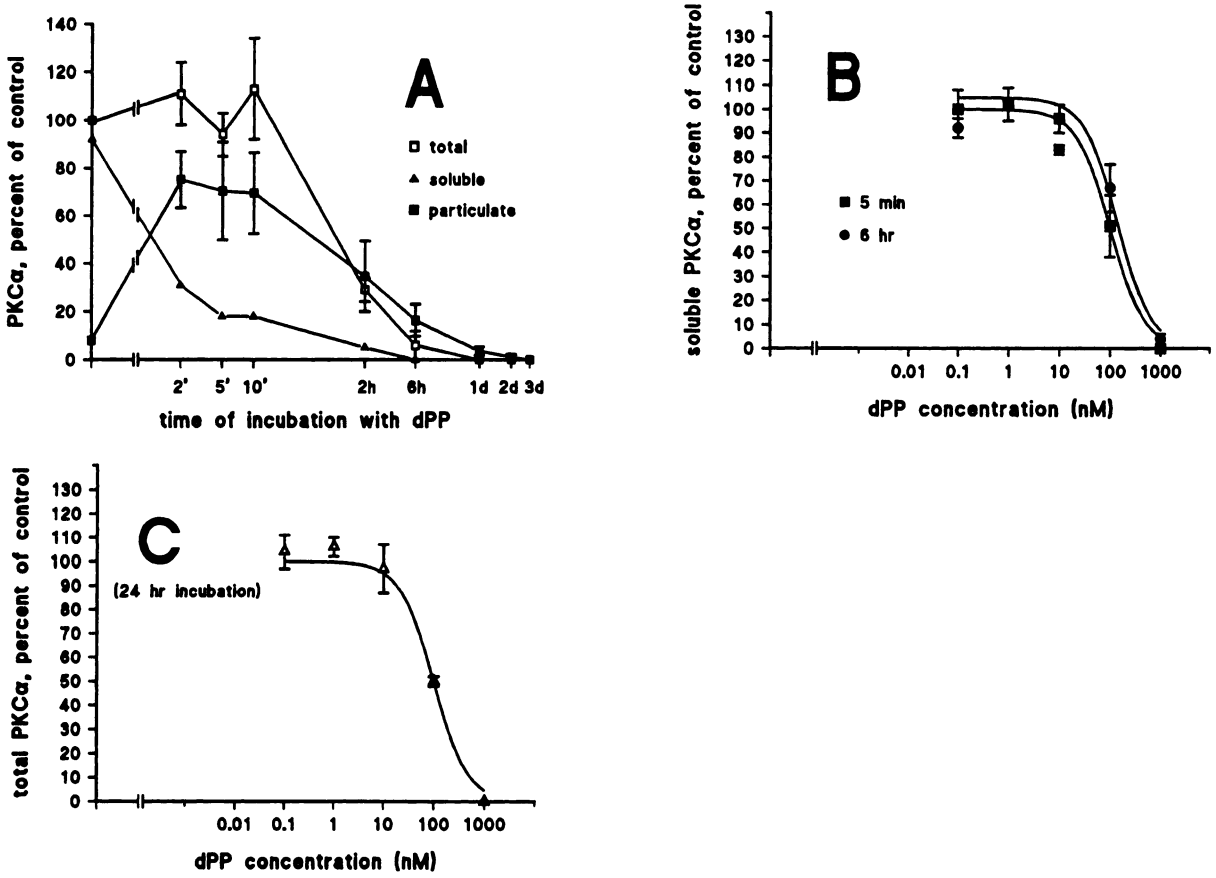

Fig. 2. A, Translocation and down-regulation of PKC-α induced by 1 μM dPP. Primary mouse keratinocytes were treated with 1 μM dPP for 2, 5, or 10 min or 2, 6, 24, 48, or 72 hr. Samples for SDS-PAGE were prepared and Western immunoblotting was performed as described in Experimental Procedures. The amount of the enzyme was quantitated in the total, soluble, and Triton X-100-soluble particulate fractions by densitometry and is expressed as a percentage of the total amount of isozyme present in control cells. Time is plotted on a logarithmic scale. Each *point* is the average ± standard error of three independent measurements. B and C, dPP-induced changes in the levels of PKC-α in the soluble (B) and total (C) fractions of primary mouse keratinocytes. Primary mouse keratinocytes were treated with the indicated doses of dPP for 5 min or 6 hr (B) or 24 hr (C). Samples for SDS-PAGE were prepared and Western immunoblotting was performed as described in Experimental Procedures. The amount of the enzyme was quantitated by densitometry and is expressed as a percentage of the amount of isozyme present in the soluble fraction (B) or in the total fraction (C) from control cells. Each *point* is the average ± standard error of three independent sets of experiments.

TABLE 1

Stability of radioactively labeled dPP in mouse keratinocyte cultures

Mouse keratinocytes were treated with 1 μM [^{3}H]dPP for periods of 5 min to 3 days, and the metabolism of dPP was assessed as described in Experimental Procedures. Each value is the mean ± range of duplicate determinations in a single experiment. A second experiment yielded similar results.

Time of treatment	[^{3}H] dPP remaining
	%
5 min	93 ± 5
2 hr	101 ± 4
6 hr	98 ± 4
1 day	91 ± 5
2 days	98 ± 2
3 days	98 ± 4

ethyl acetate, and chromatographed on normal phase thin layer chromatography plates (with aluminum backing) (EM Science, Gibbstown, NJ), using ethyl acetate as the solvent (the relative mobility of dPP under these conditions was about 0.5). The thin layer chromatography plates were cut into strips corresponding to individual samples, each strip was cut into 10 equal pieces that were transferred into scintillation vials, Aquasol was added, and the radioactivity was measured in a Pharmacia Biotech 1218 scintillation counter.

Results

We have previously reported the amounts and distribution of PKC isozymes α, δ, ϵ, and ζ found in primary mouse keratinocytes (8). The availability of new antibodies now makes it possible to quantitate the amount of PKC-η.

dPP caused rapid translocation of PKC-α from the soluble fraction to the Triton X-100-soluble particulate fraction (Figs. 1 and 2A).[2] In 2–10 min 85% of the soluble PKC-α was translocated. At these early time points no loss in the total amount of PKC-α was observed and the amounts of the isozyme disappearing from the soluble fraction could be recovered from the Triton X-100-soluble particulate fraction in a quantitative fashion. The dose-response curve for translocation was determined using the amount of isozyme remaining in the soluble fraction after 5-min treatment (Fig. 2B). The level of PKC-α steadily decreased with time, reaching maximal down-regulation at 24 hr after treatment. No restoration of the isozyme level was observed for 72 hr. Over this time period, dPP was

[2] dPP very quickly dissociates from its receptor, leading to immediate redistribution of PKC isozymes α and δ to the soluble fraction. To maintain the dPP concentrations used for the treatments, we included an equimolar concentration of dPP in every washing buffer and homogenizing solution for all preparative steps (32).

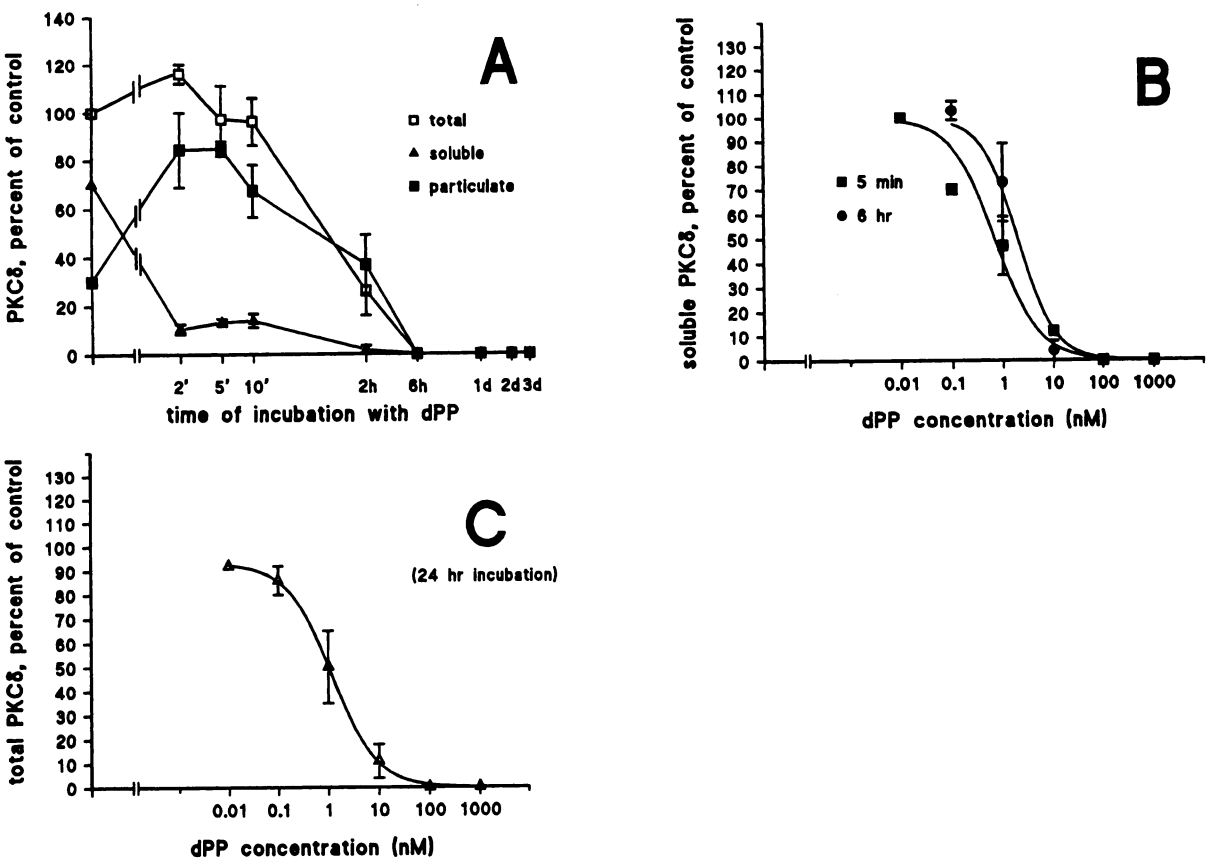

Fig. 3. A, Translocation and down-regulation of PKC-δ induced by 1 μM dPP. Primary mouse keratinocytes were treated with 1 μM dPP for 2, 5, or 10 min or 2, 6, 24, 48, or 72 hr. Samples for SDS-PAGE were prepared and Western immunoblotting was performed as described in Experimental Procedures. The amount of the enzyme was quantitated in the total, soluble, and Triton X-100-soluble particulate fractions by densitometry and is expressed as a percentage of the total amount of isozyme present in control cells. Time is plotted on a logarithmic scale. Each *point* is the average ± standard error of three independent measurements. B and C, dPP-induced changes in the levels of PKC-δ in the soluble (B) and total (C) fractions of primary mouse keratinocytes. Primary mouse keratinocytes were treated with the indicated doses of dPP for 5 min or 6 hr (B) or 24 hr (C). Samples for SDS-PAGE were prepared and Western immunoblotting was performed as described in Experimental Procedures. The amount of the enzyme was quantitated by densitometry and is expressed as a percentage of the amount of isozyme present in the soluble fraction (B) or in the total fraction (C) from control cells. Each *point* is the average ± standard error of three independent sets of experiments.

not metabolized, as was determined by extraction of radiolabeled dPP and analysis by thin layer chromatography (Table 1).

The potency of dPP for down-regulation was quantitated at 24 hr, the time giving maximal down-regulation, using the amount of PKC-α remaining in the total fraction (Fig. 2C). We also determined the potency of dPP to remove PKC-α from the soluble fraction at an intermediate time point, i.e., 6 hr (Fig. 2B). Previously we found that in the case of PMA and bryostatin 1 there was a marked shift in the dose-response curves for removal of PKC from the soluble fraction determined at shorter (5–20-min) and longer (6-hr) time points (8). This pattern of slow translocation might produce down-regulation of PKC with very low levels of activation. For dPP the dose-response curves determined at 5 min and 6 hr were practically identical (ED_{50} at 5 min, 100 ± 5 nM; at 6 hr, 140 ± 10 nM), as was expected because of the very fast on-rate of this ligand. The dose-response curves for translocation and down-regulation were the same, giving an ED_{50} of approximately 100 nM for both effects.

High doses of dPP translocated PKC-δ from the soluble fraction to the Triton X-100-soluble fraction very rapidly, within 2–5 min (Figs. 1 and 3A). The dose-response curve for translocation of PKC-δ was determined at 5 min after treatment, using the amount of isozyme remaining in the soluble fraction (Fig. 3B). As with PKC-α, we also determined the potency of dPP to remove PKC-δ from the soluble fraction at 6 hr after treatment (Fig. 3B). The two dose-response curves showed no significant shift, indicating the fast on-rate of the ligand (ED_{50} at 5 min, 0.8 ± 0.1 nM; at 6 hr, 2 ± 0.1 nM). The amount of PKC-δ translocated to the Triton X-100-soluble particulate fraction was subsequently down-regulated by 24 hr after treatment (Fig. 3A). The dose-response curve for down-regulation was determined at this time point, using the amount of isozyme remaining in the total fraction (ED_{50}, about 1.1 ± 0.1 nM) (Fig. 3C). The potency of dPP for translocating and down-regulating PKC-δ was thus 2 orders of magnitude higher than that for PKC-α.

PKC-ε, similarly to PKC-α and PKC-δ, was quickly translocated from the soluble fraction to the Triton X-100-soluble particulate fraction. Two minutes after treatment with 1 μM dPP, no isozyme was left in the soluble fraction (Figs. 1 and 4A). We determined the dose-response curve for translocation at 5 min after treatment, using the amount of the isozyme remaining in the soluble fraction (Fig. 4B), and the dose-response curve for down-regulation at 24 hr after treatment,

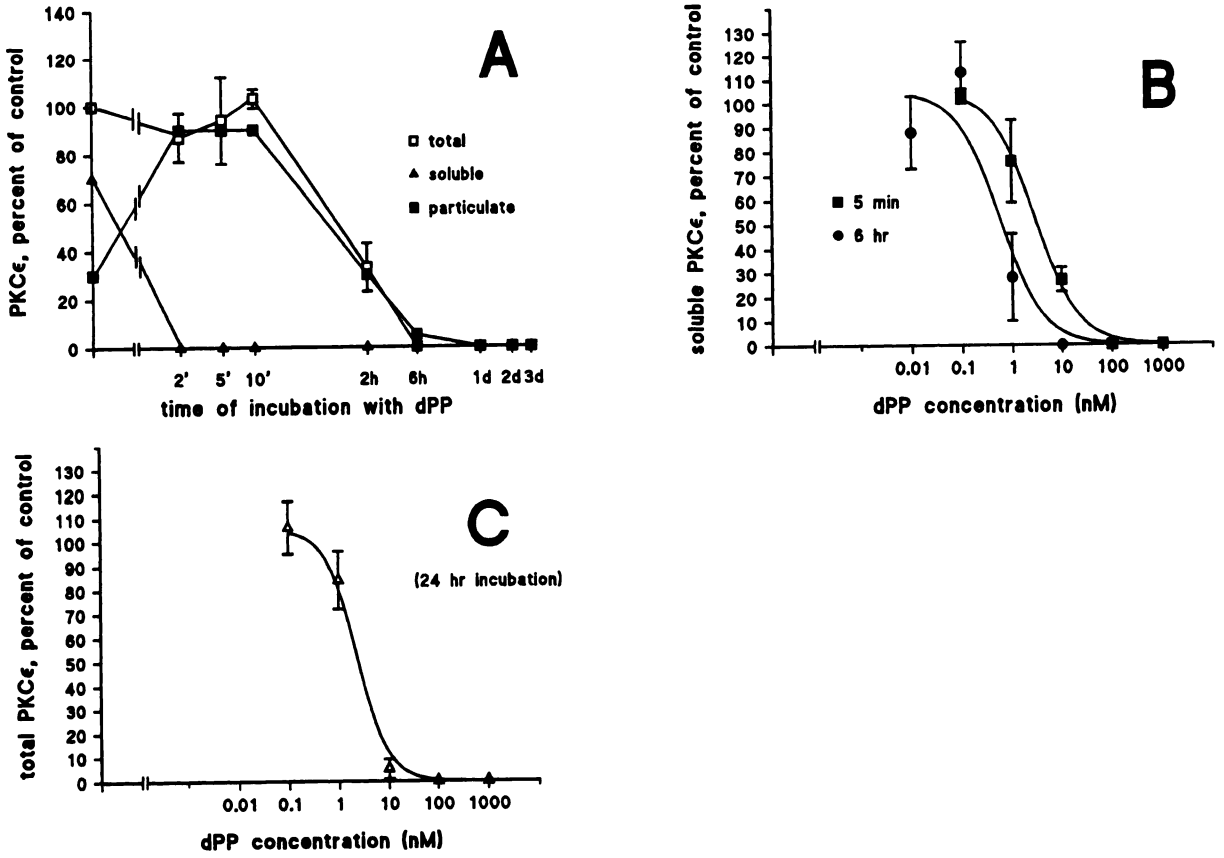

Fig. 4. A, Translocation and down-regulation of PKC-ε induced by 1 μM dPP. Primary mouse keratinocytes were treated with 1 μM dPP for 2, 5, or 10 min or 2, 6, 24, 48, or 72 hr. Samples for SDS-PAGE were prepared and Western immunoblotting was performed as described in Experimental Procedures. The amount of the enzyme was quantitated in the total, soluble, and Triton X-100-soluble particulate fractions by densitometry and is expressed as a percentage of the total amount of isozyme present in control cells. Time is plotted on a logarithmic scale. Each *point* is the average ± standard error of three independent measurements. B and C, dPP-induced changes in the levels of PKC-ε in the soluble (B) and total (C) fractions of primary mouse keratinocytes. Primary mouse keratinocytes were treated with the indicated doses of dPP for 5 min or 6 hr (B) or 24 hr (C). Samples for SDS-PAGE were prepared and Western immunoblotting was performed as described in Experimental Procedures. The amount of the enzyme was quantitated by densitometry and is expressed as a percentage of the amount of isozyme present in the soluble fraction (B) or in the total fraction (C) from control cells. Each *point* is the average ± standard error of three independent sets of experiments.

when down-regulation was maximal, using the amount of PKC-ε remaining in the total fraction (ED_{50}, 2 ± 0.1 nM) (Fig. 4C). We also determined the potency of dPP for removing PKC-ε from the soluble fraction at an intermediate time point, 6 hr after treatment, which yielded a dose-response curve similar to that for translocation at 5 min (ED_{50} at 5 min, 3 ± 0.3 nM; at 6 hr, 1 ± 0.2 nM) (Fig. 4B). Similarly to PKC-δ, PKC-ε was translocated and down-regulated with 2 orders of magnitude higher potency than PKC-α.

We found significant breakdown of PKC-η during preparation of the soluble and Triton X-100-soluble particulate fractions, precluding precise quantitation in fractionated cells. Therefore, we measured the amount of PKC-η in the total fraction after harvesting and immediate boiling in SDS sample buffer. The down-regulation of PKC-η was somewhat slower and less complete than that of any of the other isozymes (Fig. 5A). About 85% down-regulation was reached 24 hr after treatment, with a dose-response curve similar to that of PKC-δ and -ε (ED_{50}, 1 ± 0.1 nM) (Fig. 5B), and further down-regulation was not seen at later times.

In the case of PKC-ζ we did not detect any translocation or down-regulation upon dPP treatment (data not shown).

We determined the potency of dPP for inducing the following biological responses associated with PKC activation in keratinocytes: cornified envelope formation (a measure of differentiation in keratinocytes), induction of transglutaminase activity, induction of ornithine decarboxylase activity, and down-regulation of EGF binding. dPP at 1 μM induced all four responses to the same extent as did 1 μM PMA. The ED_{50} for cornified envelope formation was determined at 48 hr, giving a value of about 100 nM (Fig. 6A), transglutaminase activity was maximal at 6 hr after treatment, giving an ED_{50} of 140 ± 20 nM (Fig. 6B), ornithine decarboxylase activity was maximal at 3 hr after treatment, with an ED_{50} of 120 ± 20 nM (Fig. 6C), and down-regulation of ^{125}I-EGF binding was determined at 1 hr and 3 hr after treatment, giving an ED_{50} of 2 ± 0.2 nM at 1 hr and 1.5 ± 0.3 nM at 3 hr (Fig. 6D).

Discussion

The phenomenon of differential regulation of PKC isozymes by PKC activators with distinct, sometimes opposing, biological activities has been previously described in our laboratory (8, 9). We wished to determine whether another PKC activator, dPP, with unique biological and anti-tumor-promoting activity also showed a unique translocation and down-regulation pattern for individual PKC isozymes in primary mouse keratino-

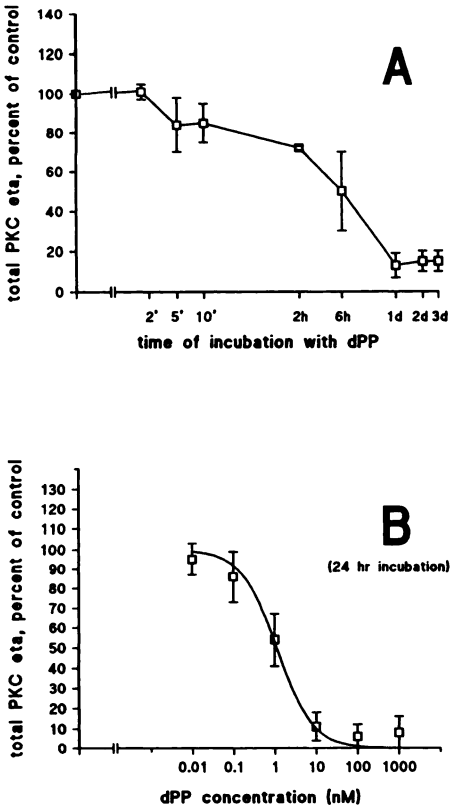

Fig. 5. A, Down-regulation of PKC-η induced by 1 μM dPP. Primary mouse keratinocytes were treated with 1 μM dPP for 2, 5, or 10 min or 2, 6, 24, 48, or 72 hr. Samples for SDS-PAGE were prepared and Western immunoblotting was performed as described in Experimental Procedures. The amount of the enzyme was quantitated in the total fraction by densitometry and is expressed as a percentage of the total amount of isozyme present in control cells. Time is plotted on a logarithmic scale. Each *point* is the average ± standard error of three independent measurements. B, dPP-induced changes in the levels of PKC-η in the total fraction of primary mouse keratinocytes. Primary mouse keratinocytes were treated with the indicated doses of dPP for 24 hr. Samples for SDS-PAGE were prepared and Western immunoblotting was performed as described in Experimental Procedures. The amount of the enzyme was quantitated by densitometry and is expressed as a percentage of the amount of isozyme present in the total fraction from control cells. Each *point* is the average ± standard error of three independent sets of experiments.

cytes. Our results reveal three major differences in the regulation of the PKC isozymes of keratinocytes by the antipromoting phorbol derivative dPP, compared with that found previously for the typical, tumor-promoting, phorbol ester PMA. First, dPP very quickly translocates (and presumably activates) all three of the PKC isozymes α, δ, and ϵ, whereas we previously showed that PMA and bryostatin 1 translocate PKC-δ with significantly slower kinetics (8). This difference is illustrated in Fig. 7. As pointed out previously, very slow translocation could lead to down-regulation of PKC with a very low level of effective activation (8). Conversely, it has been documented that PKC isozymes may have antagonistic effects for inducing certain biological responses (17, 18). For mutually antagonistic PKC isozymes, the first to be activated might determine the final response. We have suggested that the presence and/or activation of PKC-δ could be of importance for keratinocyte differentiation (8). Our data suggest that dPP might be a more potent and faster activator of PKC-δ than is PMA and consequently might induce the differentiation of keratinocytes more efficiently.

The second major difference between PMA and dPP is related to the relative potencies for translocation of the different PKC isozymes. PMA was only 5-fold more potent for translocating PKC-δ than PKC-α, whereas it was 60-fold more potent for translocating PKC-ϵ than PKC-δ, so the dose-response curves for activation of the classical and novel PKC isozymes in keratinocytes were not well resolved (8). On the other hand, dPP was clearly at least 2 orders of magnitude more potent for translocating both PKC-δ and PKC-ϵ than PKC-α and was at least 2 orders of magnitude more potent for down-regulating PKC-δ, -ϵ, and -η than PKC-α. This is in good correlation with the fact that 12-deoxyphorbol monoesters were able to distinguish between subclasses of receptors in particulate preparations from mouse skin (19). dPP thus shows a degree of selectivity, at least in keratinocytes, for the novel PKC isozymes comparable to that of okadaic acid for protein phosphatase 2A, compared with protein phosphatase 1 (20). dPP is thus a powerful tool to dissect the pathways inducing biological responses associated with the classical or novel PKC isozymes. We took advantage of this observation and showed that the dose-response curves for the induction of cornified envelope formation, transglutaminase activity, and ornithine decarboxylase activity show excellent correlations with the dose-response curve for PKC-α translocation, whereas the dose-response curve for EGF binding down-regulation correlates with the dose-response curves for the translocation of novel PKC isozymes (Table 2). We are in the process of confirming the association of individual PKC isozymes with the aforementioned biological reponses by transfecting activated PKC isoforms into mouse keratinocytes. It should be noted that a recent publication suggests that the endogenous PKC activator diacylglycerol shows similar selectivity between PKC-α and the novel isozymes PKC-δ and PKC-ϵ in Swiss 3T3 fibroblasts (21), suggesting a parallel between dPP and the physiological activator of PKC. We previously observed that for PMA the structure-activity relations for PKC isozymes varied with the cell type (8, 9). The selectivity of dPP in other cell types remains to be determined.

The third difference is related to the regulation of PKC-ϵ. Neither PMA nor bryostatin 1 down-regulated PKC-ϵ associated with the Triton X-100-soluble particulate fraction (8). In contrast, dPP translocated and down-regulated PKC-ϵ very effectively. Based on multiple examples, it seems that whether PKC-ϵ can be down-regulated depends on both the cell line and the ligand applied (for review, see Ref. 8). The specific down-regulation of PKC-ϵ by dPP but not by PMA is an attractive explanation for the antipromoter effect of dPP, because the repeated treatment during the tumor promotion protocol would lead to the elimination of PKC-ϵ by dPP, blocking the constant activation by PMA. It is perhaps relevant that the overexpression of PKC-ϵ and the presence of constitutively active catalytic fragments of PKC-ϵ have been found to be tumorigenic in several systems (22–24). A detailed analysis of PKC isozyme regulation in mouse skin during tumor promotion treatment is underway in our laboratory and, it is hoped, will provide an answer to this question. dPP provides another example, in addition to PMA and bryostatin, of a PKC activator that shows significant selectivity between PKC isozymes *in vivo* in keratinocytes but basically similar affinities for all PKC isozymes,

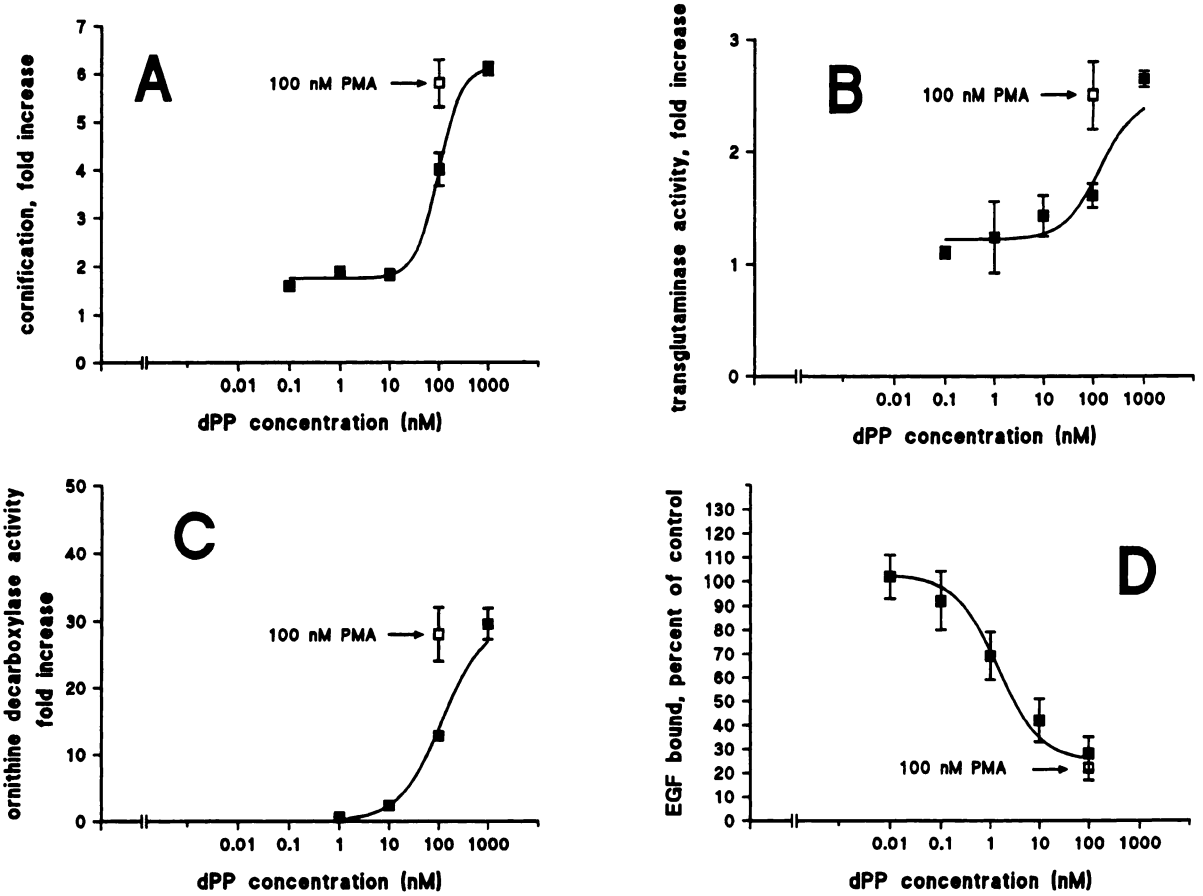

Fig. 6. dPP-induced changes in levels of cornification (A), transglutaminase activity (B), ornithine decarboxylase activity (C), and inhibition of EGF binding (D). Cells were treated with different doses of dPP for 48 hr, 6 hr, 3 hr, and 3 hr in A, B, C, and D, respectively. Cornification, enzyme activities, and EGF binding were determined as described in Experimental Procedures. Each *point* is the average ± standard error of three samples. Two more experiments yielded similar results. In A, B, and C, the *y*-axis represents the fold increase over vehicle-treated samples. In D, 100% represents the vehicle-treated value.

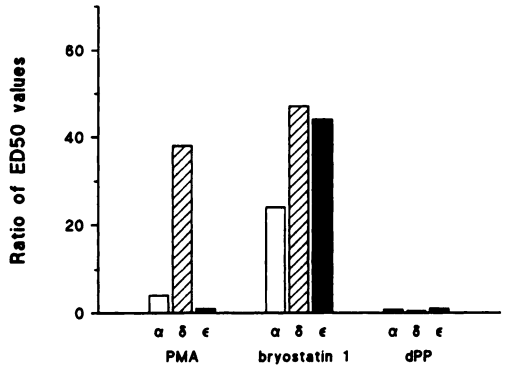

Fig. 7. Comparison of the ratios of the ED_{50} values of dPP, PMA, and bryostatin 1 for translocating and removing the individual PKC isozymes from the soluble fraction at 5 min and 6 hr. Each value represents the ratio of the ED_{50} values determined for translocation at 5 min and removal from the soluble fraction at 6 hr for an individual isozyme, for dPP, PMA, or bryostatin 1. The values for PMA and bryostatin 1 are taken from our previous publication (8).

TABLE 2

Potency of dPP for inducing translocation and down-regulation of PKC isozymes and biological responses

ED_{50} values for the dPP-induced effects were determined as described in the text.

	ED_{50} (dPP)
	nM
Translocation of PKC-α (at 5 min)	100 ± 5
Removal of PKC-α from the soluble fraction (at 6 hr)	140 ± 10
Down-regulation of PKC-α (at 24 hr)	100 ± 5
Translocation of PKC-δ (at 5 min)	0.8 ± 0.1
Removal of PKC-δ from the soluble fraction (at 6 hr)	2 ± 0.1
Down-regulation of PKC-δ (at 24 hr)	1 ± 0.1
Translocation of PKC-ε (at 5 min)	1 ± 0.2
Removal of PKC-ε from the soluble fraction (at 6 hr)	3 ± 0.3
Down-regulation of PKC-ε (at 24 hr)	2 ± 0.1
Down-regulation of PKC-η (at 24 hr)	1 ± 0.1
Cornified envelope formation (at 48 hr)	100 ± 1
Induction of transglutaminase activity (at 6 hr)	140 ± 20
Induction of ornithine decarboxylase activity (at 3 hr)	120 ± 20
Down-regulation of EGF binding (at 1 hr)	2 ± 0.2
Down-regulation of EGF binding (at 3 hr)	1.5 ± 0.3

as well as the distinct phorbol ester receptor n-chimaerin, in *in vitro* assays (9, 25, 26). The lack of metabolism of dPP rules out conversion of dPP to an isozyme-selective ligand as an explanation for this difference. The basis for the differences in selectivity is unknown; the differences presumably reflect the complex interactions between the PKC isozymes, the ligand, and such factors as the state of phosphorylation, membrane lipid composition, or associated proteins.

Our findings emphasize that *in vitro* binding assays have little predictive power regarding the *in vivo* potencies of PKC activators. In addition, our previous studies clearly showed (8, 9) that the *in vivo* potencies and selectivities of PKC activators

depended on the specific cell lines evaluated. It is therefore inappropriate to extrapolate from our current findings in keratinocytes to assume similar selectivity of dPP in other cell lines. On the other hand, the concept that ligand selectivity is cell context dependent has great importance for drug development, because it provides additional opportunities for the establishment of selectivity for a specific pathway.

In light of its central role in tumor promotion and signal transduction, PKC is an attractive target for cancer therapy (27). There is increasing evidence that the individual PKC isozymes play different, sometimes opposing, roles in biological processes (17, 18, 22), providing two obvious directions for pharmacological exploitation. One is the design of specific (preferably isozyme-specific) inhibitors of PKC (28, 29). This approach has mainly focused on inhibition of the catalytic domain of PKC; a complication with the approach is that the catalytic domain is not the domain primarily responsible for the isotype specificity of PKC (30, 31). The other approach is to develop isozyme-selective PKC activators. These may provide a way to override the effect of other signal transduction pathways with opposite biological effects. Alternatively, by inducing down-regulation of PKC after acute activation, PKC activators may cause long term antagonism. dPP and bryostatin (8, 9) provide cogent examples for the existence of such isozyme-selective activators. Both strategies face problems of secondary targets. Inhibitors directed at the catalytic domain of PKC may interact with others of the many kinases in the cell. Agents directed at the regulatory domain may also affect the phorbol ester receptors distinct from PKC, such as the chimaerins and unc-13. Depending on the specific system, the approaches may thus be complementary.

Acknowledgments

We thank Nancy E. Lewin for purification of [20-^{3}H]dPP.

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